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Development of Genome-wide Simple Sequence Repeat Markers from Whole-genome Sequence of Mungbean (*Vigna radiata*)

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ABSTRACT

Background: Mungbean is an important pulse crop and it is mainly cultivated in Asia for human consumption. Its small genome and diploid nature make it a well-suited model organism among legume crops. Thus, cost-effective, reliable and highly polymorphic molecular markers distributing the whole genome are needed for diversity, mapping and functional genomics studies in this model species.

Methods: The whole-genome sequence of mungbean was obtained and used as a source of identification of simple sequence repeats (SSR). The sequence reads were aligned and SSRs detection was performed using the Phobos plugin tandem repeat finder in the Geneious software program. A total of 12 mungbean genotypes were selected to validate the newly developed SSR markers.

Result: In the present study, a total of 12, 49,774 and 11, 86, 386 perfect and imperfect SSR repeats were identified from the mungbean genome. The tri-repeats were the most abundant (26.10%), followed by hexa (20.82%), penta (20.45%), tetra (17.65%) and di-repeats (14.95%). We designed 1330 SSR primers based on the genomic sequence of flanking perfect SSRs (Di and tri-repeats). Among them, 50 SSR primers uniformly distributed across the 11 mungbean chromosomes were selected and used to validate 12 mungbean genotypes. The newly developed genomic SSR markers generated in the present study are a valuable genomic resource for the mungbean breeding programs.

Key words: Genetic diversity, Mungbean, Simple sequence repeats, Whole-genome sequence.

Mungbean (*Vigna radiata*) is an important grain legume crop in Asian agriculture, particularly in India and is becoming popular in Asia's contiguous areas. It is well suited to many cropping systems and constitutes an essential source of cereal-based diets worldwide, covering more than six million hectares per annum (Karthikeyan *et al*., 2014). Mungbean originated in India and is predominantly grown in Asian countries such as China, India, Pakistan and Thailand. India is the world's largest producer and the total area covered under mungbean in India was 3.44 million ha with a total production of 1.78 million tonnes in 2019-2020. Mungbean seed has 24% digestible protein with low flatulence and is rich in vitamin A, iron, calcium and zinc. It is a drought-resistant crop suitable for dryland farming and predominantly used as an intercrop with other crops. Diverse uses of mungbean make it a more widely desired crop plant and are rapidly increasing its demand. Therefore, it is necessary to improve traits with economic value, including yield potential, protein content and biotic and abiotic stress resistance, to enhance mungbean potential as a crop (Nair *et al.,* 2019). Different breeding strategies have been used to improve the economically important traits in the mungbean. However, the success is limited due to the precision in breeding programs. Advances in sequencing technology have provided a powerful set of genetic tools which can facilitate the establishment of genomics-based

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breeding programs. Plant geneticists believe that molecular markers are useful genomics tools in plant breeding programs to make the selection more efficient. With the advent of molecular markers, selection decisions were made by integrating information from molecular markers and phenotypic data through marker-assisted selection (MAS) (Fujino *et al*., 2019).

At present, simple sequence repeats (SSR) and single nucleotide polymorphism (SNPs) play an important role in many types of genetic analyses, including the construction of linkage maps, QTL mapping, diversity assessment of germplasm and identification of molecular markers for MAS in different crops. However, only a few hundred mungbean SSR markers have been developed for QTL mapping and linkage maps associated with important traits like resistance to abiotic and biotic stress and yield (Kitsanachandee *et al*., 2013; Tangphatsornruang *et al.,* 2009; Kaewwongwal *et al*., 2017). Owing to its small genome size and evolutionary divergence, the mungbean is a potentially important model plant among the legumes. The publishing of the whole genome sequence (WGS) in 2014 (Kang *et al*., 2014) has accelerated mungbean research in diverse ways. The availability of WGS provides information not only for the identification of genes, study of the evolution of species, genome structure but also is an ideal resource for the genome-wide identification of SSRs.

With this backdrop, we have developed a new set of SSR markers for augmenting the genomic resources especially, to assist the genetic and molecular dissection of mungbean genes that encode traits with economic value, including quantitative traits. This was achieved by using the following procedures: (i) identification of the SSRs from mungbean WGS; (ii) design of primer pairs in regions flanking the SSR motifs from WGS; (ii) experimental validation of representative SSR markers using fragment analysis among 12 mungbean genotypes.

