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Mapping of genomic regions associated with dwarfing and the determinate growth habit in horsegram (*Macrotyloma uniflorum*)

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Abstract: Horsegram (*Macrotyloma uniflorum*) – an important, self-pollinated food legume, however due to limited genomic and genetic resources the genetic improvement could not be achieved as compare to other major legumes. Our work aims at finding novel microsatellite markers and their use for the construction of a linkage map from 157 individuals of F_9 recombinant inbred lines (RILs) of horsegram. The determinate growth habit and plant height are important traits for its suitability for different cropping systems. The genotypic data were generated by screening 2 395 molecular markers, of which 600 (25.05 %) polymorphic markers were selected. Two-hundred eighty-seven markers were mapped on ten linkage groups (LGs) at a log of odds (LOD) of 3.5 straddling 796.76 cM with 2.78 cM of marker density. For the identification of the quantitative trait loci (QTLs), the phenotypic data recorded on the RILs for the plant height and growth habit were analysed using the statistical tools JoinMap® and Windows QTL cartographer, based on the composite interval mapping (CIM) technique. Across the ten linkage groups, we detected four QTLs (LOD ≥ 2.5) for four traits. All the traits were major QTLs as indicated by the percentage of phenotypic variance (PVE) (≥ 10%) that ranged from 13.5% to 40.3%, therefore, this is very important information which can be used in marker-assisted selection (MAS). The present genomic information generated in this orphan crop, thus, provides the base for genetic improvements by devising molecular breeding strategies.

Keywords: determinate growth habit; dwarfing; horsegram; linkage map; *Macrotyloma uniflorum*

Horsegram, *Macrotyloma uniflorum* (Lam.) Verdc, ($2n$ = 20) earlier known as *Dolichos bioflorus*, is an underutilised pulse crop that can be grown extensively after some architectural modification. It is an arid food legume that grows in a variety of climates across the Indian sub-continent, from hot and tropical climates to moist temperate regions (Reddy et al. 2005). The benefits of this crop centre around its nutritive and medicinal properties as its seeds are used to cure kidney stones, to treat calculus afflictions, corpulence, hiccups and worms in traditional Indian medicine (Chunekar et al. 1998; Neelam 2007; Perumal & Sellamuthu 2007; Ravishankar & Vishnu Priya 2012). Being a drought resistant crop, many breeders have targeted at improving the crop traits by improving the unfavourable attributes, *viz.*,

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twisting growth practice, longer and asynchronous maturity, light-sensitive and indeterminate growth pattern using conventional breeding techniques. The difficulty in starting a successful breeding programme for modification of architectural traits is due to the lack of these traits in the Indian germplasm (Chahota et al. 2005). The long thin stem generally twinning around any support and an indeterminate growth habit (flowering progressed from the base to tip of the plant with continuous growth of the apical and auxiliary buds) accompanied by asynchronous maturity are the other major constraints in this crop. The mutation breeding approach using gamma radiation was employed to develop semi-dwarf, dwarf and determinate genotypes of horsegram having synchronous maturity (Chahota et al. 2013). Due to the immense significance of these traits, the present study is focused on providing insight into the genetic control of these traits, which will help in molecular breeding over the longer term (linkage study, QTL analysis, mapping studies, etc). Thus, the present study was conducted on the mining and validation of new microsatellite markers from genomic sequences and utilised them along with validated markers for mapping the genomic regions associated with dwarfing and the determinate growth habit traits. The information generated will not only helpful to understand the genetic make-up of these traits, but will also help us to identify the genomic region responsible for the expression of these traits, which can be used in marker assisted breeding programmes of this crop.

MATERIAL AND METHODS

Mining of SSRs and primer designing. The sequencing data were retrieved from the database of the Kazusa DNA Research Institute (KDRI), Chiba, Japan for the mining of novel simple sequence repeat (SSR) markers. From the Illumina *hiseq* sequencing data, full-fledged paired ends having 375 million sequences of the "HPK4" genotype were assembled into a total of 186 445 scaffolds, which were further used to detect and develop the genomic SSRs. The obtained DNA sequences (FASTA Format) were further explored for identification of the simple sequence repeats using the online software SSRIT (Simple Sequence Repeat Identification Tool), http://www.gramene.org/db/markers/ssrtool. Primer pairs were designed by using the Batch Primer3 software (http://probes.pw.usda.gov/cgi-bin/batchprimer3/batchprimer3.cgi) on the sequences having an SSR and further oligos were synthesised from Sigma Aldrich Chemicals Pvt. Ltd and Eurofins Genomics India Pvt. Ltd. A total of 1 600 SSR motif repeats were identified in the sequences from the genomic data, out of which, 384 newly designed primers were validated on 157 recombinant inbred lines (RILs) after testing them on two parents (HPKM249 and HPK4) for amplification and polymorphism.

