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Research article

Leveraging barrel medic genome sequence for the development and use of genomic resources for genetic analysis and breeding in legumes

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ABSTRACT

Background: A total of 62,591 cowpea expressed sequence tags (ESTs) were BLAST aligned to the whole-genome sequence of barrel medic (*Medicago truncatula*) to develop conserved intron scanning primers (CISPs). The efficacy of the primers was tested across 10 different legumes and on different varieties of cowpea, chickpea, and pigeon pea. Genetic diversity was assessed using the same primers on different cowpea genotypes. Single-nucleotide polymorphisms (SNPs) were detected, which were later converted to length polymorphism markers for easy genotyping. CISPs developed in this study were used in tagging resistance to bacterial leaf blight disease in cowpea.

Results: A total of 1262 CISPs were designed. The single-copy amplification success rates using these primers on 10 different legumes and on different varieties of cowpea, chickpea, and pigeon pea were approximately 60% in most of the legumes except soybean (47%) and peanut (37%). Genetic diversity analysis of 35 cowpea genotypes using 179 CISPs revealed 123 polymorphic markers with PIC values ranging from 0.05 to 0.59. Potential SNPs identified in cowpea, chickpea, and pigeon pea were converted to PCR primers of various sizes for easy genotyping. Using the markers developed in this study, a genetic linkage map was constructed with 11 linkage groups in cowpea. QTL mapping with 194 F_3 progeny families derived from the cross C-152 × V-16 resulted in the identification of three QTLs for resistance to bacterial leaf blight disease.

Conclusions: CISPs were proved to be efficient markers to identify various other marker classes like SNPs through comparative genomic studies in lesser studied crops and to aid in systematic sampling of the entire genome for well-distributed markers at low cost.

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1. Introduction

In agricultural importance, legumes are second only to cereal crops in cropped area and production. Legumes play diverse roles in human nutrition, ranging from providing dietary protein to health-promoting secondary compounds [1]. Legumes complement cereals for the source of protein in the human diet and livestock feed. Molecular phylogeny within the legume family [2,3,4] has revealed high synteny at the genome level among tropical [5] and temperate legumes [6]. Until recently, legumes were regarded as "orphan crops," as there was not much progress in genetic research, and also, the availability of genomic resources was meager among primary legume crops [7]. The complete genomic sequences of cereal crops and their comparative genomic analyses have significantly contributed to their improvement [8]. Comparative genomic analysis is a cost-effective tool that can speed up gene identification in species that either are less studied at the genomic level or have large genomes [9,10,11,12]. Similar efforts were made in our study to foster genetic research in economically important legumes. With the availability of the complete genome sequence of model legume barrel medic (*Medicago truncatula*) and expressed sequence tag (EST) sequences

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Table 1
CISPs designed from ESTs of cowpea.

Alignment	No. of ESTs	No. of primer pairs	Distribution on *Medicago* genome	
			Chromosome	No. of primers
Cowpea – Barrel medic	62,591	1262	1	163
			2	147
			3	158
			4	199
			5	213
			6	54
			7	167
			8	159
			0	2
			Total	1262

of legumes, we were able to generate genomic resources for both resource-rich legumes such as pigeon pea, chickpea, and soybean, and less studied legumes such as black gram, horse gram, and field bean.

For significant application of genomic tools across taxa at low cost and for genetic diversity studies, there is a need to identify conserved genome sequence as well as variation at the DNA level [13]. On the basis of this understanding, a marker system, conserved-intron scanning primers (CISPs), was developed in our study that detects variation in introns, usually in terms of length polymorphism and point mutations, necessarily SNPs. The gene-based single-nucleotide polymorphisms (SNPs) are becoming the first-choice markers in genomics-assisted breeding [14,15]. SNPs and insertion–deletions (INDeLs) are essentially the inexhaustible source of highly stable markers that often contribute to phenotype [16]. There are numerous ways for SNP identification in plants, namely, whole-genome sequence alignment and EST clustering, which requires available genomic resources. In the last decade, plant biologists have been keen on capitalizing the advancements of sequencing technologies in generating genomic resources for "orphan" species for which minimal genomic data exist [17].

In the present study, we used ESTs generated from cowpea to develop intron-flanking markers for genetic analysis in the legume family. These markers also helped in the detection of potential SNPs and/or INDeLs in pigeon pea, chickpea, and cowpea, and attempts were made to convert SNPs into PCR-based primers for their use in enriching existing genetic maps. These markers were used to construct a molecular map and tag resistance to bacterial leaf blight in cowpea, as it is a very devastating disease causing severe grain yield loss of more than 64% [18]. Some of the studies revealed single dominant gene-controlled resistance to this disease [19], and recent literature indicated quantitative inheritance necessitating QTL identification [20]. One of the major QTL was introgressed into C-152, a superior high yielding but susceptible variety, through marker-assisted backcrossing (MABC).

2. Materials and methods

2.1. Development of conserved intron-scanning primers (CISPs)

We used 62,591 ESTs from drought stressed and nonstressed genotypes of cowpea; these ESTs were downloaded from the website https://www.ncbi.nlm.nih.gov in this study and were BLAST ($E < 1 \times 10^{-10}$) aligned with the barrel medic genome (TIGR ver. 3.0; http://www.tigr.org). PCR primer pairs were designed from highly conserved (0–1 mismatch) alignments. The criteria for primer design involved that the PCR amplicon spanned at least one intron, 17–22 bp long primer sites, 250–1500 bp predicted amplicon size, and importantly, the designed primer sets should amplify single-copy amplicon in barrel medic. The amplification region on the barrel medic genome was checked, and redundant primers that amplify the same region were removed.

2.2. Plant material and DNA extraction

Sampling for the PCR amplification across legumes included one genotype from barrel medic (*M. truncatula* Gaertner., EC 547749), pigeon pea [*Cajanus cajan* (L.) Millsp., BRG2], chickpea (*Cicer arietinum* L., Annigeri-1), cowpea [*Vigna unguiculata* (L.) Walp., KBC2], common bean [*Phaseolus vulgaris* (L.), ArkavSuvidha], soybean [*Glycine max* (L.) Merr., MAUS2], black gram [*Vigna mungo* (L.) Hepper. TAU1], horse gram [*Macrotyloma uniflorum* (Lam.) Verdc., PHG9], field bean [*Lablab purpureus* (L.) HA4], and peanut [*Arachis hypogaea* (L.) GPBD4]. For polymorphism identification, PCR was carried out on eight varieties of pigeon pea (BRG1, BRG2, BRG3, TTB7, ICPL 87119, ICPL 8863, GS1, and ICPL 7034), eight varieties of Chickpea [ICCV4958, JG62, ICCV2, K850, BG256, WR315, Annigeri-1 (A-1), KAK2], and four varieties of cowpea (KBC2, IT 38956–1, C-152, and IC-219607) based on their importance in breeding programs. Pigeon pea, chickpea, cowpea, horse gram, field bean, peanut, and soybean genotypes were obtained from the respective All India Coordinated Research Projects, University of Agricultural Sciences, GKVK, Bangalore. The common bean genotype Arka Suvidha was procured from the Indian Institute of Horticultural Research, Bangalore, and barrel medic seeds from the Indian Grassland and Fodder Research Institute, Jhansi. Tified CTAB method [21] and the quality and quantity of extracted DNA was checked on a 0.8% agarose gel. A mapping population was developed by crossing high-yielding and susceptible cultivar C-152 with resistant genotype V-16 to detect QTL regions for BLB resistance. The F_1 individuals were selfed to produce F_2 population. Leaf samples were collected from individual F_2 plants and advanced to generate $F_{2:3}$ progenies for disease phenotyping.