SSR identification and primer design

The mungbean WGS (Vr 1.0), which is publicly available at https://legumeinfo.org/gbrowsevigra1.0 (Verified 28th Feb 2018), was obtained and used as a source of identification of SSRs with di-, tri- and tetranucleotide repeats. Geneious software (Kearse *et al*., 2012) was used to screen the mungbean genome sequences. The parameter used for SSRs identification in this study was from 2-8 bp motifs and mononucleotide was not considered due to the difficulty of distinguishing real SSRs from sequencing or assembly error. DNA sequences were searched for perfect and compound or imperfect SSRs, with a basic motif for 2-8 bp. Di-, tri- and tetranucleotide SSRs with a repeat number of five or greater were identified. The presence of repeat regions (SSRs) in the mungbean genome and their positions were determined through the Phobos plugin available in the Geneious software. The results from the PHOBOS output were filtered to identify only the perfectly matching SSRs. Primer premier 3.0 (PREMIER Biosoft International, Palo Alto, California USA) (Rozen and Skaletsky, 2000) was used to design primer pairs from the genomic sequence flanking perfect SSRs only.

Plant genetic materials and genomic DNA isolation

The seeds of the 12 mungbean genotypes used in this study were obtained from the National Pulses Research Center, Tamil Nadu Agricultural University, Vamban, Tamil Nadu and India. The genomic DNA was isolated from 10-day-old young leaves of mungbean genotypes using the cetyltrimethyl ammonium bromide (CTAB) method (Sudha, 2009). The quantity and quality of the genomic DNA were confirmed using UV Bio-spectrophotometer (Eppendorf, Germany) and agarose gel (0.8%). The final concentration of all the samples was adjusted to 50 ng/µl Biospectrophotometer).

PCR analysis and validation of selected SSRs

A total of 50 SSR markers, at least four SSR markers per chromosome, covering the eleven mungbean chromosomes were selected and used for validation in mungbean genotypes. The PCR analysis for SSR markers and gel electrophoresis conducted following the methodology adopted by Karthikeyan *et al*. (2012) and Sudha *et al*. (2013). Briefly, PCR was conducted in a total volume of 10 µl, including 1 µl of genomic DNA, 0.5 µl of the forward primer and 0.5 µl of reverse primer (Eurofins Genomics India, Bangalore, India), 7.0 µl 1X master mix (Amplicon, Denmark) and 1 µl of ddH_2O. Amplification was carried out in a thermal cycler (Eppendorf, Germany) programmed to run the following temperature profile: 3 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55-60°C and 1 min at 72°C. The final elongation step was extended to 10 min at 72°C and finally maintained at 4°C. The five microliters of PCR products were separated by Agarose gel (3%) electrophoresis in 1X TBE bufier with 120 V for 2.0 h and the amplified bands were visualized in the gel documentation system (Gel Doc XR + Imaging system, BIO-RAD, USA). The general scheme for developing SSR markers from WGS and their validation in mungbean genotypes presented in Fig 1.

The Mungbean crop is valued for its nutritional and agronomic benefits. However, mungbean breeding programs have stagnated due to limited funding from various funding agencies and research organizations. Thus, more breeding programs integration with genomic tools have to be planned for crop improvement. Molecular markers are powerful genomics tools to study the genetic variation and identify the genomic regions/ genes associated with a specific trait in plants. Among the various marker systems, SSR was the best choice for various applications, primarily in marker-assisted breeding (MAB) programs. Compare to other markers, SSR markers are reliable and relatively inexpensive molecular markers. Because they use PCR, are codominant and have high levels of allelic diversity. Unfortunately, the number of markers from mungbean on a public platform is very limited. Primarily, random markers and SSR markers from closely related species, including adzuki bean and soybean were used in mungbean genetic

studies (Isemura *et al.,* 2012; Sudha *et al*., 2013; Kitsanachandee *et al*., 2013). The first version of the mungbean genome sequence was recently published and it is a game-changer to mungbean molecular breeding programs. It facilitated to development of genome-wide markers and cost-effective genotyping platforms. The availability of WGS of mungbean offers the chance to develop genome-wide SSRs.

Discovery and mining of genomic SSR loci from WGS have had fruitful applications in many plant species, including soybean (Song *et al*., 2010), peanut (Lu *et al*., 2019), castor bean (Tan *et al.,* 2014). In the present study, a total of 12, 49,774 and 11, 86, 386 perfect and imperfect SSR repeats with di-, tri-, tetra-, penta and hexanucleotide repeats equal to, or longer than 8, 5, 4, 3 and 2 repeat units were identified from the mungbean genome. This study examined the frequency and distribution of SSRs with motifs of 2–8 bp long and the least lengths of 18 bp in the mungbean genome. The standard we used was based on the point that polymorphism levels and mutation rate correlate positively with the number of repeat units. As a result, a higher polymorphic ratio is expected for these SSR markers developed in this study. Frequency analysis of various nucleotide repeats in mungbean shown that tri-repeats were the most abundant (26.10%), followed by hexa (20.82%), penta (20.45%), tetra (17.65%) and di-repeats (14.95%). There were 186839 di-repeats, 326232 tri-repeats, 220922 tetra-repeats, 255544 penta-repeats and 260237 hexa-repeats (Fig 2 and Table 1). These results agree with the reports of Katti *et al*., 2001; Lawson and Zhang, 2006; Bhandawat *et al*., 2016, who described that tri repeats were more abundant in various crops.