Plant materials. The RILs of the F_9 generation consisting of 157 genotypes resulted from the intraspecific cross of HPKM249 and HPK4, which were utilised for the construction of the genetic linkage map (Table 1). After the hybridisation generations were advanced by following the single seed descent (SSD) method up to F_7. Thereafter, the RIL population was maintained in the Department of Agricultural Biotechnology CSK HPKV, Palampur, Himachal Pradesh, India situated at 1 290 m a.s.l. with latitude and longitude of 32.11°N and 76.53°E, respectively.

Table 1. Morphological variations in the parents

S. No.	Trait	HPKM249 (P_1)	HPK4 (P_2)
1	growth	bush type	twining
2	flowering time (days)	30–40	55–65
3	growth habit	determinate	indeterminate
4	maturity (days)	80–85	110–115
5	photosensitivity	photoinsensitive	photosensitive
6	plant height (cm)	45.0	110.0
7	maturity	synchronous	asynchronous
8	seed characteristics	medium seed size	bold seed size
10	drought stress	susceptible	tolerant
12	stem pigmentation	absent (green)	purple
13	number of pods/plant	< 15	> 35

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Genotyping studies. The genomic DNA was isolated from the plant leaves (weighing 1 g) from all 157 RILs along with the parents (HPK4 and HPKM249) using the modified cetyltrimethylammonium bromide (CTAB) method (Murray & Thompson 1980). Electrophoresis was performed on an agarose gel to check the DNA quality, whereas the DNA quantification was carried out on a micro-volume spectrophotometer (Biospec-nano, Shimadzu Biotech, Japan). The polymorphic markers identified on the parental lines were utilised to generate the genotypic data on the 157 RILs mapping population. The newly designed 384 primers were screened on the parental lines, of which, 106 (27.60%) were found to be polymorphic (Table 2). For the amplification of the genomic DNA, polymerase chain reaction (PCR) plates were occupied with the reaction mixture (10 µL) containing 10× buffer (10 mM of Tris-HCl, 50 mM of KCl, pH 8.3), 25 mM of $MgCl_2$, 10 mM of dNTPs, 0.5 mM of each primer, 5U *Taq* of the DNA polymerase along with the DNA template (13 ng/µL). The amplification profile consisted of 1 cycle at 94 °C/5 min; 35 cycles at 94 °C/1 min, 40 to 60 °C/1 min and 72 °C/1 min; 1 cycle at 72 °C/7 min; hold at 4 °C/∞ in a Veriti 384® (Applied Biosystems, USA) and a 2720 Thermal Cycler (Applied Biosystems, USA) The PCR products were separated on agarose gel (4%) and visualisation was performed on a Gel-Documentation Unit (ENDURO™ GDS Gel Documentation System, USA). A 100-bp DNA ladder was used to measure the size of the alleles (Fermentas, ThermoFisher Scientific, Lithuania).

Phenotyping studies. A population of 157 RILs along with the parents was evaluated under controlled conditions for the plant height and determinate growth habits. For phenotypic data lines were evaluated with two replications in an environment under the summer season of the North-western Himalayan region. Plants were grown in 12-inch pots under polyhouse conditions by following all the packages and practice for cultivation. Phenotyping was undertaken for various morphological traits, *viz*., plant height (PH), growth habit (GHT), the phenological traits (days to flowering) and yield related traits, seed index/100-seed weight (SI, g) and seed yield per plant (SY, g) by following standard procedures to record data on these traits.