2.3. PCR conditions

PCR conditions were the same for all primers. Reaction mixtures included 30 ng of genomic DNA, 0.5 mM dNTPs (Sigma-Aldrich), 1

Table 2
PCR results of CISPs in selected legumes.

Alignment	No. of primers	Legumes	PCR success			% success	Single-copy amplification (%)
			Blank	Single	Multiple		
Cowpea – *Medicago*	384	Barrel medic	44	259	81	88.5	67.4
		Cowpea	49	246	89	87.2	64.1
		Chickpea	43	258	83	88.8	67.2
		Pigeon pea	37	212	135	90.4	55.2
		Horse gram	62	242	80	83.9	63.0
		Field bean	50	236	98	87.0	61.5
		Soybean	64	182	138	83.3	47.4
		Groundnut	77	90	73	67.9	37.5
		Black gram	70	216	50	79.2	64.3
		Common bean	75	227	82	80.5	59.1

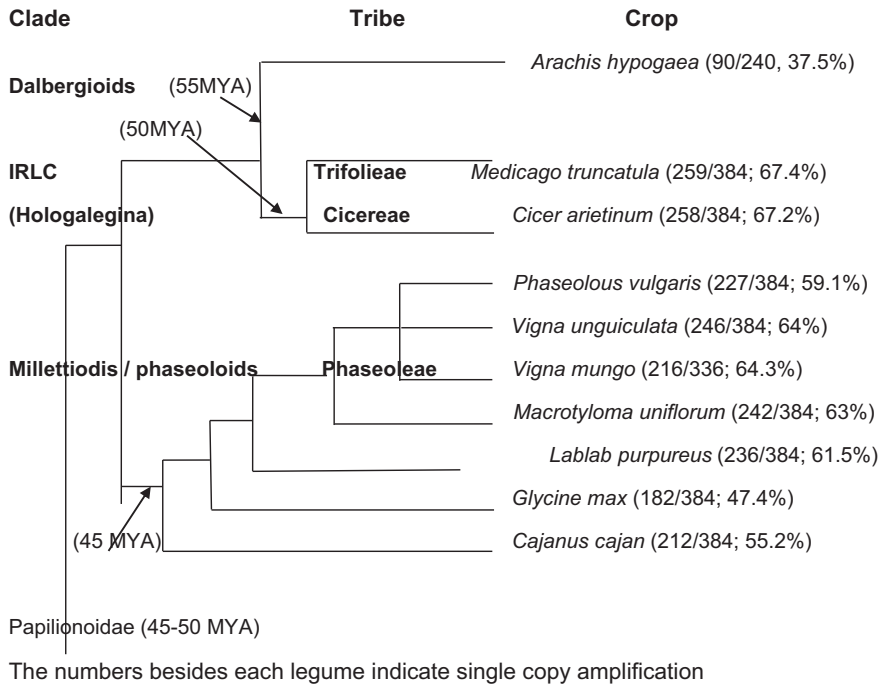

Fig. 1. PCR success rates (single-copy amplification) of 384 primers in different legumes. Comprehensive legume tree adapted from Gepts et al. 2005.

unit of *Taq* DNA polymerase (Sigma-Aldrich), 0.0626 U PR Polymerase (Bangalore Genei), 15 pmol each of forward and reverse primers, and 1× PCR buffer (Sigma-Aldrich) in a total reaction volume of 30 μl. PCR (Eppendorf Mastercycler) cycling parameters were 94°C for 5 min, followed by 94°C for 30 s, 61°C (-0 .2°C/cycle) for 45 s, 72°C for 60 s for 16 cycles, followed by 94°C for 30 s, 57°C for 45 s, 72°C for 60 s for 30 cycles, and a final extension at 72°C for 10 min. PCR products were visualized on a 1.5% agarose (Sigma) gel stained with ethidium bromide. Loci were classified (0–2) according to whether they yielded no product (0), a single band (1), and two or more bands (2).

2.4. Scoring of CISPs on 35 cowpea genotypes and data analysis

The genotype profiles produced by intron-flanking primers were scored manually. Each allele was scored as present (1) or absent (0) for each of the loci. A total of 179 markers, which worked on the cowpea genotype KBC-2, were used for genotyping 35 cowpea varieties comprising advanced breeding lines, released varieties, and local collections in the state of Karnataka, India. Markers were scored for variation in amplicon size and the data analyzed for PIC using the formula described by Bornstein et al. [22] for their informativeness.

$$PIC = 1 - \Sigma\ Pi^2$$

where, *Pi* is the frequency of the i^{th} allele in the set of genotypes analyzed, calculated for each locus. The genetic similarity between any two genotypes was estimated on the basis of Jaccard's similarity coefficient [23]. All the 35 genotypes were clustered with the UPGMA analysis using NTSYS-PC v2.10t [24].

2.5. Sequencing of PCR products

PCR amplified products were purified using the column-based QIA quick PCR purification kit. After purification, all the products (4 μl) with the Fermentas GeneRuler 1 Kb DNA ladder were run on a 2.0% agarose gel and documented. Sequencing of all the purified PCR products was performed with respective forward primer using the ABI 3730XL sequencer (Ocimum Bio-Sciences, Hyderabad, India). Purified high-quality PCR products were amplified using the ABI Big Dye 3.1 cycle sequencing kit (Applied Biosystems). Cycle sequencing PCR parameters consisted of 24 cycles of 96°C for 10 s, 50°C for 5 s, 60°C for 4 m, and finally 4°C for 10 m. After cycle sequencing completion, PCR products were purified and dissolved in Hi-Di formamide and run in ABI 3730XL sequencer. The results were subjected to Phred-Phrap (www.phrap.org/phredphrapconsed.html) analysis. The sequences were BLAST aligned with the barrel medic genome to check whether orthologous regions were amplified and whether the amplicons derived from the same primer hit the intended genomic region. Sequences hitting the intended genomic region from different varieties of pigeon pea, chickpea, and cowpea were aligned using ClustalW2 (www.ebi.ac.uk/clustalw) to identify putative SNPs. Only those regions having a base score of more than 20 were used in the identification of SNPs/INDeLs.

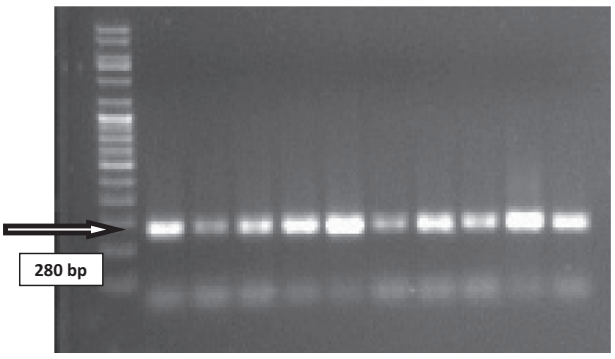

Fig. 2. Intron length conservation across 10 legumes generated by the marker VuMt_427. L-Low Range Ruler; 1—Medicago; 2—Commonbean; 3—Cowpea; 4—Chickpea; 5—Pigeon pea; 6—Soybean; 7—Field bean; 8—Horse gram; 9—Black gram; 10—Groundnut.

