GENETIC DIVERSITY ANALYSIS IN RELATION TO FLOWERING BEHAVIOR IN MUNGBEAN *(Vigna radiata)*

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ABSTRACT

Mungbean (*Vigna radiata* L.) is an important food legume that is widely cultivated in tropical and subtropical regions of the world. Flowering over an extended period leading to asynchrony in pod maturity is a major problem in mungbean. Designing the plant architecture with synchronous pod maturity can be achieved by mapping genes for flowering related traits followed by breeding. In this regard, to evaluate the diversity in flowering related traits, 20 mungbean accessions were grown in the field and fourteen flowering related phenotypic characters were measured to analyze their variations. Results revealed that flowering duration leading to asynchronous pod maturity is significantly positively correlated with days to first flower, number of pods at maturity, plant height, number of leaves at first flowering, number of pod clusters per plant, number of primary branches, number of flowers and yield per plant at first harvest. Successful PCR amplification was obtained with four Simple Sequence Repeat (SSR) markers and molecular genotyping was conducted with twenty accessions. A dendrogram was drawn based on SSR marker polymorphism using Jaccard's similarity coefficient. It has separated 20 mungbean cultivars into six clusters at the

similarity level of 15, showing suitable parents with maximum intra-cluster distance to be selected for gene mapping studies based on synchrony of pod maturity.

Keywords: Genetic Diversity, Mungbean, SSR Markers, Synchronous Pod Maturity, Vigna radiata

INTRODUCTION

Mungbean (*Vigna radiata* L.) is an important legume crop which is widely cultivated in Asia. It is self-pollinating diploid crop (2n=2x=22) with a genome size of 579 Mb/1C (Sehrawat *et al.*, 2014). Seeds, young pods and fresh sprouts are consumed as human food while plant parts are used as forage for livestock and green manure. Mungbean serves as inexpensive vital source of vegetable protein (19.1-28.3%), mineral (0.18-0.21%) and vitamins (Singh *et al.*, 2013).

Mungbean is a tropical crop requiring 90–120 days from planting to maturity which can be grown under low moisture and low fertile soil conditions. Mungbean is one of the important grain legumes in the rain fed farming system in Dry and Intermediate Zones of Sri Lanka (Ranawake *et al.*, 2011). Mungbean is grown about 15,722ha in Sri Lanka with an average yield of 1.16 mt/ha. Production level of mungbean in the year 2011 was approximately 10,838mt which is far below the national requirement (Annual performance report, 2012).

Asynchrony in pod maturity is one of the major problems faced by famers in harvesting because of consuming time and adding cost for repeated hand picking of pods. There are three harvesting periods in mungbean and first harvest occurs at 70-75 days after sowing (DAS) giving 65% of pods. About 18% of the pods can be harvested from the second harvest at 75-80 DAS and about 17% of the pods can be harvested from the third harvest at 90-95 DAS (Mondal *et al.*, 2011). If the cultivar produces more than 90% of the total harvest from the first harvest, the trait of the cultivar could be considered as ideally synchronous. Therefore, flowering behavior and morphological traits related to flowering of the cultivar need to be assessed to predict the pod maturity synchrony and final yield in order to use them in successful breeding programs.

Hence, this study was conducted aiming at understanding the flowering behavior of some Sri Lankan mungbean cultivars leading to synchrony of pod maturity. Also molecular analysis was conducted to assess the genetic diversity among cultivars to identify suitable parental lines for mapping of genes on synchrony of pod maturity.

MATERIALS AND METHODS

Experimental Location

This experiment was conducted in field plots and the laboratory of Department of Biotechnology, Wayamba University of Sri Lanka, Makandura, Gonawila (NWP) in 2016.

Plant Material

A total of twenty mungbean accessions with genetically diverse background including four traditional varieties were used in this study. These mungbean accessions were obtained from the Plant Genetic Resource Center, Gannoruwa and from Field Crop Research and Development Institute, Mahailluppallama, Sri Lanka.

Experimental Design

The experiment was carried out in a randomized block design containing 15 plants from each accession. Seeds were sown in ridges with the spacing of 30×10cm. The general management practices were carried out as recommended by the Department of Agriculture, Sri Lanka.