Linkage map and QTL analysis. The amplified PCR products were visualised as bands on a Gel-documentation system and scored manually and coded as 'A' for genotypes following the banding pattern of parent HPKM249, 'B' for the HPK4 type banding pattern and H for the heterozygous one, if any. The JoinMap® 4.1 (van Ooijen 2006) application was used to construct the genetic linkage map by utilising a scored genotypic data file. The grouping of the markers was undertaken using a minimal independence log of odds (LODs) threshold of 3.0 and a maximum of 8.0 with a step up of 0.5 to find the linkage groups. The quantitative trait loci (QTL) analysis was carried out with the phenotypic data on the 157 F_9 individuals for the plant height, type of growth habit and the genotypic data, which consisted of 600 mapped markers in ten linkage groups of horsegram. QTL Cartographer V2.5 was further employed to detect the QTLs using an analysed linkage map and the phenotypic data of the plant height and growth habit. A statistical approach involving composite interval mapping (CIM) was used to calculate the QTL frequency using Zmapqtl standard model 6 with a window size of 10 cM along with a walking speed of 2 cM (Zeng 1993, 1994; Wang et al. 2012). Cofactors were found using the forward regression algorithm. To map the QTLs on the horsegram linkage groups, a LOD threshold score of ≥ 2.5 with 1 000 permutations were considered significant (5% level of significance). One LOD interval surrounding the QTL peak designate the QTL locations at 95% of the composite interval (CI) (Mangin et al. 1994;

Table 2. List of newly designed SSRs and % polymorphism

S. No.	Name of primers	Total No.	Polymorphic markers	% Polymorphism
1	MUGR 3001 to MUGR 3096	96	11	11.45
2	MUGR 3401 to MUGR 3448	48	17	35.41
3	MUGR 3501 to MUGR 3596	96	47	48.96
4	MUGR 4001 to MUGR 4048	48	14	29.16
5	MUGR 4101 to MUGR 4148	48	9	18.75
6	MUGR 4501 to MUGR 4548	48	8	16.66
Total		384	106	27.60

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Doerge & Churchill 1996). The R^2 value from this analysis was accepted as the percentage of phenotypic variance (PVE) explained by each locus.

RESULTS

Mining and validation of new microsatellites. The Illumina *hiseq* sequencing data of 5 754 sequences were used for the identification of the SSRs. Of the 1 600 potential SSR motifs identified by SSRIT, 384 markers were designed using the Primer3 software and were validated on the mapping population. All the newly designed markers are of di-repeat motifs and, of these, 106 markers were found to be polymorphic on the parental lines (SA1).

Intraspecific linkage map construction. A genetic linkage map was constructed from the genotyping data employing 600 polymorphic markers using JoinMap software (Ver. 4.0). A total of 287 SSR markers were assigned positions on ten linkage groups (LGs) at an LOD of 3.5 (Figure 1) based on the number of chromosomes of *Macrotyloma uniflorum* ($2n = 20$, $n = 10$). The generated linkage map of horsegram using these markers spanned a 796.76 cM distance with a 2.78 cM marker density (Figure 2). The maximum number of markers were plotted on LG1, which harboured 141 markers with the average marker density of 1.23 cM and the minimum markers were plotted on LG7, which harboured only three markers with an average marker

