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Validation of QTLs for earliness and plant type traits in pigeonpea (*Cajanus cajan* (L.) Millsp.)

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Abstract

Validation of QTLs recently reported in pigeonpea for plant type and earliness traits *viz*., plant height, number of secondary branches, number of pods per plant, days to flowering and days to maturity was carried out using RILs derived from the cross Pusa Dwarf x H2001-4. RILs exhibited significant phenotypic variation for the all the traits. Using Interval mapping analysis QTLs for these traits except for plant height were successfully validated in the RILs in the marker interval ASSR100 and ASSR 206. Contribution of enhancing alleles by female parent Pusa Dwarf for the traits was also confirmed. QTLs for earliness traits qFL5.1 and qMT5.1 explained 15.3% and 11.6% phenotypic variation respectively while QTLs for plant type characteristics qSB5.1 and qPD5.1 exhibited phenotypic variation of 18.1 % and 11.2 % respectively.

Key words: Recombinant inbred lines, simple sequence repeats, interval mapping, validation

Introduction

Pigeonpea (*Cajanus cajan* (L.) Millsp.), a diploid legume crop species (2n=2x= 22), belongs to the tribe Phaseoleae of family Leguminosae. Globally, pigeonpea is cultivated on 5.3 m ha with an annual production of 4.3 mt and productivity of 813 kg/ha [1]. It is the sixth most important legume crop in the world. India is the primary centre of origin and largest producer of pigeonpea in the world sharing approximately 62% of the production and covering 73% of the area. It is the chief source of protein for more than a billion people in the developing nations. It is fairly drought tolerant. It has diverse uses such as food, fodder and fuel. It plays important role in soil conservation by fixing atmospheric nitrogen and improving soil structure by its deep root system [2]. Despite its importance in food and nutritional security there is a gap in the potential and actual productivity in pigeonpea accounting to the several factors. These include growing of pigeonpea in low-input and marginal environments, various biotic and abiotic stresses low levels of genetic diversity in the cultivated varieties [3] and availability of limited genomic resources [4]. Recently with the publication of draft genome sequence and deep transcriptome studies, the stage has been set to expedite the process of enriching genomic resources to aid molecular breeding in pigeonpea [5-7].

Development of molecular markers served several functions in pigeonpea including, genetic diversity analysis using restriction fragment length polymorphism (RFLP) [8], amplified fragment length polymorphism (AFLP) [9], random amplification of polymorphic DNA (RAPD) [10], microsatellite markers [11] and DArT [3], characterization of hybrid parents and purity assessment, gene tagging for Fusarium wilt resistance [12], mapping for various traits *viz*., (drought tolerance [13], determinacy [14], sterility mosaic disease [15], fertility restoration [4]) and development of linkage maps [3, 15-17].

In pigeonpea, traits days to flowering and days to maturity determine the earliness and these traits are important with respect to increasing cropping intensity with pigeonpea – wheat rotation. Recently

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Kumawat *et al.* [17] identified the QTLs for plant type and earliness traits *viz.*, plant height (qPH5.1), number of secondary branches/plant (qSB5.1), number of pods/plant (qPD5.1), days to flowering (qFL5.1) and days to maturity (qMT5.1) in the $F_{2:3}$ mapping population derived from the cross Pusa Dwarf x HDM04-1. All these five QTLs have been mapped on the linkage group 5 (LG_5) in the marker interval ASSR100-ASSR 206. QTLs for earliness traits qFL5.1 and qMT5.1 showed phenotypic variance of 8.7% and 25.9%, respectively, whereas QTLs for plant type traits qPH5.1, qSB5.1 and qPD5.1 explained 27.5%, 10.4% and 18.9 % of phenotypic variance, respectively. These QTLs need to be validated in different mapping populations and germplasm lines for their effective use in Marker Assisted Selection for improvement of the respective traits.

In an attempt to validate these identified QTLs we have used recombinant inbred lines mapping population derived from the cross between Pusa Dwarf x H2001-4.

Materials and methods

A total of five QTLs *viz*., qFL5.1, qMT5.1, qPH5.1, qSB5.1 and qSB5.1 were investigated in segregating RIL population for validation in the present study using two genic SSR markers. List of markers and traits studied has been given in Table 1.

