Genetic and QTL Analysis for Hilum-Eye Types in Cowpea (*Vigna Unguiculata* L. Walp)

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Abstract

Cowpea is an important food legume widely grown in the semi-arid tropics and serves as a main source of dietary protein, minerals, and vitamins. However, varieties differ from region to region based on the consumer's preference for seed types determined by seed size, seed coat texture, seed color, and hilum-eye types. The genetics of seed size, seed color, and seed coat texture have been well documented, but the hilum-eye types have not been studied well because they represent seven different types with complex interactions. We studied the genetic segregation for hilum-eye types and determined the number of genes involved in a recombinant inbred line (RIL) population derived from a cross between a small eye parent 'GEC' and a Watson eye parent 'IT98K-476-8'. The results demonstrated a three-gene model, W (Watson), S (small), and R (large), for cowpea seed hilum-eye type pattern and the interaction of these three genes, W, S, and R, resulted in five phenotypes, viz. self, Watson, small, large, and ring hilum-eye types. Moreover, we also mapped the RILs for hilum-eye types, identified three quantitative trait loci (QTLs), and aligned to the cowpea reference genome as QTL *qHilum7.1, qHilum9.1, and qHilum10.1*, corresponding to these three genes, Ring type (R), Watson type (W), and Small type (S) hilum-eye type patterns, respectively. Therefore, there was a complete agreement between the genetic analysis and QTL mapping for the number of genes controlling the hilum types in cowpea.

Introduction

Cowpea [*Vigna unguiculata* (L.) *Walp*.] is an important food legume cultivated in over 65 countries in the semi-arid regions of the tropics and sub-tropics (Singh 2014, 2016). It has a diverse growth habit, plant type, pod type, seed type, and maturity date. Therefore, cowpea is grown and consumed in a variety of ways, based on the local preference, especially for seed types. Consumers make decisions on the acceptability, quality, and presumed taste of cowpea, depending on its seed color, hilum type, and texture, which vary across and within markets based on different uses (Singh 2014). For example, in West Africa preferred varieties are with large white and brown seeds and a rough seed coat. In contrast, countries in Central America and the Caribbean prefer varieties with red, black, or white seeds and a smooth coat. The preference in Mexico, Guatemala, Nicaragua, Costa Rica, and Cuba is for black seeds, while in Honduras, El Salvador, Venezuela, and Jamaica it is for red. In East Africa and Asia, any color other than black is acceptable. Tan and red are preferred in East Africa and white and cream in Asia. In the United States, tight back eyes, popularly known as "black eyed peas", is preferred (Fery 1985). Nutritional value of cowpea seeds also varies by seed color. Brown and black color seeds are higher in proximate, vitamins, and minerals compared to white seeds (Alfa et al. 2020).

The diverse hilum-eye type patterns in cowpea is result of a combination of different seed colors and hilum-eye type genes (Fery and Singh 1997; Singh and Ishiyaku 2000; Singh 2002, 2014). Within each seed color group (normally black, brown, tan, cream, white, and green), the hilum-eye may be colorless (eyeless), have a narrow band of color around hilum (ring eye type), small eye pattern color (small eye), small eye color pattern but diffused edge on one side (Watson eye), large eye color pattern (large eye), and very large eye color pattern covering the entire seed surface (self-colored). These hilum-eye types within the brown color group are shown in Fig.1. Most of the genetic studies on cowpea seed color and hilum-eye types have been conducted on individual types because the parental varieties have one or the other type of hilum-eye (Spillman 1911; Harland 1919; Fery and Singh 1997; Singh and Ishiyaku 2000; Singh 2002). Spillman (1911) and Harland (1919) studied inheritance of cowpea seed color and reported three major genetic factors for cowpea seed color. Spillman and Sando (1930) reported a three-locus model for cowpea seed color and it was later confirmed by Saunders (1960). In modern studies, Herniter et al. (2019) reported a detailed study on seed coat color pattern in cowpea. They demonstrated different seed patterns and inheritance of seed pattern, and proposed genes controlling seed coat color pattern. They identified three major genes, with each on Vu07, Vu09, and Vu10. They used a binary phenotyping method. Recently, Lonardi et al. (2019) sequenced the cowpea acc. IT97K-499-35 genome as its reference genome and re-sequenced six additional cowpea diverse accessions. Genes on the reference cowpea genome were identified and are available with physical distances. The genomic resources facilitate gene mining within a specified region of the reference genome.

In our study, these resources were used to physically locate the mapped QTLs in the cowpea genome and explored potential candidate genes for the hilum-eye type trait.