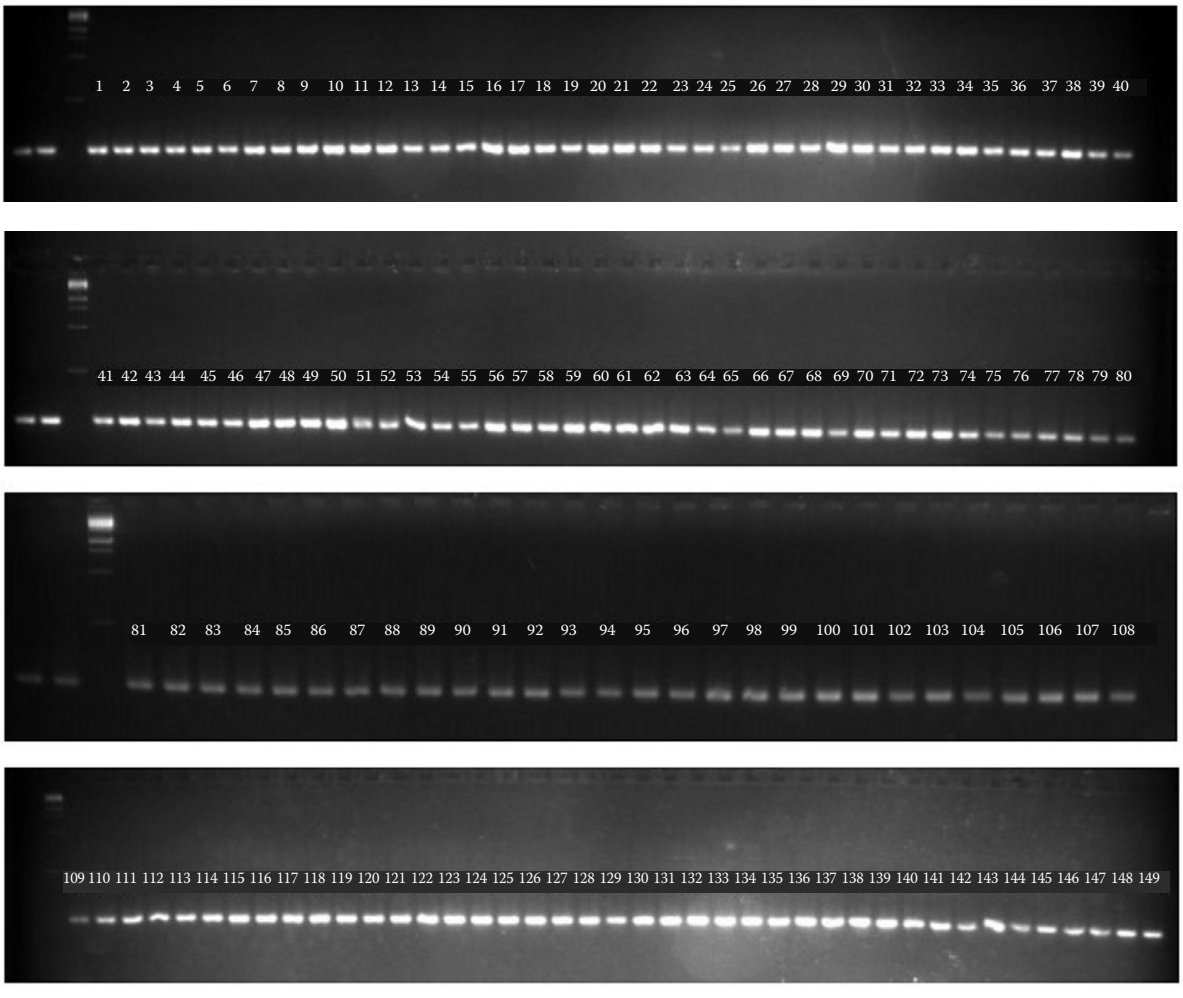

Figure 1. Simple sequence repeat (SSR) banding profile using the MUGR 3410 primer on parents P_1 (HPKM249), P_2 (HPK4) and 157 F_8 recombinant inbred lines (RILs)

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density of 5.65 cM (SA1). The variation in the linkage groups length ranged from 16.95 cM in LG7 to 173.36 cM in LG1. The marker density varied from 1.23 to 17.41 cM, with an average of 2.78 cM indicating the differing degrees of saturation of the different LGs (Table 3).