Table 3
Number of plant regulatory elements predicted in our study.

No. of Alignments	CISPs screened			Promoters/Enhancers from ILPs			Motifs of regulatory elements			% Success		
	Chickpea	Cowpea	Pigeon pea	Chickpea	Cowpea	Pigeon pea	Chickpea	Cowpea	Pigeon pea	Chickpea	Cowpea	Pigeon pea
VuMt	22	112	66	3	16	52	13	95	14	73	99	100

2.6. Designing of dCAPS

For designing derived cleaved amplified polymorphic sequence (dCAPS) primers, identification of restriction endonuclease recognition sites and accompanying primer mismatches is needed [25]. For this identification, we used a web browser-based program, dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html). Two haplotypes with approximately 25 nucleotides on each side of the SNP were entered in the program, and with no mismatches in the query, dCAPS Finder 2.0 was used to determine whether the SNP in the query generates a RFLP-based CAPS marker. Rerunning the program with one or more mismatches allowed in the design of dCAPS primer. All of the potential primer sequences for dCAPS analysis were identified, including the highlighted mismatches used to generate the restriction endonuclease recognition site.

Table 4
Details of 300 intron-flanking primers showing polymorphism among 35 cowpea cultivars.

Sl. no.	Locus	PIC	Sl. no.	Locus	PIC	Sl. no.	Locus	PIC	Sl. no.	Locus	PIC
1	VuMt-193	0.282	55	VuMt-364	0.1445	109	VuMt-438	0.3772	163	VuMt-502	0.1945
2	VuMt-194	0	56	VuMt-365	0.182	110	VuMt-440	0.3732	164	VuMt-503	0.2631
3	VuMt-195	0.248	57	VuMt-366	0.054	111	VuMt-442	0	165	VuMt-504	0.2437
4	VuMt-197	0.2149	58	VuMt-367	0.1019	112	VuMt-443	0.1019	166	VuMt-505	0
5	VuMt-198	0.3688	59	VuMt-371	0.054	113	VuMt-444	0.3091	167	VuMt-506	0.3608
6	VuMt-199	0.2149	60	VuMt-376	0.2437	114	VuMt-445	0.3091	168	VuMt-507	0.054
7	VuMt-200	0	61	VuMt-378	0.2484	115	VuMt-446	0.4011	169	VuMt-508	0.1046
8	VuMt-201	0.3437	62	VuMt-379	0	116	VuMt-447	0	170	VuMt-509	0.2391
9	VuMt-202	0	63	VuMt-380	0.44	117	VuMt-448	0.3135	171	VuMt-511	0
10	VuMt-203	0.1445	64	VuMt-381	0.054	118	VuMt-449	0.2241	172	VuMt-512	0.2149
11	VuMt-204	0.248	65	VuMt-382	0	119	VuMt-450	0.1445	173	VuMt-513	0.2149
12	VuMt-205	0.148	66	VuMt-383	0	120	VuMt-451	0.3741	174	VuMt-514	0.1065
13	VuMt-206	0.1019	67	VuMt-384	0.0555	121	VuMt-452	0.3091	175	VuMt-518	0.2149
14	VuMt-207	0	68	VuMt-389	0.3091	122	VuMt-453	0.1019	176	VuMt-519	0.2905
15	VuMt-208	0	69	VuMt-390	0.1445	123	VuMt-454	0.3135	177	VuMt-520	0
16	VuMt-209	0	70	VuMt-391	0.2743	124	VuMt-455	0.4018	178	VuMt-521	0
17	VuMt-210	0	71	VuMt-392	0.2484	125	VuMt-456	0.1074	179	VuMt-522	0
18	VuMt-211	0.3749	72	VuMt-393	0.0605	126	VuMt-457	0.2905			
19	VuMt-213	0	73	VuMt-396	0.3972	127	VuMt-459	0			
20	VuMt-214	0.054	74	VuMt-397	0	128	VuMt-460	0			
21	VuMt-215	0	75	VuMt-399	0.2437	129	VuMt-461	0.3491			
22	VuMt-217	0.2688	76	VuMt-400	0	130	VuMt-462	0			
23	VuMt-331	0	77	VuMt-401	0.4372	131	VuMt-463	0			
24	VuMt-332	0	78	VuMt-402	0.1445	132	VuMt-464	0.3648			
25	VuMt-333	0	79	VuMt-403	0	133	VuMt-465	0			
26	VuMt-334	0	80	VuMt-404	0.2149	134	VuMt-466	0.3698			
27	VuMt-335	0.2688	81	VuMt-405	0.5914	135	VuMt-467	0.231			
28	VuMt-336	0	82	VuMt-406	0.374	136	VuMt-468	0.0644			
29	VuMt-337	0	83	VuMt-407	0	137	VuMt-469	0.3249			
30	VuMt-338	0.182	84	VuMt-408	0	138	VuMt-470	0			
31	VuMt-339	0.1445	85	VuMt-409	0.4365	139	VuMt-471	0.1019			
32	VuMt-340	0	86	VuMt-410	0.329	140	VuMt-472	0			
33	VuMt-342	0.1019	87	VuMt-411	0	141	VuMt-473	0.2861			
34	VuMt-343	0.318	88	VuMt-412	0.3491	142	VuMt-474	0.282			
35	VuMt-344	0	89	VuMt-413	0.1445	143	VuMt-475	0.2194			
36	VuMt-345	0	90	VuMt-414	0	144	VuMt-476	0.2149			
37	VuMt-346	0	91	VuMt-416	0.054	145	VuMt-477	0.054			
38	VuMt-347	0.2688	92	VuMt-417	0.2688	146	VuMt-478	0.2905			
39	VuMt-348	0	93	VuMt-418	0	147	VuMt-479	0.2437			
40	VuMt-349	0.1019	94	VuMt-419	0.1445	148	VuMt-480	0			
41	VuMt-350	0.2688	95	VuMt-420	0.1019	149	VuMt-481	0			
42	VuMt-351	0.3692	96	VuMt-421	0.3524	150	VuMt-482	0.4812			
43	VuMt-352	0.1445	97	VuMt-422	0.1445	151	VuMt-483	0.3484			
44	VuMt-353	0.3381	98	VuMt-426	0.3203	152	VuMt-484	0.2951			
45	VuMt-354	0.2149	99	VuMt-427	0.1861	153	VuMt-485	0.3491			
46	VuMt-355	0.054	100	VuMt-428	0.2735	154	VuMt-486	0.3419			
47	VuMt-356	0	101	VuMt-429	0.1555	155	VuMt-487	0.3194			
48	VuMt-357	0.0555	102	VuMt-430	0.0555	156	VuMt-488	0.054			
49	VuMt-358	0	103	VuMt-432	0.2437	157	VuMt-492	0			
50	VuMt-359	0.2735	104	VuMt-433	0	158	VuMt-495	0			
51	VuMt-360	0.054	105	VuMt-434	0.4485	159	VuMt-498	0.3381			
52	VuMt-361	0	106	VuMt-435	0	160	VuMt-499	0.2437			
53	VuMt-362	0.3748	107	VuMt-436	0	161	VuMt-500	0.1445			
54	VuMt-363	0.2905	108	VuMt-437	0.3732	162	VuMt-501	0			