Data Recording

Field was monitored regularly and thirteen flowering related phenotypic characters were assessed from each plant. Flowering duration (FD), pod length (PL), days to first flowering (DFF), number of pods at 80%maturity (NPM), plant height at 1st flowering (PH), number of leaves at 1st flowering (NLFF),number of primary branches per plant (NPB), number of pod clusters per plant (NCPP), number of seeds per pod (NSPP), 100-seed weight (HSW), number of pods per cluster (NPPC), number of flowers per plant (NF) and yield per plant (YPP) were recorded. Yield per plant was measured by dry weight of seeds at the first harvest.

Genomic DNA Extraction

Extraction of DNA was done according to the general CTAB extraction method. A 0.125g of young leaf sample from one week old seedling was collected and homogenized with 800µL of pre warmed CTAB extraction buffer (1.4MNaCl, 0.02M EDTA, 0.1M TrisHCl, 2% CTAB, 0.2% β-mercapto-ethanol). The contents were incubated in a water bath at 60°C for 30 min followed by centrifugation at 3,500rpm for 15 min and Phenol: chloroform: isoamyl alcohol (25:24:1 v/v) extraction was performed by adding with the supernatant and then the DNA was precipitated with cold isopropanol. The contents were incubated at -20°C for 2 h. The tubes were centrifuged at 13000 rpm for 10 min to pellet out DNA. DNA pellet was washed with 70% ethanol and then the pellet was dissolved in 200 µL TE buffer.

DNA samples were incubated with 2 µL of RNase ONE (10U/µL) at 37 °C for 1 h. Phenol: chloroform: isoamyl alcohol (25:24:1 v/v) extraction was performed and the DNA was precipitated with cold isopropanol as mentioned above followed by washindfg with 70% ethanol. The Tubes were air dried and the pellet was dissolved in 100 µL of 1/10th TE buffer.

Selection of Microsatellite Markers

The SSR markers namely, CEDAAG002, CEDG037 and CEDG014 which are near to the flowering time QTL, qDFL2.1, qDFL6.1 and qDFL5.1 respectively reported by Somta *et al.*, (2015) and another SSR marker CEDG044 reported by Bajracharya *et al.*, (2008) were used for the present study.(Table 01)

Table 1. Primer sequences and annealing temperatures

Code	Forward Primer (5'-3')	Reverse Primer (5'-3')	Annealing Tem.(^{0}C)
CEDAAG002	GCAGCAACGCACAGTTTCATGG	GCAAAACTTTTCACCGGTACGACC	58
CEDG014	GCTTGCATCACCCATGATTC	AAGTGATACGGTCTGGTTCC	57
CEDG037	GAAGAAGAACCCTACCACAG	CACCAAAAACGTTCCCTCAG	55
CEDG044	TCAGCAACCTTGCATTGCAG	TTTCCCGTCACTCTTCTAGG	55

Table 2. Correlation coefficient among traits

TRAIT	FD	PL	NSPP	DFF	NPM	PH	NLFF	NPPC	NPB	NCPP	HSW	NF	YPP`
FD	1	-.085	.354	.531*	.566**	.444*	.571**	-.092	.824**	.751**	-.331	.849**	.521*
PLs			.191	-.371	-.387	-.151	-.295	-.315	-.246	-.295	.348	-.339	-.168
NSPP				.472*	.397	.596**	.405	.344	.346	.472*	-.548*	.349	.513*
DFF					.820**	.610**	.814**	.421	.633**	.842**	-.576**	.709**	.679**
NPM						.680**	.844**	.616**	.698**	.895**	-.413	.825**	.804**
PH							.594**	.540*	.443	.656**	-.403	.669**	.943**
NLFF								.451*	.773**	.730**	-.303	.726**	.759**
NPPC									.142	.395	-.411	.257	.540*
NPB										.784**	-.235	.845**	.612**
NCPP											-.461*	.898**	.758**
HSW												-.403	-.232
NF													.755**
YPP													1

* Significant at 5% and ** Significant at 1% level. Flowering duration (FD), Pod length (PL), Number of seeds per pod (NSPP), Days to first flowering (DFF), Number of pods at 80% maturity (NPM), Plant height at 1st flowering (PH), Number of leaves at 1st flowering (NLFF), Number of pods per cluster (NPPC), Number of primary branches per plant (NPB), Number of pod clusters per plant (NCPP), 100-seed weight (HSW),Number of flowers per plant (NF) and Yield per plant at first harvest(YPP)