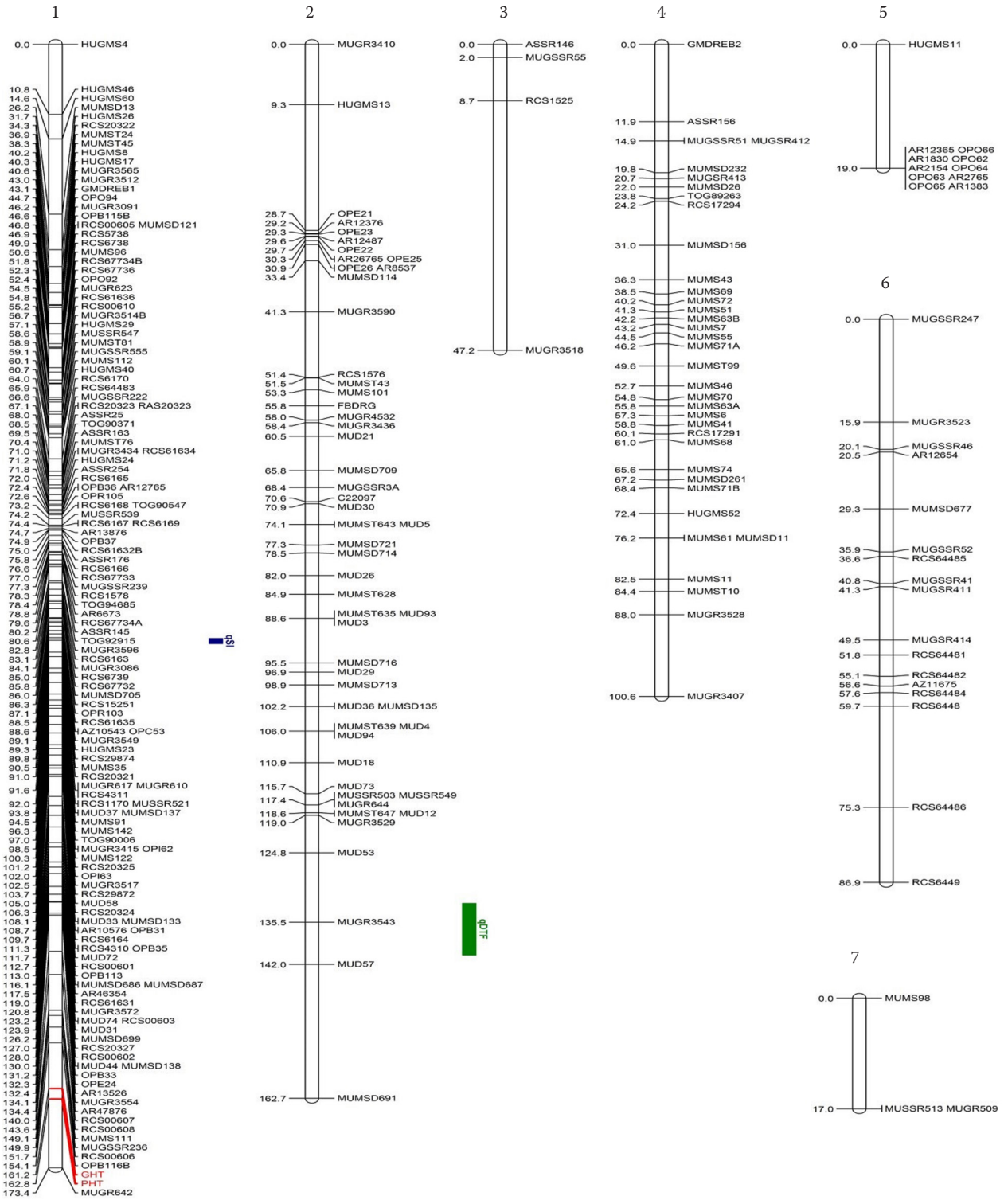

Figure 2. Likelihood intervals for the quantitative trait loci (QTLs) associated with the plant height and determinate growth habit traits in the recombinant inbred line (RIL) mapping population

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Table 3. Distribution of 287 markers on ten linkage groups of an intra-specific linkage map of horsegram

LGs	Markers mapped	Map length	Average marker density
		(cM)	
LG1	141	173.36	1.23
LG2	53	162.74	3.07
LG3	4	47.19	11.80
LG4	36	100.58	2.79
LG5	11	19.03	1.73
LG6	17	86.89	5.11
LG7	3	16.95	5.65
LG8	11	69.27	6.30
LG9	7	51.08	7.30
LG10	4	69.67	17.41
Total	287	796.76	2.78

LG – linkage group

QTL identification. A total of four QTLs (LOD ≥ 2.5) were identified across the ten linkage groups in the RIL population (Figure 2, Table 3). All the QTLs found were major QTLs as PVE ≥ 10%. On LG1, one QTL each for the plant height trait (*qPHT*), growth habit trait (*qGHT*) and seed index (*qSI*) were found, while one QTL for the days to flowering (*qDTF*) was found on LG2. The QTL spotted for the plant height (*qPHT*) on LG1 at the position of 161.2 cM was fringed by OPB116B-MUGR 642 at the distance of 19.3cM, explaining 39.74% of the PVE at an LOD value of 22.09 with an additive effect of 9.78 contributed by the allele of the HPKM249 parent. The second QTL detected for the growth habit trait (*qGHT*) on LG1 at the position of 162.8 cM was flanked by OPB116B-MUGR 642 at the interval of 19.3cM, explaining 40.32% of the PVE at an LOD value of 22.55 with an additive effect of 51.56. The third QTL for the days to flowering (*qDTF*) was found on LG2 at the position of 135.51 cM explaining 13.70% of the PVE at an LOD value of 5.84 with an additive effect of –2.88. The flanking markers for this trait are MUD53 and MUGR3543 covering the distance of 10.7 cM. The fourth QTL for the seed index (*qSI*) was found on LG1 with the position of 92.01 cM explaining 13.52% of the PVE at an LOD