Materials And Methods

Mapping population

A population consisting of 164 Recombinant Inbred Lines (RILs) at F_7 was used for this study. The RIL population was developed from a cross between two varieties, 'Golden Eye Cream (GEC)' that is heat tolerant with small eye and brown color and 'IT98K-476-8' that is heat susceptible with Watson eye and brown color (Fig.1) primarily for QTL mapping of the genes for heat tolerance in cowpea. However, during development of the RIL population from F_1 onwards, we noticed complementary gene action in F_1 showing self-colored hilum type and a transgressive segregation from F_2 onwards for ring type, small eye, large eye, Watson eye, and self-colored hilum-eye types. The population was advanced to F_7 by the single seed descent method and used to elucidate the genetics as well as QTL mapping of different hilum-eye types in cowpea.

Phenotypic data collection

The RILs were planted in multi-row replicated trials on July 16, 2014 at an agronomy farm, College Station, Texas and on June 3, 2015 at an agronomy farm, Corpus Christi, Texas. Three representative plants from each RIL were hand harvested and visually classified in five hilum-eye types - Ring (1), Small (2), Watson (3), Large (4), and Self (5) as shown in Fig.1. Since both parents had brown color eyes, all seeds had brown color eye and no segregation for seed color was observed. The hilum-eye type score was assigned based on the area of brown color noticed around the hilum of the seeds. RILs that were assigned "5" had the highest area of brown color coverage (Self-type: whole seed was brown) and RILs that were assigned "1" had the lowest area of brown color (Ring-type: only a brown ring around the seed eye was observed) on the seeds. The remaining RILs were assigned to "2", "3", or "4" that have different areas and patterns of brown color around the seed eye.

QTL mapping

A cowpea SNP linkage map had previously been constructed with the IciMapping software V4.1 using the RILs used for this study and was used for QTL mapping of cowpea flowering data and heat tolerance (Angira et al. 2020). This SNP linkage map was used to map the QTLs for hilum-eye types. Briefly, extracted DNA of RILs and parents was double-digested with *Bam*H I and *Mlu*C I, constructed into ddRAD-seq (double digested restriction site-associated DNA sequencing) libraries, barcoded and sequenced to 6.1x – 33.6x. SNPs genotypic data were called using the STACKs software (Catchen et al. 2011). The SNPs with a minor allele frequency of <0.05, a missing data rate of >0.20, a nucleotide calling quality of <Q30, and a significant genetic segregation distortion were filtered. After these filtering criteria, 4,154 quality SNPs remained for the SNP linkage map construction. The map was constructed using the IciMapping software (Meng et al. 2015). The map consisted of 531 bins containing 4,154 SNPs grouped into 11 linkage groups and spanned over 1,084.7 cM, thus having a density of one SNP in 0.26 cM or 149 kb. Each bin had an average of 7.8 SNPs that were co-segregating in the population. All the 11 linkage groups of the map were aligned to the 11 chromosomal pseudomolecules of the cowpea reference genome (Lonardi et al. 2019) using two or more single-copy sequence SNPs selected from each linkage group of the map. In this study, the IciMapping software V4.1 (released January 2016) was also used to conduct inclusive composite interval mapping (ICIM-ADD) for hilum-eye types, as described by Meng et al. (2015). QTL mapping parameters, Step size = 1 cM and PIN = 0.001, were used for the mapping. Logarithm of the odds (LOD) scores for the threshold level of a QTL was generated using 1,000 permutations and 0.05 Type I error. Only the QTLs mapped with a higher LOD score than the threshold LOD score were reported.

Gene mining

The sequence of SNP markers (Supp. Table S1) flanking the mapped QTLs were searched using BLAST (Basic Local Alignment Search Tool) to locate their physical positions on the cowpea reference IT97K-499-35 genome (Lonardi et al. 2019; phytozome.jgi.doe.gov/pz/portal.html). The genes in the mapped QTLs were identified using the Annotation *Vigna unguiculata* v1.2 version and the tools available at https://mines.legumeinfo.org/cowpeamine/begin.do (Lonardi et al. 2019) (Supp. Table S2, S3, and S4).