Polymerase Chain Reaction (PCR) Amplification

Amplification was carried out in 12μL reaction mixture by using BioRad (My CyclerTM). Reaction mixture contained 2μL (15ng/μL) of DNA, 1.2μL of 10X PCR buffer, 1.2μL of 2.5mMdNTP, 1.1μL of 20pmol/μL primer and 0.2μL of 5U/μLTaq DNA polymerase. Amplification conditions were, initial denaturation at 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 1 minute, annealing optimum temperature for 30 seconds and extension 72°C for 1 minute and a final extension at 72°Cfor5 minutes. PCR amplified products were subjected to gel electrophoresis in a 3.5% agarose gel stained with ethidium bromide.

Statistical Analysis

Analysis of variance (ANOVA) was done to study the variations on phenotypic traits among the genotypes. Correlation between pairs of each trait was estimated by using the Pearson correlation coefficient. Cluster analysis was done using SSR markers based on Jaccard's similarity coefficient following the unweighted pair group method with arithmetic mean (UPGMA) by SPSS 16.0 version to investigate distance, similarity and relatedness of genotypes.

RESULTS AND DISCUSSION

Field Data Analysis and Correlation between Traits

The analysis of variance showed significant differences among the genotypes for all the measured characters at $P<0.001$ level, except DFF ($P<0.05$) and NPB. Most of the correlation coefficients between traits were significant (Table 2). Asynchrony in flowering was indicated by higher flowering duration. Flowering duration showed strong positive correlation ($r=0.824$) with number of primary branches and number of flowers per plant

(r=0.849). It also showed significant positive relationship (r=0.521) with the yield at the first harvest. This information revealed that plants which have extended flowering period were bearing branches with higher number of flowers. Days to first flowering was strongly positively correlated with number of pods at maturity (r=0.820), plant height (r=0.610), number of leaves at first flowering (r=0.814) and number of flowers (r =0.709). However hundred seed weight was negatively correlated (r=-0.576) with the days to first flowering significantly.

Similar relationships have been reported in the study conducted by Somta *et al.* (2015) on identifying QTL for seed weight and days to flowering in mungbean. Scatter plot (Figure 01) illustrates positive association between flowering duration and yield per plant. Accessions with high yield tended to have long flowering duration. When considering the scatter plot drawn between flowering duration and the number of flowers (Figure 01), there was a strong positive correlation. Accessions with long flowering duration tended to have more numbers of flowers and it could be the reason for showing high yield in those accessions. Hundred seed weight and the pod length were negatively associated with the flowering duration.

As indicated in Table 3, phenotypic data revealed that most of the accessions produce 80% of the total flowers within 10 days after first flowering (DAF) and ceased the flowering after 15 days. Accession AC0099 produces 98% of the total flowers within 10DAF while AC3022, AC8416, AC8433, MI5 (a recommended variety) and AC0993 (a traditional variety) produced >90% of total flowers within 10 days. Lowest percentage of flowers (50%) obtained within 10DAF records by the accession AC0219. According to the previous studies, genotypes which produce maximum opened flowers at 10th day and ceased flowering within 15th day were considered as synchrony in pod maturity (Mondal *et al.*, 2013 and Fakir *et al.*, 2011).