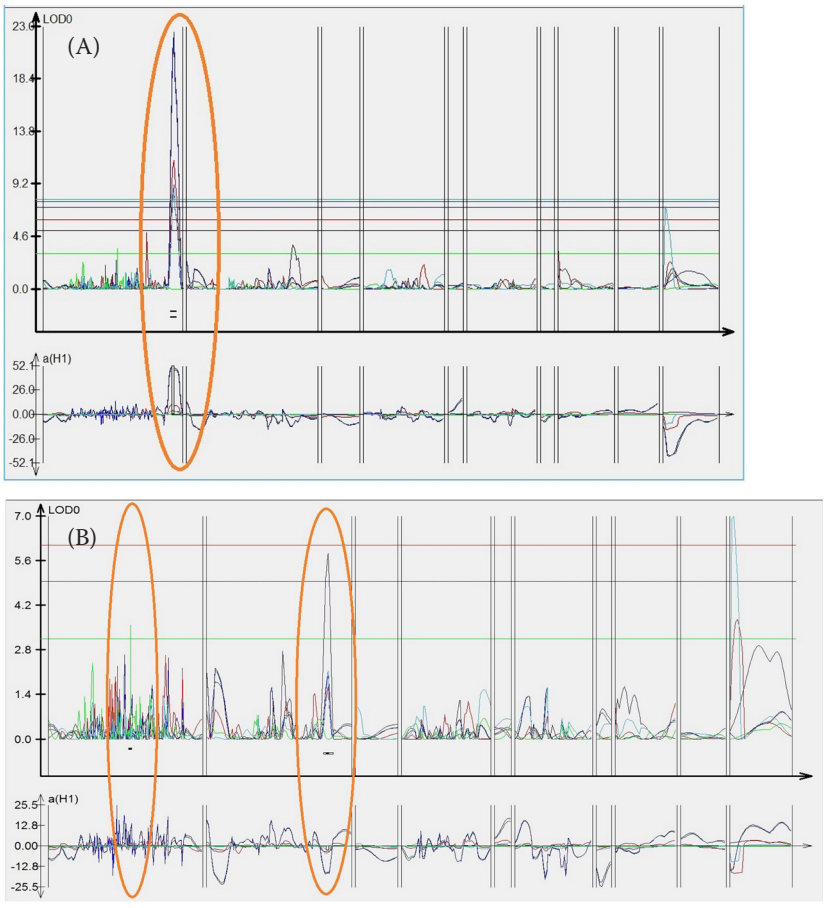

Figure 3. Position of the QTLs for the plant height trait (PHT) and growth habit trait (GHT) on ten linkage groups of horsegram (A) and position of the QTLs for the days to flowering (*qDTF*) and seed index (*qSI*) on ten linkage groups of horsegram (B)

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Table 4. Quantitative trait loci (QTLs) for various traits identified using QTL Cartographer

S. No.	Trait name	QTL name	Chr. No.	QTL position (cM)	Marker interval	Distance (cM)	LOD	Additive effect	PVE (%)
1	plant height	q*PHT*	1	161.2	OPB116B- MUGR642	19.3	22.09	9.78	39.74
2	type of growth habit	q*GHT*	1	162.8	OPB116B-MUGR642	19.3	22.55	51.56	40.32
3	days to flowering	*qDTF*	2	135.51	MUD53-MUGR3543	10.7	5.84	–2.88	13.70
4	seed index	*qSI*	1	92.01	MUGR617- RCS1170	0.40	3.59	0.31	13.52

LOD – logarithm of the odds; PVE – percentage of phenotypic variance

value of 3.59 with an additive effect of 0.31. This QTL was very important in that two flanking markers, namely MUGR617 and RCS1170, were found very close to each other covering the distance of 0.40 cM (Figure 3A, B; Table 4). Therefore, these markers can be used for the marker assisted selection for the seed index in this crop.