Development of mapping population

A recombinant inbred line mapping population was developed from a cross between pigeonpea inbred lines 'Pusa Dwarf' and 'H2001-4' (Fig. 1). The parents were contrasting for earliness and plant type characters. Parent H2001-4 is early in flowering and maturity than the Pusa Dwarf. H2001-4 produces high number of pods per plant compared to Pusa Dwarf. Pusa Dwarf is shorter in height and having more number of secondary branches per plant than the

Fig. 1. Field photograph of the parents. a-Pusa Dwarf (P_1), b-H2001-4 (P_2)

H2001-4. Single seed descent method with strict selfing was followed to develop the recombinant inbred lines mapping population. Seven generations of selfing were done to obtain the F_8 generation recombinant inbred lines. The field evaluation was carried out during *kharif* 2012-13 at IARI, New Delhi. A total of 300 recombinant inbred lines along with parents and six check varieties (Pusa 991, Pusa 992, Pusa 2001, Pusa 2002, UPAS 120 and AL 201) were evaluated for plant type and earliness traits using Augmented field design-II [18] in which parents and check varieties were replicated. Each RIL, parental lines and check varieties were grown in single row with row to row and plant to plant spacing of 60 and 20 cm, respectively. Observations were recorded for earliness traits *viz*., days to flowering, days to maturity and plant type characters *viz*., plant height, number of secondary branches per plant and number of pods/plant. Observations were recorded on

Table 1. Trait wise details of QTLs and markers used for validation in recombinant inbred lines mapping population derived from Pusa Dwarf x H2001-4

S.No.	Trait	QTL	Marker interval	Position (cM)	PVE (%)	Reference
1	Days to flowering	qDF5.1	ASSR100-ASSR206	79.3	8.7	[17]
2	Days to maturity	qMT5.1	ASSR100-ASSR206	81.3	25.9	[17]
3	Plant height	qPH5.1	ASSR100-ASSR206	79.3	27.5	[17]
4	Number of secondary branches/plant	qSB5.1	ASSR100-ASSR206	85.3	10.4	[17]
5	Number of pods/plant	qPD5.1	ASSR100-ASSR206	85.3	18.9	[17]

ten randomly selected plants from the middle of each row.

Genomic DNA isolation and validation of SSR markers

Young and fresh leaves were collected from one month old recombinant inbred lines and both of the parents. Genomic DNA was isolated from 300 recombinant inbred lines and parental genotypes following CTAB method of Murray and Thompson [19].

The quantification of DNA was carried out with 0.8 % agarose gel electrophoresis using lambda DNA as standard and diluted to concentration of approximately 50 ng/µl of PCR analysis. Genic microsatellite markers ASSR100 and ASSR206 were used for genotyping of the recombinant inbred lines (Table 2).

The PCR reactions were performed using PTC225 Gradient Cycler (MJ Research). Each PCR reaction consisted of 1.0µl of 10 × buffer, 0.15mM dNTPs, 0.2 pmol each of forward and reverse primers, 0.2µl of template genomic DNA (50ng/µl), 0.2 units of Taq DNA polymerase (5U/µl) (Vivantis Technologies) in a final reaction volume of 10µl. The PCR reaction profile followed was, DNA denaturation at 94^{o}C for 5 minutes followed by 36 cycles of denaturation at 94^{o}C for 1 minute, annealing at 55^{o}C for 1 minute and extension at 72^{o}C for 1 minute. Finally, final extension at 72^{o}C for 10 minutes. The amplified products were resolved by electrophoresis in 4% Metaphor agarose gels (Lonza, Rockland ME USA) containing 0.1 µg/ml ethidium bromide in 1x TBE buffer at 120 V for 3 h. After electrophoresis, the DNA fragments were visualized and documented using ALPHA IMAGER gel documentation system (Alpha Innotech, USA) which was used for marker scoring. Gels were scored using A/B method where in A corresponds to the allele contributed by parent Pusa Dwarf and B corresponds to allele contributed by parent H2001-4. Allelic segregation at each of the marker loci was analysed for deviation from the expected 1:1 ratio in the recombinant inbred line population using χ^2 test using the formula

$$\chi^2 = \Sigma (O - E)^2/E$$

Where O is observed value and E is the expected value.