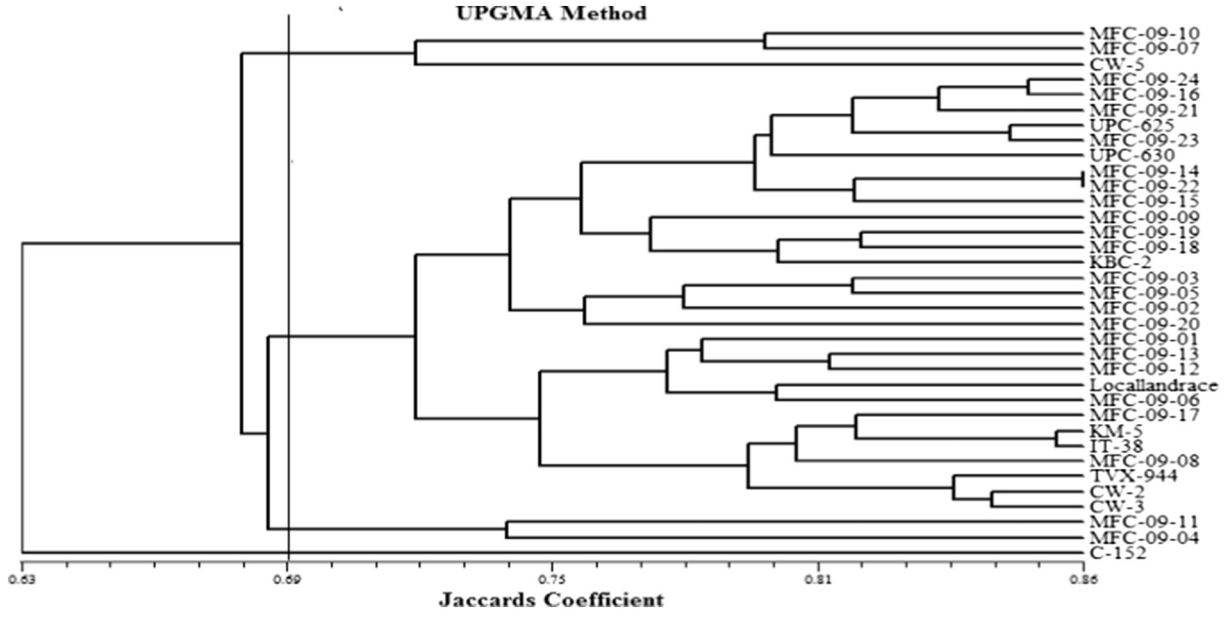

Fig. 3. UPGMA-based genetic clustering of selected 35 cowpea genotypes with the aid of CISPs.

2.7. Phenotyping of F_3 plants for BLB

Seeds of F_3 progenies were planted in polythene bags (12″ × 18″) filled with soil in randomized complete block design with two replications during rainy season of 2013. Ten plants were retained per polythene bag and were watered at regular interval. The plants in F_3 progenies were inoculated with the bacterial suspension at a concentration of 2×10^6 (CFU/ml), and disease development was ensured.

2.8. Construction of linkage map

A set of 189 SSR primer combinations and 271 CISPs developed in our study were used to detect polymorphism between C-152 and V-16. A total of 194 F_2 plants were genotyped using 96 markers (79 SSR and 17 CISP), which were found to be polymorphic. The linkage analysis was performed using JoinMap (version 4.0) [26].

2.9. QTL detection

A total of 194 $F_{2:3}$ plants were used for QTL mapping using WinQTL Cartographer version 2.5 [27].

2.10. Marker-assisted introgression of QTL region controlling BLB resistance

MABC was carried out with the resistant parent V-16 (as male) and high-yielding susceptible cowpea variety C-152 to transfer a major QTL. Twenty-seven F_1 plants were screened with the marker VuMt 338, and 19 true F_1 plants were identified. The F_1 plants were backcrossed with C-152 and 63 BC_1F_1 seeds were planted and subjected for foreground selection with flanking markers of the major QTL on LG 8. Among them, 17 heterozygous plants were selected. A BC_2F_1 plant population was developed by crossing 17 heterozygous plants with C-152. We raised 118 BC_2F_1 plants, and foreground selection was performed. Among the 118 plants, 51 heterozygous plants were identified and selfed to produce BC_2F_2 plants. These progenies, each consisting of ten plants, were planted, and 31 progenies homozygous for flanking markers were identified by foreground selection and phenotyped for disease resistance and subjected to both foreground and background selection. Background selection was performed using 40 markers from 11 linkage groups.

3. Results

A total of 2086 conserved primer pairs were designed from cowpea-barrel medic alignments. A nonredundant set of 1262 primer pairs was selected from the original set of 2086 primer pairs (Table S1). The number of primers in chromosome 6 was less.

3.1. Distribution of CISP loci in Medicago genome

The probable distribution of *in silico*-developed *Vigna–barrel medic* (VuMt) CISPs was studied. The study revealed the distribution of the highest number of loci on chromosomes 5 and 4, while chromosome 6 has the lowest number of CISP loci on *M. truncatula* (Table 1).

Out of 1262 intron-flanking markers, 384 primers from cowpea-barrel medic alignments were synthesized and tested on 10 members of the legume family, namely, barrel medic, pigeon pea (BRG-2), cowpea (KBC-2), chickpea (A1), common bean (Arka Suvidha), soybean (MAUS-2), horse gram (PHG-9), black gram (TAU-1), field bean (HA-4), and peanut (TMV-2) (Table 2). On the source taxa barrel medic (67.4%) and cowpea (64.1%), a high percentage of primer sets were amplified. Equally, a high percentage of primer sets were amplified in six other legumes, namely, chickpea (67.2%), pigeon pea (55.2%), horse gram (63%), field bean (61.5%), black gram (64.3%), and common bean (59.1%). Out of the six legumes mentioned above, chickpea belongs to galegoid clade and the rest belong to phaseoloid clade, implying high cross-species transferability of the primers. The percentage success rate was relatively low in soybean (47.4%) and groundnut (37.5%) (Fig. 1), accounting for the presence of chromosomal duplications, resulting in multiple amplicons in soybean genome, and groundnut is an allotetraploid with a complex genome and a distant relative belonging to dalbergiod clade. High-quality sequences generated from barrel

Table 5
Intron length polymorphism in cowpea.

Legume	Parents	No. of CISPs tried	Polymorphic CISPs
Cowpea	C-152 × V-16	206	49
	C-152 × V 57817	206	45

medic, pigeon pea, chickpea, and cowpea were examined for AT and GC contents. In all these legumes, the AT fraction was found to be more than the GC fraction.

3.2. Intron size conservation

Out of 384 primers tried on 10 taxa, 68 primers exhibited conserved intron length (Fig. 2). The PCR products of all 10 legumes amplified by 10 primers designed to generate uniform and varying intron sizes across legumes were sequenced and aligned using ClustalW, which revealed that nucleotide variations were not correlated with implied intron size, and the polymorphism rates did not show statistically significant difference between introns that showed the same length and those that showed different lengths across taxa. Furthermore, it was observed that intron position and approximate length were strongly conserved features, even across longer evolutionary distances.

3.3. Presence of regulatory elements in the introns

Intron length conservation, particularly in the first intron of the gene across taxa, has been reported to be due to the presence of regulatory elements such as promoters, enhancers, and motifs found in plant cis-acting regulatory DNA elements. These elements are involved in complex gene regulation mechanisms by acting as either enhancers or silencers. In this study, the majority of intron sequences subjected to regulatory elements prediction tools (TSSP and NSITEMP) revealed the presence of regulatory elements (Table 3).