Table 3. Flowering behavior and seed yield of mungbean accessions

Genotype	1-5 DAF	6-10 DAF	Cumulative flowers in 10DAF	Percentage of flowers till 10 DAF	11-15 DAF	16-20 DAF	21-25 DAF	Open flowers/ plant	Seed yield per plant at first harvest (g)
MI6	8.54	6.00	14.54	78.74	3.64	0.29	0.00	18.46	2.60
MI5	8.50	8.50	17.00	93.10	1.26	0.00	0.00	18.26	3.20
AC8419	10.94	10.78	21.72	87.68	3.05	0.00	0.00	24.77	2.05
AC0099	14.10	12.78	26.88	98.61	0.38	0.00	0.00	27.26	1.13
MIMB908	9.47	5.28	14.75	74.38	1.88	1.30	1.90	19.83	2.03
AC3022	11.20	7.09	18.29	91.84	1.48	0.15	0.00	19.92	2.62
AC0259	9.18	5.26	14.44	77.66	3.56	0.59	0.00	18.60	2.50
AC0219	9.80	7.94	17.74	50.56	6.90	5.31	5.14	35.08	4.66
AC8432	7.40	6.08	13.48	76.89	2.55	1.50	0.00	17.53	1.62
AC8416	9.55	8.45	18.00	92.07	1.55	0.00	0.00	19.55	2.72
AC8446	8.50	7.61	16.10	81.52	3.65	0.00	0.00	19.75	2.96
AC8433	6.97	7.18	14.15	95.41	0.68	0.00	0.00	14.83	1.91
AC8436	9.24	9.95	19.19	84.11	3.63	0.00	0.00	22.81	3.98
AC8425	8.99	9.24	18.23	83.77	3.53	0.00	0.00	21.76	2.02
*AC2958	14.53	13.76	28.29	68.54	8.14	3.30	1.55	41.27	3.26
*AC8002	8.07	9.62	17.68	71.86	6.74	0.18	0.00	24.60	3.99
*AC0993	8.81	8.27	17.08	93.87	1.12	0.00	0.00	18.20	1.75
MIMB921	9.13	10.20	19.33	82.05	4.23	0.00	0.00	23.56	4.55
MIMB924	8.87	7.09	15.95	85.53	2.70	0.00	0.00	18.65	2.62
*AC8310	7.23	8.05	15.28	81.36	3.50	0.00	0.00	18.78	2.32