Table 2. Sequence information of two genic SSR markers used for validation of QTLs

SSR marker	Forward primer	Reverse primer
ASSR-100	AACTTAGACTCTG ACCCATTTC	CAAGTTGAACCAC TAGACACAC
ASSR-206	GGAAGGGAAAAC TTAGAGAGAG	ATATCTTCCCTACG TGTGTGTC

Statistical analysis of the phenotypic data was performed using SPAD (Statistical Package for Augmented Design) software. ANOVA were analysed at a P = 0.05 level of significance. The interval QTL analysis was performed using adjusted means of the phenotypic trait value and genotyping data using QTL cartographer 2.1 software employing the full-QTL model. The interval mapping method was adopted as the two markers used in the present study were part of the set of markers used for identification of QTLs in the same mapping population. As the QTLs selected for the validation were identified in the maker interval ASSR 100 and ASSR 206 by performing interval mapping, single marker analysis separately for each marker was not conducted. The test window size was set to 5 cM with a walk speed of 2 cM and a cut-off probability of 0.05 for deciding the significance of the QTL. LOD threshold for declaring a QTL significant was determined by permutation tests using 1,000 reiterations.

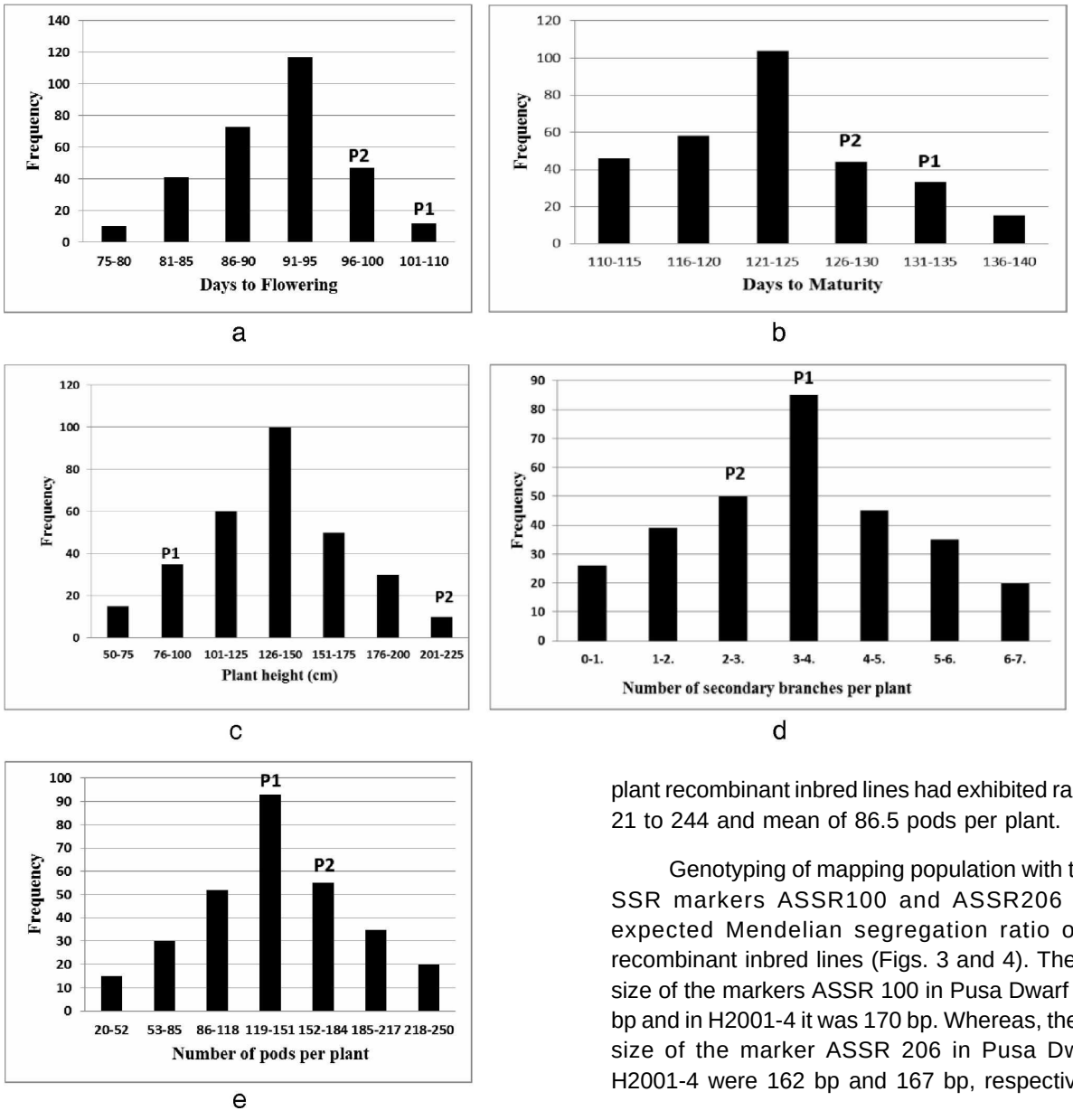
Results and discussion

Phenotypic data for five traits *viz*., days to flowering, days to maturity, plant height, number of secondary branches/plant and number of pods/plant were recorded in recombinant inbred line population. ANOVA as per augmented design was performed to test the variation in trait expression among recombinant inbred lines. Significant variation was observed among recombinant inbred lines for all the traits studied (Table 3). The phenotypic variation for all the five traits showed normal frequency distribution (Fig. 2). The two parents, Pusa Dwarf and H2001-4 showed significant variation for all the traits considered. Pusa Dwarf had exhibited more number of