3.4. Assessment of genetic diversity among cowpea genotypes using CISPs

The intron-flanking markers, which were designed from cowpea-barrel medic alignments and were used to amplify PCR products in the pigeon pea variety KBC 2, were used to assess the genetic diversity in a set of 35 genotypes of cowpea consisting of advanced breeding lines, local landraces, and released varieties. Out of 179 markers tested, 123 markers were found to be polymorphic with a PIC value ranging from 0.05 to 0.59 and with an average PIC of 0.24 (Table 4). On average, two alleles per locus were amplified among the 35 genotypes for 123 polymorphic markers. A dendrogram was constructed to identify the genetic relationship among 35 genotypes (Fig. 3). A majority of cowpea genotypes (29) clustered in one group (Cluster III) out of four cluster groups formed at a similarity coefficient of 0.69. At 0.75 similarity coefficient, cluster III was subdivided into four subclusters containing seven, five, four, and thirteen genotypes.

3.5. Intron length polymorphism

To study whether these intron-flanking markers generate length polymorphism on agarose gel, we tried them on three different genotypes of cowpea involved in the development of mapping population to tag resistance to leaf blight and cowpea yellow mosaic virus. Out of 206 markers, 49 generated polymorphism between C-152 and V-16 and 45 between C-152 and V 57817 (Table 5).

3.6. SNP discovery in pigeon pea, chickpea, and cowpea

Primer pairs producing prominent single bands were selected in pigeon pea, chickpea, and cowpea. The primer pairs were then tested with different varieties of these three legumes, and high-quality PCR products among them were sequenced. The PCR products of 296 primer pairs were sequenced and aligned. Manual mining for SNP/INDeL was done considering only those bases with a quality score of more than 20. This revealed the presence of 76 SNPs and 25 INDeLs from alignments of 100 primers in cowpea. Similarly, 27 primer pairs in pigeon pea revealed 21 SNPs and six INDeLs, and in chickpea, 16 SNPs and 17 INDeLs were detected from 33 primer pairs (Table S2, Table S3 and Table S4). Average SNP frequency was 5.73/kb in cowpea, 1.91/kb in chickpea, and 1.56/kb in pigeon pea. Transitions and transversions were found in equal proportion in the SNPs identified in cowpea, whereas transversions were more in chickpea than transitions and *vice versa* in pigeon pea (Table 6).

3.7. Conversion of SNPs into PCR-based markers

The web browser-based program dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html) was used to develop PCR-based markers for utilization of the identified SNPs in crop improvement programs. We have designed 33 dCAPS primers in cowpea, four each in pigeon pea and chickpea (Table 7). The primer pairs were synthesized and tried on lines from which SNPs were identified, and the amplified products were digested using respective enzymes and separated on 4% high-resolution agarose gel to detect polymorphism between the lines (Fig. 4). The lines were perfectly distinguished on the gel reflecting on the efficacy of dCAPS primers in genetic studies. We utilized web-based program BIO-EDIT and identified 20, 3, and 12 restriction sites in the region of SNPs, which can be used directly as CAPS markers in cowpea, chickpea, and pigeon pea, respectively (Table 8).

3.8. Genetic linkage map, detection of QTL, and marker-assisted introgression of QTL on LG8 for resistance to bacterial leaf blight

A genetic linkage map was constructed using 79 SSRs and 17 CISPs that were found to be polymorphic between parents. The map comprised 11 linkage groups (Fig. 5), and QTL mapping was carried out on 194 F_3 progeny families derived from the cross C-152 × V-16. Three QTLs were detected, one on linkage group 8 and other two on linkage group 11. The QTL on LG8 was flanked by the markers VuMt 401 and VuMt 397 and, on LG 11, by VuMt 338 and VuMt 337 and VuMt 338 and VuMt 252, respectively. The highest phenotypic variation of 30.58% was explained by the QTL located on linkage group 8.

4. Discussion

The completely sequenced plant genomes permit development of an array of genomic resources for crop improvement. In this study, gene coding (thus, relatively conserved) sequences located near exon–intron boundaries were identified for designing primers, which specifically amplified the more rapidly evolving introns. A nonredundant set of 1264 primer pairs was designed from cowpea-barrel medic alignments. The number of primers resulted are low because more than 40% of the ESTs failed to find near-perfect hits (<70% conservation) on barrel medic genome, while more than 35% were found to be redundant. Such limited synteny results were also observed with legume family members earlier by Cannon et al. [28] while comparing a few members of legume family with either barrel medic or birdsfoot trefoil or among

Table 6
Polymorphisms detected in three different legumes from VuMt Primers.

	No. of primers	Transitions	Transversions	Total no. of good sequences	Primers with polymorphisms	No. of SNPs	No. of INDeLs	No. of SNPs/kb
Chickpea	33	14	19	252	33	4	17	1.91
Cowpea	100	40	39	753	100	33	25	5.73
Pigeon pea	27	12	7	607	27	4	6	1.56

Table 7
dCAPS identified in *Vigna – Medicago* CISPs in pigeon pea, cowpea, and chickpea.