Results

Phenotypic data genetic analysis

The hilum-eye types in parents, F_1, and RILs are presented in Fig.1 and the segregation data in different classes in Table 1. As evident, the hilum-eye type of seeds in GEC was 'Small eye'; in IT98K-476-8 was 'Watson eye type'; and in F_1 was self-colored, indicating a complementary gene action. The segregation ratio of the seed hilum-eye type in the RILs was 23 Self: 52 Watson: 27 Small: 44 Ring: 24 Large hilum-eye type and all had brown color as expected since both parents had brown color. The data fitted well to a three gene model with two alleles (W-w, R-r, and S-s) giving different phenotypes based on their interactions. Thus, all the plants with dominant homozygotes at all three loci (*WWSSRR*) were Self hilum-eye type; the genotypes *WWSSrr, WWssRR, and WWssrr* exhibited Watson hilum-eye type; the genotypes *wwSSRR* exhibited Small hilum-eye type; the genotype *wwssRR* exhibited Large hilum-eye type, and the genotype *wwssrr* exhibited Ring hilum-eye type (Table 1). All the three genes were inherited independently, and the observed data fitted well to the expected segregation as confirmed by the Chi-square test. The total calculated Chi-square value of 3.381 is less than Chi-square table value of 9.49 for 4 degrees of freedom at 0.05 alpha level (95% probability), thus confirming the three gene segregation model (Table 1).

QTL mapping

We mapped QTLs for the hilum-eye type of seeds. We identified three major QTLs for hilum-eye phenotype (Fig.2, Table 2), defined *qHilum7.1*, *qHilum9.1*,and *qHilum10.1*. SNP markers, SNP6683 and SNP32139, with a LOD of 37.4, flanked *qHilum7.1* and its LOD score peak was positioned at the 57 cM position, within the physical region of 18.9 to 20.2 Mbp on Chr7. This QTL explained the highest phenotypic variation (38.43%) of the cowpea seed hilum-eye type. Parent GEC, which had Small-type hilum-eye type, donated the alleles for hilum-eye color in this QTL. *qHilum9.1* was mapped at the 52 cM position, within the physical region of 30.5 to 30.8 Mbp on Chr9 and flanked by SNP3899 and SNP26097, with a LOD score of 18.1. This QTL explained 13.6% of the seed hilum-eye type phenotype. Parent IT98K-476-8 contributed the alleles for hilum-eye color for this QTL (Table 2). *qHilum10.1* was mapped at the 5 cM position, within the physical region of 38.4 to 38.9 Mbp on Chr10, with a LOD score of 28.5. It was flanked by SNP32261 and SNP17347 and explained 25.2% of the seed hilum-eye type phenotype. Parent IT98K-476-8, with Watson-type hilum-eye color, contributed the alleles of this QTL.

Determination of the seed hilum-eye type genes and their corresponding QTLs

Both genetic analysis and QTL mapping consistently showed that three major genes: *R*, *W*, and *S*, or three QTLs: *qHilum 7.1*, *qHilum 9.1*, and *qHilum 10.*1, controlled the seed hilum-eye type variation in the RIL mapping population. Nevertheless, what was the relationship between the three genes and three QTLs? To determine their correspondence, we extracted the genotypes of the SNPs immediately flanking each QTL and their corresponding seed hilum-eye type phenotypes, and conducted association analysis between the QTL flanking SNPs and seed hilum-eye types by Chi-square Test. We found that both SNP 6683 and SNP 32139 flanking *qHilum 7.1* were associated with the Ring hilum-eye type seeds ($P < 0.0001$), while none of the SNPs flanking either *qHilum 9.1* or *qHilum 10.1* was associated with the Ring hilum-eye type seeds ($P > 0.05$). Similarly, both SNP 3899 and SNP 26097 were associated with the Watson hilum-eye type seeds ($P < 0.0001$), and both SNP 32261 and SNP 17347 were associated with the Small hilum-eye type seeds ($P < 0.0001$). These results indicated that *qHilum7.1* corresponded to the *R* gene; *qHilum9.1* corresponded to the *W* gene; and *qHilum10.1* corresponded to the *S* gene (Table 2).

Gene mining

Physical location of the SNP markers flanking the mapped three QTLs was identified using BLAST tool available at phytozome.jgi.doe.gov/pz/portal.html using IT98K-499-35 reference genome (Supp. Table S1). Genes within the mapped QTLs *qHilum7.1* (Supp. Table S2), *qHilum9.1* (Supp. Table S3),and *qHilum10.1* (Supp. Table S4) were mined using tools available at https://mines.legumeinfo.org/cowpeamine/begin.do (Lonardi et al. 2019). Number of genes discovered under QTL *qHilum7.1*, *qHilum9.1*, *qHilum10.1* were 53, 23, and 43, respectively. QTL *qHilum10.1* harbors eight genes, close to SNP32261, produce iron binding protein and two other genes in the QTL – Vigun10g165300 and Vigun10g165400, are myb transcription factors (chromatin binding protein) may play role in iron deficiency resistant (Supp. Table S2, S3, and S4).