Secondary branches per plant. H2001-4 exhibited early flowering and maturity compared to the Pusa Dwarf. H2001-4 showed more number of pods per plant than the Pusa Dwarf. In recombinant inbred lines, plant height ranged from 54.7 cm to 209.9 cm with mean plant height of 166 cm whereas number of secondary branches/plant ranged from 0 to 7 with mean of 3.2 secondary branches/plant. Days to flowering and days to maturity ranged from 78 days to 100 days and 113 days to 138 days with means of 94.2 days and 122.9 days, respectively. For number of pods per

Table 3. Descriptive statistics of the traits for parents and the recombinant inbred line population

S.No.	Trait	Parents		t-value	RILs	
		Pusa Dwarf	H2001-4		Mean ± SD	Range
1	PH	81.9	203.2	58.9**	166.0 ± 19.7	54.7-209.9
2	SB	3.6	2.7	4.89**	3.2 ± 0.6	0-7
3	PD	130.7	155.3	6.39**	86.5± 12.2	21-244.8
4	FL	110	99	5.34**	94.2±7.1	78-110
5	MT	135	128	4.44**	122.9±6.0	113-138

PH=Plant height (cm); SB=Number of secondary branches/plant; PD=Number of pods/plant; FL=Days to flowering; MT=Days to maturity



plant recombinant inbred lines had exhibited range from 21 to 244 and mean of 86.5 pods per plant.

Genotyping of mapping population with the genic SSR markers ASSR100 and ASSR206 showed expected Mendelian segregation ratio of 1:1 in recombinant inbred lines (Figs. 3 and 4). The product size of the markers ASSR 100 in Pusa Dwarf was 160 bp and in H2001-4 it was 170 bp. Whereas, the product size of the marker ASSR 206 in Pusa Dwarf and H2001-4 were 162 bp and 167 bp, respectively. The

Fig. 2. Frequency distribution pattern for days to flowering (a), days to maturity (b), plant height (c), number of secondary branches (d) and number of pods per plant (e) in recombinant inbred lines

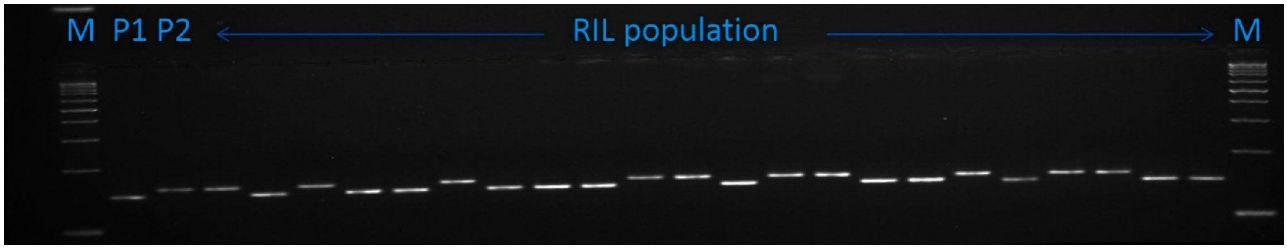


Fig. 3. Genotyping of recombinant inbred lines using polymorphic marker ASSR 100 on 4 % Metaphor gel electrophoresis. M-100 bp ladder. P1- Pusa Dwarf, P2-H2001-4