Sl. no.	Primer no.	Chro. no.	SNP	dCAPS primer	Enzyme	Recognition site
Pigeon pea						
1	VuMt-235	1	G/A	ACAATAATTTATGCATTAGCAACTA AACCCGTACACGCATCATTA	*Mae*I	CTAG
2	VuMt-350	2	G/T	GAAGGACCCAATAACGGTGTGGTCG AATCGTTGTGGCAAGACCA	*Hae*III	GGCC
3	VuMt-427	1	G/A	ATCTGCTTGTTGCATTAAATACTCA TGCCTGAAGAGCCTGAACTC	*BseM*II	CTCAG
4	VuMt-445	2	G/A	GAGACAAATTTCATTCAGAAACAGG ATGTTTGCTCAGGGGCTTC	*BamH*I	GGATCC
Cowpea						
1	VuMt-217	8	G/A	GCATTTATCATTGGATTAAAGAAAC GGCCATACCTAGAGACTTACGC	*Bcef*I	ACGGC
2	VuMt-230	7	T/G	GTTTGTAGTGCATCATCAAACATAG TGTGTGGAAAGGACACTGTTG	*Alu*I	AGCT
3	VuMt-339	1	C/T	GTAAAGCCAATAATATGAAACCTAG CAAACGAGCACTACGCAAAA	*Alu*I	AGCT
4	VuMt-339	1	T/G	CTCTTTTTTCCTGTTTTATTGTGAA GCACATCCATCTGTTTCAGC	*Mbo*II	GAAGA
5	VuMt-349	2	T/C	ATATATTTGTTTGTTTATTATATGA TAGAGGCAGGCCAGGTAATG	*Mbo*I	GATC
6	VuMt-359	3	A/T	ATTATGTTAATTTTTACCAATTTAT GGACCAGCAGAGGTTGATCT	*Mse*I	TTAA
7	VuMt-364	3	A/T	CGTGTTAAAGATGTAGATTCTAAGC CCCTTCATTGTGGACCAGTT	*Alu*I	AGCT
8	VuMt-364	3	G/A	CCTGCAATTACCTTTGCAAGTTGCTAGC CACACACGAACACATTTTGG	*Alu*I	AGCT
9	VuMt-426	8	T/G	AATTTTGAGGCTAATGAACTTTTT ATGACACCAAAGGTCGCTTC	*Taq*I	TCGA
10	VuMt-426	8	C/T	AACATAATAAATATGTTCAAAATAG TGCCAGAGTTTCTTGAGTCG	*Bts*I	GCAGTG
11	VuMt-458	3	G/A	AACGTGAGTTAAAGTTTTACCTTGA TGTGGAACTGGCTTTTCTGA	*Bce*83I	CTTGAG
12	VuMt-465	4	C/A	AAGAACTTCTGCATAGAAATTGTTG CATGTCTTCTGTCGTGGATTG	*CviR*I	TGCA
13	VuMt-465	4	C/T	AAGCAAAAAAAAATCAATTGAAACGT ACTGGTGGCAACACAAACAA	*Bsm*AI	GTCTC
14	VuMt-468	4	C/G	CACCTGTTGCCAAGAAAATTTGCTG TACGGAGGTTTGAGAATTGG	*CviR*I	TGCA
15	VuMt-464	4	C/T	TTATAATAAAATACCATATTTTCAG CAGGAGCACATTGATTTGG	*EcoP*15I	CAGCAG
16	VuMt-513	8	T/C	TGCTTGACAAAGAGCCGTTCATAAT GGAATTGCTCGCATAGATCC	*TspE*I	AATT
17	VuMt-514	8	C/T	CTTGAATGCAAAGTTCAAAGCCTTG GTTATTTCCCCACCCAGGTT	*CviR*I	TGCA
18	VuMt-514	8	A/G	CTTATCCAAGGTTAAATGTCAACTTA TCTCAAAAACTGCTGCTGCT	*Mse*I	TTAA
19	VuMt-477	5	A/T	TCAATTGATTGGTTCTATCCTTGCT AACACCACACCTTGGAAAGC	*Mae*I	CTAG
20	VuMt-477	5	T/A	CCGCCAAGGTACGCATTATTCA GAGCCCTTTTTAGGGTCAGC	*Sca*I	AGTACT
21	VuMt-478	5	A/G	ATATTTGGTAAATACACATGAAACT ATTGAGAAGGCCCCCACTAA	*Mae*I	CTAG
22	VuMt-488	6	T/G	CAATTATTTGTTATGCAGTCTTAAT AGCAATGCAGCCGTATTAAC	*TspE*I	AATT
23	VuMt-434	1	T/C	CCAGTATTACAATCTGACATCACTT GTGATTCGTGTTTTGGCTCA	*Mse*I	TTAA
24	VuMt-429	1	C/A	TTGCCCCTCTTTACACAAAAGAT ACTCCTGAGGTGATGGTGGA	*Mbo*I	GATC
25	VuMt-445	2	A/G	TGCATAAAAGATATCCTAATGTCAAAGGACTA GTTCCCTGGTCGTCAAAAGA	*Mse*I	TTAA
26	VuMt-451	3	T/C	TCCCTAATGCACTCCAATGGAATTTTAAGTAC GTCACCACGGAGGAAATCAT	*Sca*I	AGTACT
27	VuMt-455	3	G/A	TATGTGTGTATTTACATCACAGGAT GAAGCCAAGTGCAACAAATG	*Fok*I	GGATG
28	VuMt-491	6	G/A	TTTTTGGCTAATGAGAATTGACTCA CACAGGTCATGCATTGTGGT	*BseM*II	CTCAG
29	VuMt-493	6	T/G	GAGATGTTCCAATAGTCACACCGGA GGAATCAGACAGTATGTGCATC	*Fok*I	GGATG
30	VuMt-502	7	A/G	CAATTGCTCAAGAAGACGGAGGTTA AGATTTGCTGGGCAGGAGTT	*Mse*I	TTAA
31	VuMt-502	7	A/T	GAAAAATAAACAAACACCCG GGTGTGGCTGGCTTGATATAG	*Hinf*III	CGAAT
32	VuMt-512	8	A/G	GGTGTGCACATGTTTAATAGTGTTA ACAGCGGAATATTGATCAGG	*Mse*I	TTAA
33	VuMt-521	8	A/G	ATGTTTCTGCACTTATTCAACTTCG	*Taq*I	TCGA

Table 7 (*continued*)

Sl. no.	Primer no.	Chro. no.	SNP	dCAPS primer	Enzyme	Recognition site
				TTGTGCTTCAGATGGTGGAG		
Chickpea						
1	VuMt-233	4	C/A	AGCTTTTGCTTTCTTGCAGCTATTG GCAGAAGTTGGTGAATGCAG	*Cvi*RI	TGCA
2	VuMt-233	4	A/G	AGTTCCCTCTAGAAAAACTATGTTA CCATTCGGGATTTCTGTCAA	*Mse*I	TTAA
3	VuMt-216	8	G/C	CAATACGTTTGAGACCACCCTGCTA TCGTGCAGCCTGCTGTGTAT	*Mae*I	CTAG
4	VuMt-521	8	A/G	TTCAGAGGGAACCTATAAGTTGTCAACA GTTTTCAAGCCAAACAGACC	*Mse*I	TTAA

themselves. The limited observed synteny suggests substantial genomic rearrangements shortly following the early legume polyploidy [29]. Even macrosyntenic relationships between barrel medic and phaseoloid legumes were more complicated and less informative [30]. Chromosome number 6 of barrel medic yielded very few markers in our study consistent with it being reported to be highly heterochromatic in nature [31]. One might expect a higher number of markers using soybean genome in place of barrel medic, as it is genetically closely related to cowpea (Fig. 1), but the complete genome sequence of soybean was not available when this work was initiated. Moreover, the soybean genome is more complex, and the homologous regions resulting from genome duplication are abundant [32].

Out of 384 intron-flanking markers tried on 10 diverse legume taxa belonging to both temperate and tropical legumes, the successful amplification of single-copy loci ranged from 37.5% in peanut (Dalbergioids) to 67.2% in barrel medic [Hologalegina (IRLC) clade]. In the phaseolid clade, single-copy amplification rate ranged from 55.2% in pigeon pea to 47.4% in soybean. The amplification success rate was highest in source taxa, cowpea, and barrel medic, while the average success rate was approximately 45% across different clades, which suggests that these primer sets can potentially yield genomic tags for a majority of legume crops. In similar studies, successful cross-species genetic markers were developed from EST sequence information, and these putatively orthologous markers were mapped in barrel medic and a few other legumes [33,34,35]. The proportion of primer pairs showing multiple band amplification was higher in some members like field bean and soybean. This is probably because soybean genome underwent polyploidy approximately 13 Mya [35] and also soybean and other papilionoid legumes show evidence of an older and shared duplication estimated to have occurred approximately 59 Mya [36]. Most primers generated multiple bands in field bean (*Lablab purpureus* L.), as its genome has apparently accumulated a large number of duplications/deletions after it diverged [37]. Low single-copy PCR success rates observed for peanut (37.5%) might be attributed to its evolutionary distant relationships from the source taxa and the barrel medic, for example, among important legume crops, peanut clusters away from both the most populous papilionoid clade and dalbergioid clade. Further, the cultivated peanut is an allotetraploid ($2n = 4x = 40$), which is believed to have originated recently from a single hybridization event [38]. The sequence analysis presented in the current study supports the earlier conclusions that legume genomes are AT rich. The higher primer amplification rate despite the AT-rich genomes points to the efficiency of these intron-scanning primers in comparative genome analysis [39].