Discussion

This study successfully elucidated three major genes controlling different seed hilum-eye types in cowpea and their three corresponding QTLs. The three genes were designated as *W* (*Watson hilum-eye type*), *S* (*Small hilum-eye type*), and *R* (*Ring hilum-eye type*) and shown to have complementary and epistatic gene action, resulting in five different seed hilum-eye types:- i) Self-color hilum-eye type seeds, when all three genes were present together in dominant form indicating a complementary gene action; ii) Watson hilum-eye type seeds, when the *W* gene was dominant and one of the other two genes were either homozygous dominant or homozygous recessive, showing dominant epistasis of Watson hilum-eye type over the *S* and *R* genes; iii) in the absence of the *W* allele, the combination of the *S* and *R* dominant alleles resulted in Small hilum-eye type seeds; iv) in the absence of the *W* and *S* allele, the presence of the *R* dominant allele resulted in Large hilum-eye type seeds; and v) in the absence of the *W* allele, the presence of the homozygous recessive *r* allele gave rise to Ring hilum-eye type seeds.

This three-gene model fits perfectly with the observed ratios in this study. This is because both parents had brown color and different hilum-eye types, genetic segregation in the bi-parental population was only for hilum-eye types. In the previous studies, the parents had different seed colors as well as different hilum-eye types; therefore, genetic segregation was more complex. For example, Spillman (1911) studied different hilum-eye types and seed color patterns, and proposed three genetic factors that control seed color pattern and two factors control seed hilum-eye types, in case of Small hilum-eye type and self-color type, and these factors were independent of each other. He also proposed that there could be another factor denoted by “I” in case of Holstein and Small hilum-eye type. Harland (1919) reported three genetic factors for seed color and hilum-eye types. Similarly, Saunders (1960) and Drabo et al. (1988) reported that seed-coat patterns, Watson, Holstein, and Ring hilum-eye type were controlled by interactions between at least three genes. Herniter et al. (2019) reported three major genes for seed-coat color, but not seed hilum-eye types. Also, the material used by them was different and they used a binary phenotyping method (presence or absence of seed color). Our phenotyping method was rating the seed hilum-eye type, based on the amount of brown color visually observed in the seeds of the RIL population.

Declarations

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Conflicts of interest/Competing interests

The authors declare that they have no conflict of interest

Availability of data and material

Data included with the manuscript

Code availability – Not applicable

Authors' contributions – Dr. Brijesh Angira conducted field trials, collected data, and mapped QTLs; Dr. Yang Zhang called SNP calls from GBS data and constructed the genetic linkage map; Dr. Hong-Bin Zhang and Dr. Meiping Zhang helped and guided in marker data analysis and integration between the genetic analysis and the QTL mapping results; Dr. B.B. Singh helped in phenotyping and proposed the three gene model for cowpea seed hilum-eye type; and Dr. Dirk Hays guided and monitored the progress of the project and helped in managing field and greenhouse trials.

Ethics approval – Not applicable

Consent to participate – Not applicable

Consent for publication – Not applicable

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Tables

Table 1. Genetic segregation for hilum-eye types in a recombinant inbred line mapping population (*n* = 164) developed from a cross between GEC (Golden Eye Cream) and IT98K-476-8, both the parents had brown color on seeds.

Genotype RILs	Phenotype RILs	Observed	Expected 3 gene pairs	χ^2
WWSSRR	Self	23	21.25	0.14
WWSSrr	Watson	51	63.75	2.55
WWssRR	Watson			
WWssrr	Watson			
wwSSRR	Small	25	21.25	0.66
wwSSrr	Ring	43	42.50	0.005
wwssrr	Ring			
wwssRR	Large	22	21.25	0.026
Total		164		

GEC = wwSSRR (small); F1 = WwSsRr (self-type); IT98K- 476 = WWssrr (Watson)

Table 2. Cowpea hilum-eye color quantitative trait loci mapping in a recombinant inbred line mapping population. The recombinant inbred line population was developed from a cross between GEC (Golden Eye Cream) and IT98K-476-8.