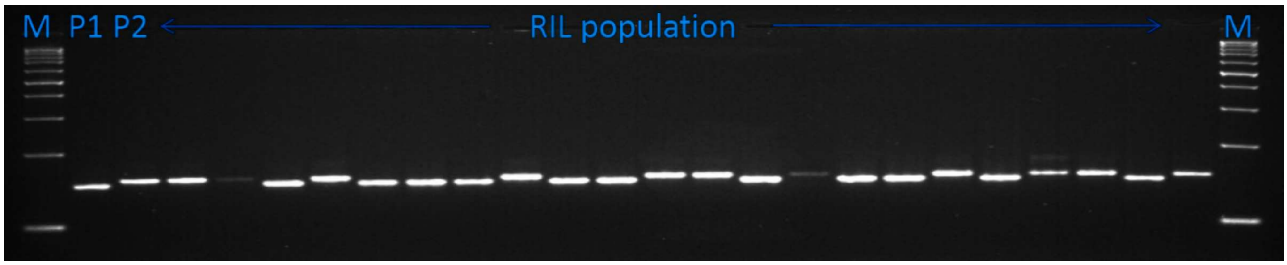


Fig. 4. Genotyping of recombinant inbred lines using polymorphic marker ASSR 206 on 4 % Metaphor gel electrophoresis. M-100 bp ladder. P1- Pusa Dwarf, P2-H2001-4

interval mapping analysis revealed the location of four QTLs qSB5.1, qPD5.1, qFL5.1 and qMT5.1 in the marker interval ASSR100-ASSR206. The QTL for number of pods/plant qPD5.1 was located at 8.1cM distance downstream from the marker ASSR100. The QTLs qMT5.1 and qFL5.1 were located at 6.2 cM and 9.2 cM distance, respectively downstream from the marker ASSR100. QTL for number of secondary branches qSB5.1 was located at the distance of 9.5 cM_upstream from the marker ASSR206. The LOD score for qPD5.1 was 3.4 and it explained 11.2% of the variation with an additive effect of 5.20. The LOD score for qSB5.1 was 4.1 with R^2 value of 18% and additive effect of 1.6.

The QTL for days to flowering qFL5.1 and days to maturity qMT5.1 had the LOD score 3.1 and 3.3, respectively. The QTLs qFL5.1 and qMT5.1 had R^2 values of 15% and 11% with additive effects of 5.86 and 1.56, respectively (Table 4). All the QTLs showed positive additive effects indicating the contribution of alleles from the female parent Pusa Dwarf. The QTL for plant height qPH5.1 could not be validated in the recombinant inbred lines mapping population used in the present study.

In pigeonpea only three QTL mapping studies have been reported so far [4, 15, 17]. Kumawat *et al*., [17] identified QTLs controlling the expression of earliness and plant type traits in pigeonpea using $F_{2:3}$ mapping population derived from the cross Pusa Dwarf x HDM04-1. Earliness traits days to flowering, days to maturity and plant type characteristics *viz*., plant height, number of secondary branches/plant and number of pods/plant are very important for the improvement of pigeonpea varieties for early maturity

Table 4. QTLs for earliness and plant type traits validated in recombinant inbred line mapping population derived from Pusa Dwarf x H2001-4

Trait	QTL	Marker	Distance (cM)	LOD	R^2 (%)	Additive effect
Days to flowering	qFL5.1	ASSR100	9.2	3.1	15.3	5.86
Days to maturity	qMT5.1	ASSR100	6.2	3.3	11.6	1.56
Number of secondary branches	qSB5.1	ASSR206	9.5	4.1	18.1	1.60
Number of pods per plant	qPD5.1	ASSR100	8.1	3.4	11.2	5.20

and high seed yield. The traits *viz*., plant height, number of secondary branches/plant and number of pods/plant are under polygenic control and response to selection in these traits through conventional selection is low. Identification of molecular markers linked to QTLs underlying the expression of these traits would immensely help in improvement of pigeonpea through Marker Assisted Selection by increasing response to selection. In the present study, the QTLs for earliness and plant type traits were validated using two genic SSR markers in recombinant inbred line mapping population. The phenotypic variation explained by the QTLs in recombinant inbred lines ranged from 11.2% to 18.1%. Validation of QTLs for grain yield component traits was also reported in rice [20-22]. All the QTLs identified by Kumawat *et al*., [17] for plant type and earliness traits, except one QTL for plant height qPH5.1, have been successfully validated in the mapping population used by us in the same marker interval. The invalidated QTL might have been specific for the original population [23, 24].

Through the present study the four QTLs qFL5.1, qMT5.1, qSB5.1 and qPD5.1 were successfully validated in the same marker interval ASSR100-ASSR206. The findings may be useful in marker assisted selection in pigeonpea to improve the pigeonpea cultivars for early maturity and plant type characteristics. These markers may be further validated in many diverse germplasm lines and mapping populations in pigeonpea for their effective utilization in Marker Assisted Selection. The validated QTLs could be used for introgression into the pigeonpea varieties to improve them for earliness and higher yield. The present investigation has clearly indicated the robustness of the genic SSR markers ASSR100 and ASSR206 in the validation of QTLs.

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