Out of 384 primer sets tested across legumes, 17.8% amplicons exhibited uniform size across the sampled taxa, and the nucleotide polymorphism rate in this set of primers was indistinguishable from that found among variable-sized introns. It has been proved that the first introns within most genes play a particularly important regulatory role that is most likely involved in transcription control [40]. The presence of regulatory elements such as promoters and enhancers and motifs found in plant cis-acting regulatory DNA elements in the introns has provided the possible explanation as to why some introns remain static in length.

Previous diversity studies with pigeon pea varieties using different marker systems reported average PIC values of 0.41–0.63 [41,42,43,44,45]. Diversity studies in cowpea varieties have been sparse, and most of the studies used RAPD markers. However, one study reported an average PIC value of 0.38 in their diversity study of 141 cowpea germplasm collection using 20 sets of SSR markers [46]. Even though genomic-simple sequence repeat (SSR) markers exhibit a higher level of polymorphism [47], intron-flanking markers developed in this study showed a comparatively high level of polymorphism, with PIC values ranging from 0.04 to 0.59. These markers have the potential to be used for comparison at the whole-genome level with the model crops, which will not only generate a valuable marker system but also be useful for the identification and selection of elite varieties for crop improvement in a cost-effective manner. Cluster analysis revealed narrow diversity in the material used with majority of the genotypes falling in the same group. Similar low genetic diversity values in the gene pool were reported in several earlier studies involving RFLP, AFLP, RAPD, SSR, and DArT markers [41,42,44,45,48,49,50,51,52,53]. The intron-flanking markers reported here open up new opportunities that can facilitate the use of candidate-gene mapping approach and linkage disequilibrium studies, thereby helping in the dissection of the complex traits.

Among various applications of the markers developed in our study, trait mapping is the most important to address issues related to both biotic and abiotic constraints in the production and crop improvement of economically important legumes. These primers have been successfully used in trait mapping of bacterial leaf blight, a devastating disease in cowpea production. A genetic map was constructed using 96 markers using 194 $F_{2:3}$ progenies derived from the cross between C-152 (susceptible) × V-16 (resistant) variety. The QTL analysis detected one major QTL on LG8 (*qtlblb-1*) and two on LG11 (*qtlblb-2* and *qtlblb-3*). The major QTL on LG8 was introgressed from cultivar V-

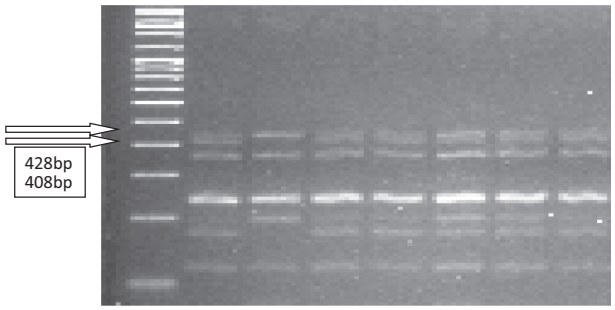

Fig. 4. Products of PCR performed with the dCAPS primer CcMt_056 on seven genotypes of pigeon pea and digested by the restriction endonuclease *Alu I* separated by gel electrophoresis.

Table 8
CAPS identified in *Vigna – Medicago* CISPs in pigeon pea, cowpea, and chickpea.

Sl. No.	Primer no.	SNP	SNP sequence	Enzyme	Recognition	RE cut
Pigeon pea						
1	VuMt_233	C/T	GTATTACCATGGCGT GTATTACTATGGCGT	*Nla*III	CATG	Wild Forward
2	VuMt_235	A/G	ATTAGCAATTAATAAT ATTAGCAATTAGTAAT	*Mse*I	TTAA	Wild Forward
3	VuMt_350	G/T	GGTCTGCCTTTTGA GGTCTTCCTTTTGA	*Mbo*II	GAAGA	Mutant Reverse
4	VuMt_350	A/C	AGGAAGAAAAAGTA AGGAAGCAAAAGTA	*Mbo*II	GAAGA	Wild Forward
5	VuMt_350	A/G	TAAAACATATCGTT TAAAACGTATCGTT	*Mae*II	ACGT	Mutant Forward
6	VuMt_427	C/T	CGTTATTGGCTAGTGGAAA CGTTATTGGTTAGTGGAAA	*Mae*I	CTAG	Wild Forward
7	VuMt_445	G/A	AAACATGGTCCAATG AAACATGATCCAATG	*Mbo*I	GATC	Mutant Forward
8	VuMt_445	G/A	AAAGGAGGACCTGC AAAGAAGGACCTGC	*Mn*lI	CCTC	Wild Reverse
9	VuMt_445	A/G	CCCAATAGCCACCC CCCAATGGCCACCC	*Hae*III	GGCC	Mutant Forward
10	VuMt_445	A/T	AAAAGCACCTCTCA AAAAGCTCCTCTCA	*Alu*I	AGCT	Mutant Forward
11	VuMt_504	G/A	GTACTTTGTCTCTT GTACTTTATCTCTT	*BsmA*I	GTCTC	Wild Forward
12	VuMt_508	C/A	CGAATACTTTTTGT CGAATAATTTTTGT	*Tsp*EI	AATT	Mutant Forward
Cowpea						
1	VuMt_217	G/A	AAAGAAAAGGCTTAATCA AAAGAAAAAGCTTAATCA	*Alu*I	AGCT	Mutant Forward
2	VuMt_230	T/A	CAGGACAGTGTTCCTGTAAGGAACACATT CAGGACAGTGTTCCAGTAAGGAACATATT	*Bsr*I	ACTGG	Mutant Reverse
3	VuMt_230	C/A	CAAACATTCCTCAACA CAAACATTCATCAACA	*Mn*lI	CCTC	Wild Forward
4	VuMt_351	A/T	AATTTAAAAACTACA AATTTAATAACTACA	*Aha*III	TTTAAA	Wild Forward
5	VuMt_359	T/A	AATTTAATAACTACA AATTTAAAAACTACA	*Aha*III	TTTAAA	Mutant Forward
6	VuMt_426	G/A	TTTGAAATATTTA TTTAAAATATTTA	*Mse*I	TTAA	Mutant Forward
7	VuMt_426	G/T	TTGACTTCGTAAAA TTGACTTCTTAAAA	*Mse*I	TTAA	Mutant Forward
8	VuMt_426	A/G	TTTAAAATATTTA TTTGAAATATTTA	*Mse*I	TTAA	Wild Forward
9	VuMt_455	A/G	TTCTCTTGCAAAACTGAGGTCATTA TTCTCTTGCAGAACTGAGGTCATTA	*BseM*II	CTCAG	mutant forward
10	VuMt_464	G/A	GAGTGATGCACACACGT GAGTGATGCACACACAT	*Mae*II	ACGT	Wild Forward
11	VuMt_464	C/A	GCTGCATCGCCTTGTCA GCTGCATAGCCTTGTCA	*Sfa*NI	GCATC	Wild Forward
12	VuMt_464	G/T	GTCACCCCGCACCGTC GTCACCCCTCACCGTC	*Aci*I	CCGC	Wild Forward
13	VuMt_464	C/G	TTTGCAGCAGTTCATAA TTTGCAGGAGTTCATAA	*Bbv*I	GCAGC	Wild Forward
14	VuMt_477	A/T	CAAGTCTCCTCCTCAA CATGTCTCCTCCTCAA	*Nla*III	CATG	Mutant Reverse
15	VuMt_478	G/A	TTTGACAAGTCTTCC TTTGACAAATCTTCC	*Bbv*II	GAAGAC	Wild Reverse
16	VuMt_488	T/G	AGTCTTATTTGCGGAA AGTCTTATTGGCGGAA	*Eci*I	GGCGGA	Mutant Forward
17	VuMt_491	C/G	TTCTGTTGATCCCAATGCCTATT TTCTGTTGATCCGAATGCCTATT	*Hinf*III	CGAAT	Mutant Forward
18	VuMt_512	A/G	TAGTGACAAAAGATGAA TAGTGACAGAAGATGAA	*Mbo*II	GAAGA	Mutant Forward
19	VuMt_513	T/C	TCTTTTGTCACTATT TCTTCTGTCACTATT	*Mbo*II	GAAGA	Mutant Reverse
20	VuMt_513	T/C	TTTCTTCGGACTTGG	*Mae*II	ACGT	Wild Forward
Chickpea						
1	VuMt_196	G/A	GTTAATGAGGATTAT GTTAATGAGAATTAT	*Tsp*EI	AATT	Mutant Forward
2	VuMt_216	C/G	TGAGACATTTTGT TGAGAGATTTTGT	*BsmA*I	GTCTC	Wild Reverse
3	VuMt_233	G/A	AAATCCCGAATGGA AAATCCCAAATGGA	*Hinf*III	CGAAT	Wild Forward