Locus	Gene name	*P*-value	Chromosome	Position	LeftMarker	RightMarker	LOD	PVE (%)	Donor
qHilum7.1	*Ring (R)*	< 0.0001	7	57	6683	32139	37.35	38.43	GEC
qHilum9.1	*Watson (W)*	< 0.0001	9	52	3899	26097	18.07	13.61	IT98K-476-8
qHilum10.1	Small (S)	< 0.0001	10	5	32261	17347	28.51	25.20	IT98K-476-8

Figures

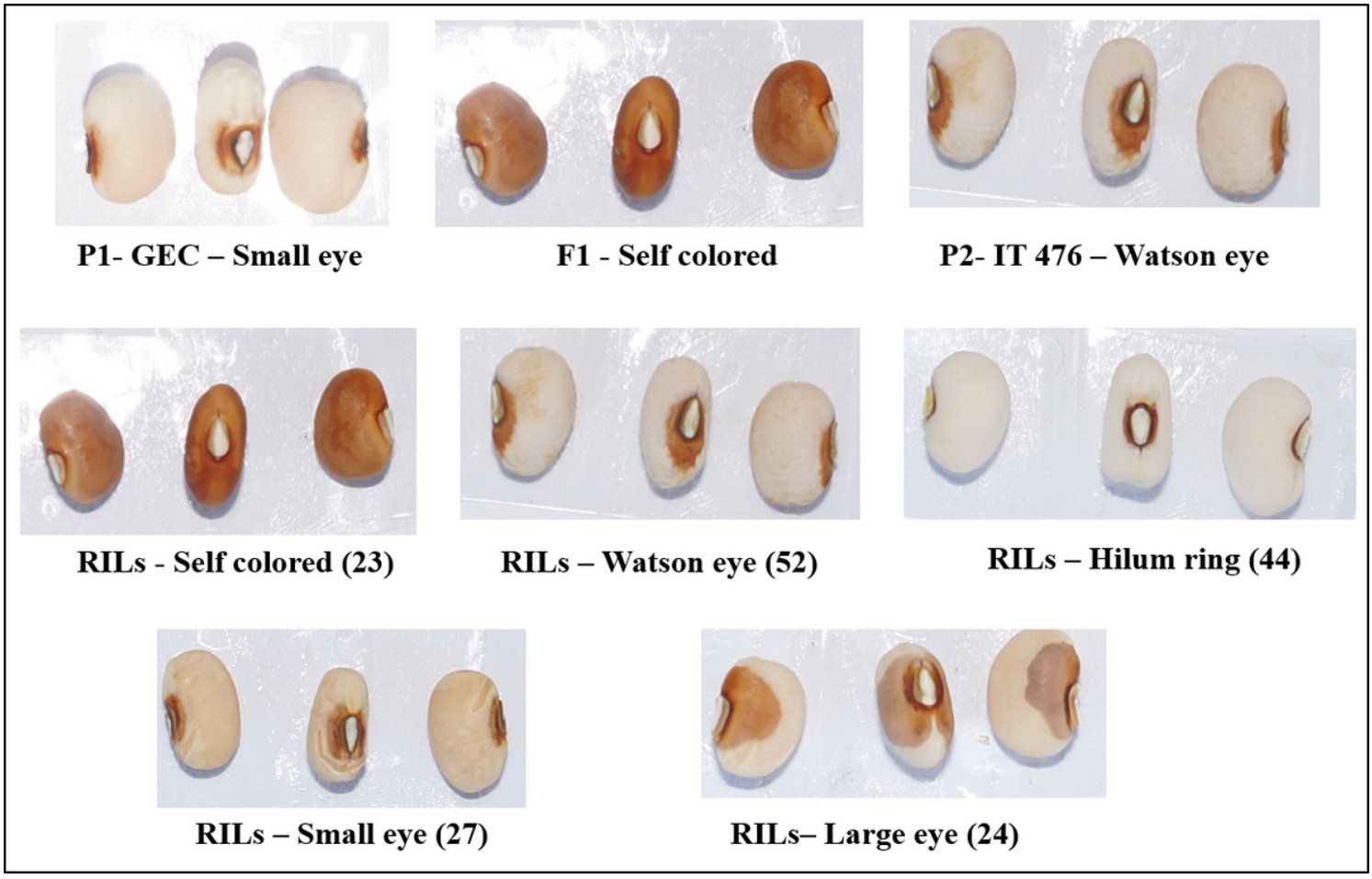

Figure 1

Segregation for hilum-eye types in the RIL population developed from a cross between GEC (Golden Eye Cream) and IT98K-476-8. Five types of seed hilum-eye phenotypes were observed in the RILs: Ring, Small, Watson, Large, and Self color. F2 seeds produced on F1 plants were all Self type because it is a maternal trait.