DAF-Days after first flowering * Traditional varieties

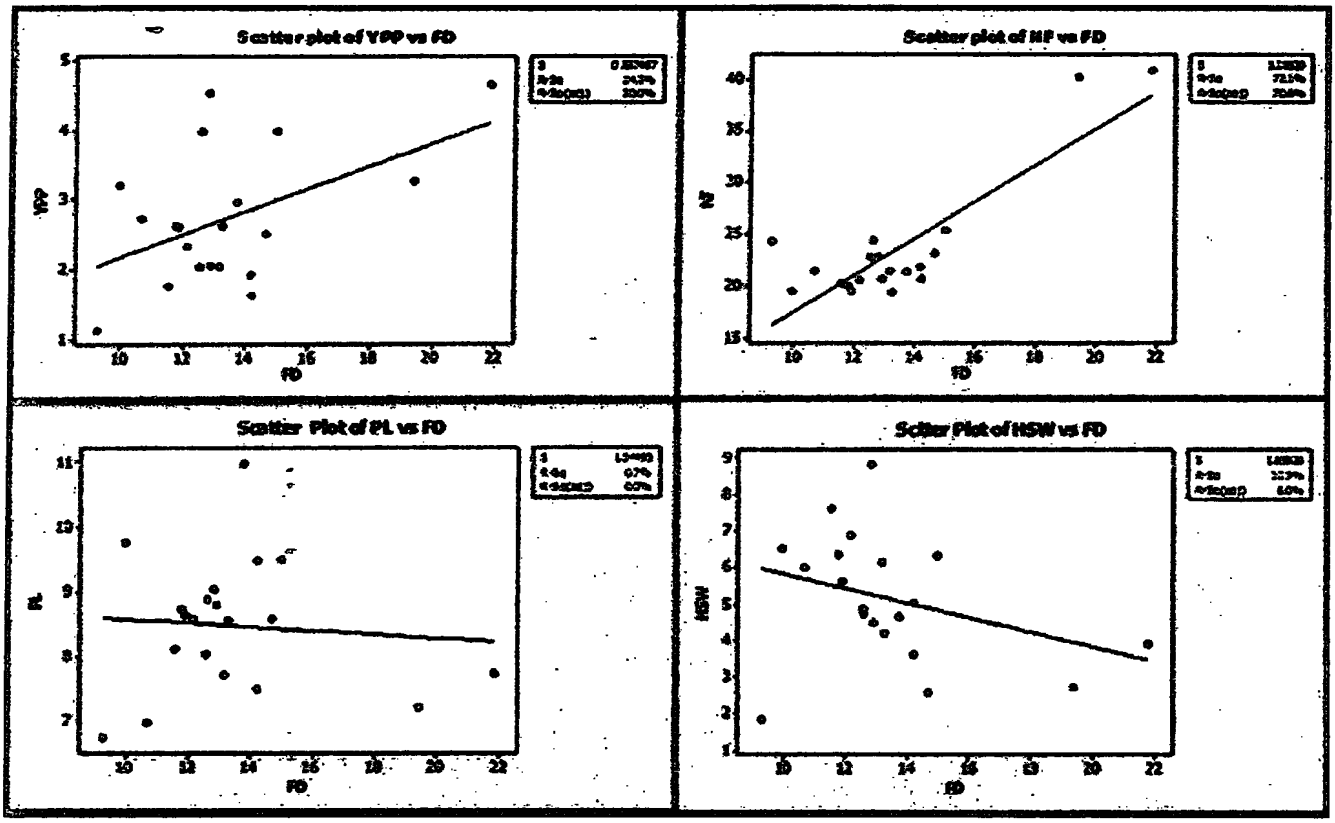

Figure 1. Relationship between flowering duration (FD) with yield per plant (YPP), number of flowers (NF), pod length(PL) and hundred seed weight (HSW) in 20 mungbean accessions

Study of the relationships between flowering traits and seed yield are very important for the breeding programs. In this study maximum yield recorded by the accession, AC0219 and this may be due to the production of higher number of flowers while AC0099 produced the lowest yield per plant at the 1st harvest. AC0099 showed synchrony in flowering but also low yield. The air temperature was the critical factor for the duration of the flowering and for pod filling of mungbean (Roknuzzaman *et al.*, 2007). Therefore, this study should be carried out in different seasons to confirm the results.

PCR Amplification and Genotyping

Quality of the DNA is crucial for the amplification of PCR and success of genotyping. Without treating with RNase, DNA could not be amplified by PCR. Finally

this DNA extraction protocol, treating with RNase was able to produce successful amplification and scorable polymorphic alleles from twenty mungbean accessions. Polymorphism status throughout the genome could satisfactorily be observed without monomorphic amplification and this may be due to the fact that primers were selected from 4 different chromosomes. All four SSR markers exhibited polymorphism among genotypes and number of alleles produced from each marker ranged from two to three. Altogether 10 different alleles were scored from four types of SSR markers used in this study and, 3 alleles amplified from marker, CEDG044 are indicated in figure 2.

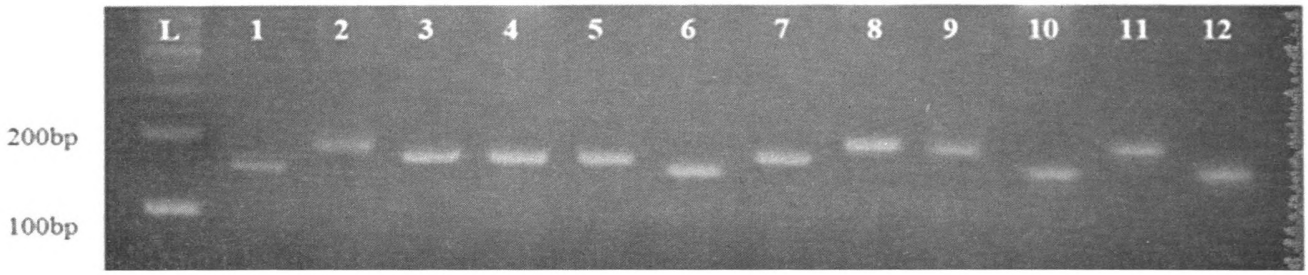

Figure 2. Polymerase Chain Reaction (PCR) amplification of twelve mungbean accessions using CEDG044 DNA marker. Lane L- 100bp ladder, Lane 1- MI6, Lane 2- MI5,Lane 3- AC8419, Lane 4- AC8432, Lane 5- AC8436, Lane 6-AC8425, Lane 7- AC2958, Lane 8- AC8002, Lane 9- AC0993, Lane 10- MIMB921, Lane 11- MIMB924, Lane 12-AC8310