DISCUSSION

The introduction of DNA based markers brought a major shift in the characterisation of quantitative traits, thus enabling the crop breeder to undertake selection for various traits based on these markers. Linkage map-based QTL analyses have been used to discover chromosomal region containing genes governing desirable qualitative and quantitative traits (McCouch & Doerge1995; Mohan et al. 1997). The present work, therefore, has been designed to enrich the existing framework linkage map of horsegram putting additional molecular markers and to identify the QTLs linked to the plant height and determinate growth habit in horsegram. The plant height and determinate growth habit are the most important traits required for the modification of the plant architecture of a crop plant making it suitable for mechanical harvesting in commercial cultivation. Various breeding techniques, such as conventional (Chahota et al. 2005), mutation (Chahota et al. 2013) and molecular (Chahota et al. 2020; Mahesh et al. 2021), are being adopted by the breeders for the successful implication of useful traits in horsegram. SSRs are present on both the coding as well as non-coding regions (Chung et al. 2006; Rajendrakumar et al. 2007) having a low degree of repetition per locus (5–100) with a high degree of length polymorphism (Tautz 1993; Zane et al. 2002; Parida et al. 2009), thus, they are the markers of choice that are used in the present study. A total of 2 395 PCR based markers were screened to identify the polymorphic primers between HPKM249 and HPK4, the parental lines of the mapping population. The polymorphic SSRs were selected for genotyping of the 157 RIL mapping population. Of these, 600 markers (27.60%) were found to be polymorphic and were used for the genotyping of the 157 individuals of the RILs. The information of these markers can be used in other legume crops for comparison and phylogenetic studies, which is especially useful for comparative genomic mapping (Grattapaglia 2000). However, there are very few reports on the development of molecular marker in horsegram. More recent efforts have been made to develop SSR markers in horsegram (Chahota et al. 2017; Kaldate et al. 2017) and a first framework linkage map was developed in horsegram spanning 1 423 cM in map length (Chahota et al. 2020). The present linkage map (Figure 2) represented the expressed regions of the horsegram genome with a map length of 796.76 cM, which is comparable to the intraspecific linkage maps of other legumes present in clade phaseoloid/millettioid with the map length of 2 458.0 cM in the soybean (Kong et al. 2018), 1 079.21 cM in the common bean (Blair et al. 2018), 1 588.7 cM in the cow pea (Somta et al. 2019) and 1 411 cM in the pigeon pea (Sheetal et al. 2017). The map length decreases with an increase in the number of mapped markers. The more markers tightly linked with the particular genes, the distance will be less, ultimately the whole map length on the chromosome decreased. Out of ten linkage groups, the maximum number of markers were mapped on LG1, which harboured 141 markers with the average marker density of 1.23 cM and a minimum were mapped on LG7 which harboured 3 markers with the average marker density of 5.65 cM (Figure 2). This inconsistency could be solved either by increasing the population size or by using a larger number of markers (SSRs and SNPs) to fill the gaps (Grisi et al. 2007). Each of the ten LGs are different from one another in terms of the marker distribution and length. Due to this, the arbitrary marker distribution has been noted in current

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work, *e.g.,* some groups were densely packed (LG1 and LG2), whereas LG3, LG7 and LG10 contained only four, three and four markers, respectively. This could be attributed to the SSRs being ubiquitous and being distributed randomly in the plant genomes (Areshechenkova & Ganal 1999; Ramsay et al. 1999, 2000; Elsik & Williams 2001). A total of four QTLs (LOD ≥2.5) were detected across the ten linkage groups fortified with 287 molecular markers for the early plant height, growth habit, days to flowering and seed index. The genomic regions controlling the plant height and determinate growth habit are adjacent to each other on LG1 and the two flanking markers, namely OPB116B & MUGR642, were identified for the mapping of these traits. The use of flanking markers/intragenic markers increased the reliability of the markers for foretelling the particular phenotype (Edward et al. 1987). Generally, the flanking markers for particular traits (morphological and architectural) give information about the sequential authentication on the chromosomes. This helps us to map the functional QTLs which ultimately determine the convoluted phenotypic variation among the specific genomic regions (Ribaut & Betran 1999). DNA markers that are closely linked to agronomically important traits are, thus, used as a molecular tool for Marker-Assisted Selection (MAS) in plant breeding (Tewodros & Zelalem 2016).

CONCLUSION

From the above discussion, it can be concluded that the present linkage map, fortified with 287 molecular markers and four QTLs would provide a means to breeders for the further genetic enhancement in this crop species. On the basis of these results, there are two flanking markers identified for the plant height trait (PHT) and growth habit trait (GHT), namely OPB116B & MUGR642 on LG1. However, a denser genetic linkage map with a large number of markers with the inclusion of SNPs would facilitate the identification of the more resolved and finer QTL positions which can significantly improve the resolution of the identified QTLs for the mapping. The identification of the QTLs controlling agronomically important traits would improve our genetic understanding of these traits and finally provide the basis for the MAS for these traits.

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