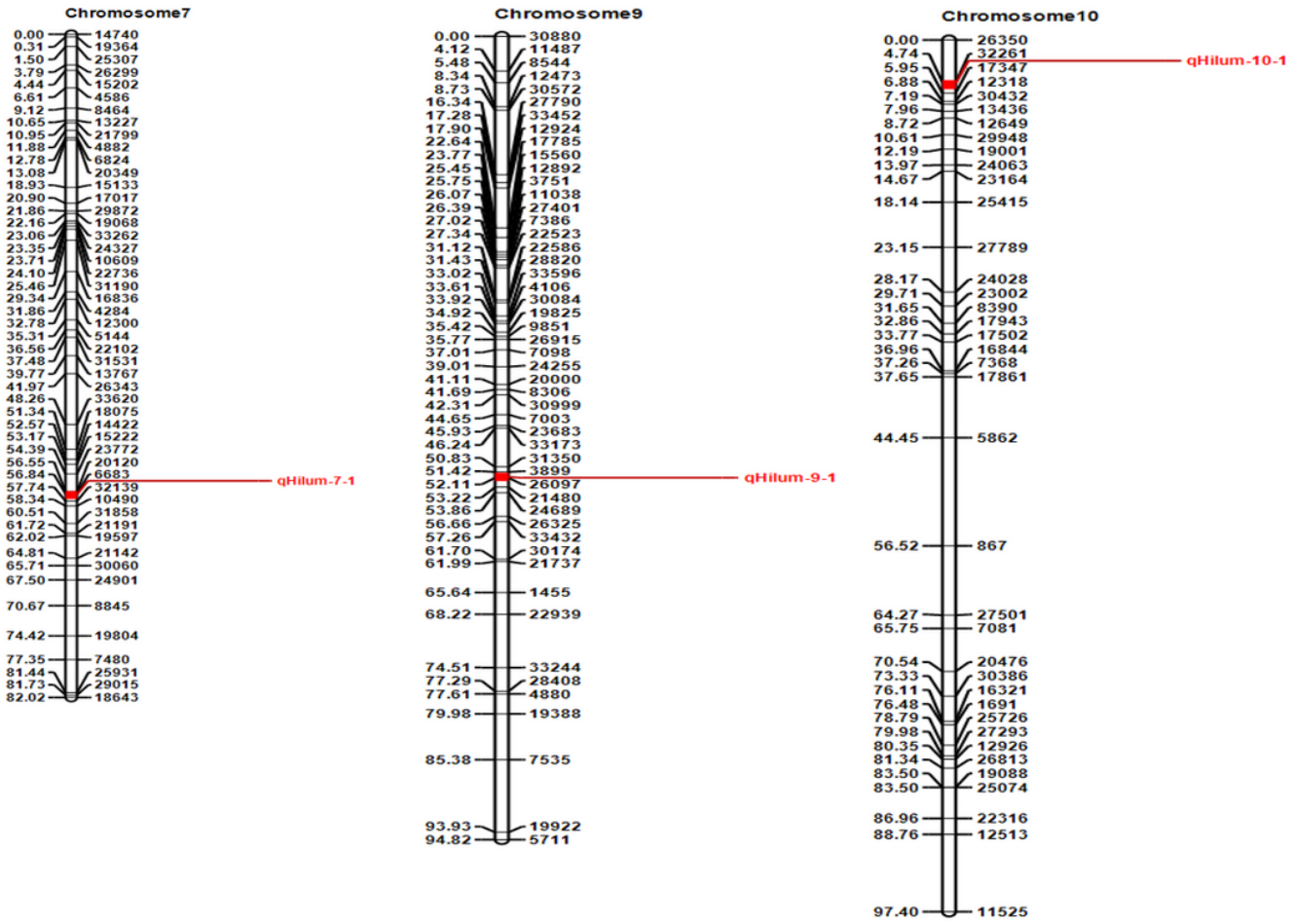

Figure 2

QTL mapping for hilum-eye types using the cowpea RIL mapping population. The RILs were developed from a cross between GEC (Golden Eye Cream) and IT98K-476-8. Three major QTLs, qHilum 7.1, qHilum 9.1, and qHilum 10.1, were detected for the seed hilum-eye types on Chromosomes 7, 9, and 10, respectively. These three QTLs together explained about 77% of the seed hilum-eye phenotypic variation.

Supplementary Files

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- Supptablemarkerseqgenemining.xlsx