Cluster Analysis

Dendrogram drawn based on Jaccard's similarity coefficient, grouped 20 mungbean genotypes into 6 sub clusters at the 15 similarity level (Figure 3). This polymorphic allele based grouping, indicated distantly related genotypes which would be useful to select parents for mapping studies on flowering related genes. Gene mapping would be the reliable and convenient method for identifying candidate genes for a targeted trait. In order to find out the candidate genes for synchronous nature of the pod maturity in mungbean, it is necessary to produce a mapping population with distantly related parents on flowering time. Although AC0099 and AC0219 showed vast differences in phenotypic traits, they cannot be considered for gene mapping for synchronous pod maturity because both resided in same sub-cluster of major cluster A indicating low level of genotipic polymorphism. The traditional variety AC8436 and improved variety, M15

which produced more than >90% of total flowers within 10 days, were located in the major cluster B, suggesting to be considered as possible parents to cross with AC0219 that produced lowest percentage of flowers.

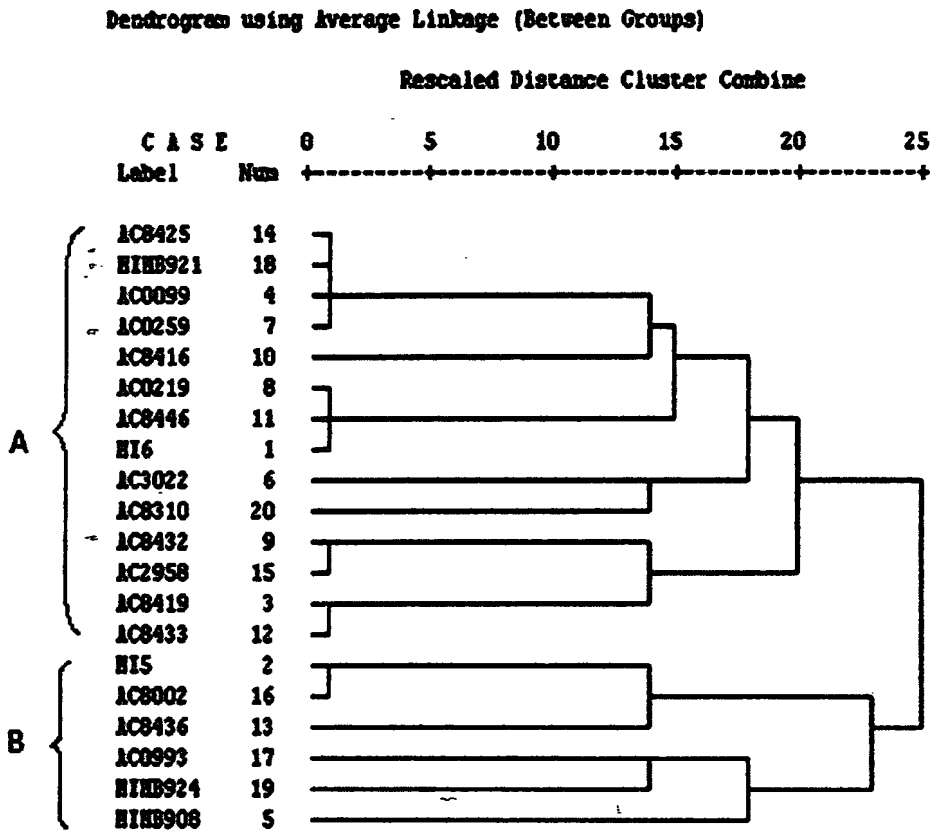

Figure 3. Unweighted pair group method with arithmetic mean (UPGMA) dendrogram drawn among twenty mung bean genotypes based on three SSR markers

CONCLUSIONS

Analysis of variance revealed significant variations among mungbean accessions for most of the observed characters with P<0.001. Mungbean accession AC0099 showed the least flowering duration while AC0219 showed longest flowering duration. Although shorter flowering duration was favorable for synchronous pod maturity, it has given a low yield in this study. Successful PCR amplification was found with four SSR makers, indicating reproducible polymorphism among 20 accessions, thereby indicating distantly related accessions to be selected as parents in gene mapping studies. Considering both

phenotypic and genotypic results of twenty mungbean accessions, it could be suggested that AC0219 would be suitable to develop a mapping population with either AC8436 or M15 accessions targeting on mapping for synchronous pod maturity.

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