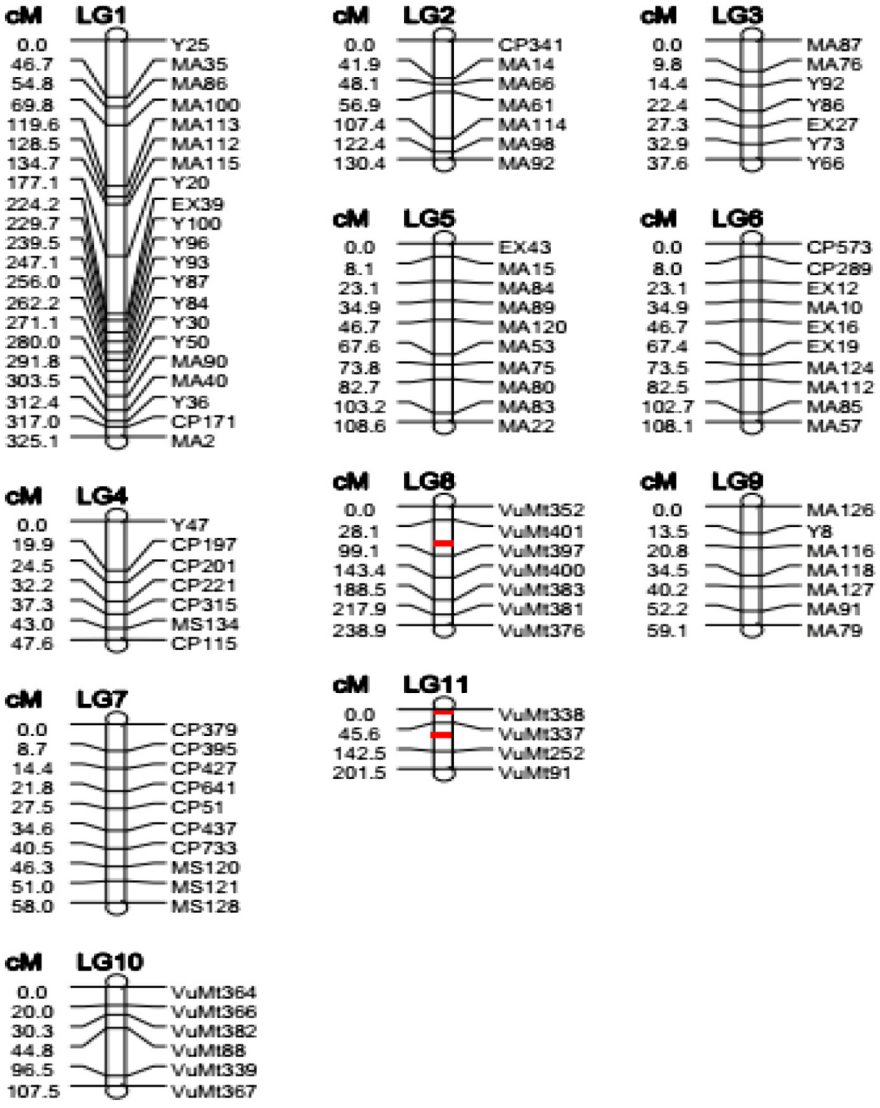

Fig. 5. SSR and CISP-based genetic linkage map of the F_2 mapping population derived from the cross C-152 × V-16 in cowpea with QTLs for BLB detected on LG8 and LG 11.

16 into C-152 through MABC [54]. Similar experiments can be explicitly carried out to obtain promising results.

The PCR products from different genotypes within a genus were orthologous, as primers were targeted to amplify conserved introns, which permitted us to align sequences for polymorphism detection. Intron-flanking primers are designed to amplify intronic regions, and hence, they should be more polymorphic than exonic regions, as introns have less evolutionary constraint. We demonstrated this in our study by studying length polymorphisms and also by detecting a relatively good number of SNPs and INDeLs in pigeon pea, chickpea, and cowpea. Both transitions and transversions were observed in equal proportions in cowpea. In pigeon pea, the proportion of transitions was more than transversions, whereas in chickpea, it was *vice versa*. We found on average 1.2 variations per unigenes in pigeon pea, 1.5 in chickpea, and 0.7 in cowpea. Genome sequencing efforts in pigeon pea [39] also revealed wide variations confirming results of this study.

Detection of restriction sites in the region of SNPs and conversion of SNPs/INDeLs into length polymorphism offers unique opportunities in the genotyping process, as it becomes simple gel-based approach. Among different techniques developed, derived cleaved amplified polymorphic sequence (dCAPS) analysis, which uses mismatches in one of the two PCR primers flanking the SNP to create or remove a restriction endonuclease recognition site in one of the two haplotypes being assayed, is widely used by the plant molecular genetics community, as it is gel-based procedure [55]. With the help of the web-based program dCAPS Finder 2.0 [55], we have successfully designed four dCAPS primers in pigeon pea, four in chickpea, and 33 in cowpea. Earlier, 13 dCAPS primers along with other co-dominant and dominant markers were successfully used in the construction of a genetic linkage map in interspecific F_2 population of *Lotus japonicas* [56]. Further, Sato et al. [57] reported a genetic map developed from 80 dCAPS markers, SSRs and SSLPs along with a combination of DNA sequencing, genomic library preparation, fluorescence *in situ* hybridization (FISH), and bioinformatics in characterizing the whole-genome structure of *L. japonicas*. The successful resolution of polymorphism in our study between parental lines in crops such as pigeon pea, cowpea, and chickpea, where absence of polymorphism is a constraint, reflects on the utility of dCAPS to improve the generation of high-density maps necessary for map-based cloning and integration of physical and genetic maps [58].

More than 23% of primer sets generated length polymorphisms between parental lines used for developing mapping population in cowpea. These primers along with the existing ones will be used for

enrichment of the genetic map, which will serve as valuable genomic resources for orphan legumes. To support this, these kinds of primers have already been mapped as intron size length polymorphisms in many crops [33,34,59].

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Supplementary material

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