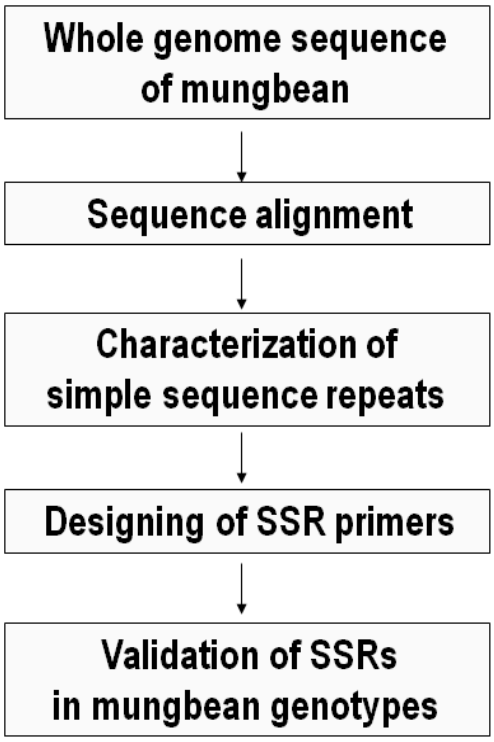

Fig 1. Schematic representation for the development and validation of mungbean SSR markers

Among the tri-repeats, (ATT) n is the most abundant (44.8%), followed by (AAG) n (23.6%), (AAC) n (13.1%), (AGG) n (4.1%), (ACT) n (3.9%), (AGT) n (3.8%), (ACC) n (3.8%), (ACG) n (1.0%), (AGC) n (1.0%) and (CCG) n (0.9%). Of the dinucleotide motifs, (AT) n is the most abundant (58.5%), followed by (AG) n (24.2%) and (AC) n (17.2%). The (GC) n motif is the least frequent (0.1%) dinucleotide in the genome. This result is consistent with other studies indicating that genomic SSRs with GC-rich repeats are rare in dicot species (Wang *et al.,* 1994; Tangphatsornruang *et al.,* 2009). Among the di repeats, the AT was the most abundant repeats. AT repeats have been reported most abundant dinucleotide repeats in many crops (Cardle *et al*., 2000; Morgante *et al*., 2002). AAT and AAAT repeats were abundant for tri and tetranucleotide repeats, respectively. ATT/AAT has been reported to be the most common tri-nucleotide motif in other crops, including soybean (Cregan *et al*., 1999), groundnut (Ferguson *et al*., 2004) and chickpea (Lichtenzveig *et al*., 2005).

SSR repeat regions in the mungbean genome and their positions were determined by the Phobos plugin available in the Geneious software. The results from the PHOBOS output were filtered to identify only the perfectly matching SSRs. Moreover, Phobos does not interact directly with Primer 3, but Phobos were used through Geneious. The results of the loci search in Phobos can be easily piped to Primer 3. We designed a set of primer pairs based on the genomic sequence of flanking perfect SSRs (Di and tri-repeats) (Table 2) only and validated a subset of primer pairs. A total of 1330 SSR primers were designed. Among them, 50 SSR primers uniformly distributed across the 11 mungbean chromosomes were selected and used to validate

Table 1. Distribution of different nucleotide repeats in the mungbean genome.

Motif length	Perfect		Imperfect	
	No. of loci identified	Frequency (%)	No. of loci identified	Frequency (%)
Dinucleotide	186839	14.95	184460	15.55
Trinucleotide	326232	26.10	259354	21.86
Tetranucleotide	220922	17.68	226674	19.11
Pentanucleotide	255544	20.45	257609	21.71
Hexanucleotide	260237	20.82	258290	21.77
Total	1249774	100	1186387	100

12 mungbean genotypes originated from India (Fig 3). Initially, these primer pairs' effectiveness was detected in mung bean genotype VRM (Gg) 1 and then tested in other genotypes. Eventually, 10 of 50 SSR primer pairs were identified as polymorphic. It shows that newly developed SSR primers had good amplification efficiency and were the potential to analyse the genetic diversity in mungbean accessions. The proportions of effectiveness and polymorphism were lower than had been observed in similar studies in mungbean and its relatives (Wang *et al*., 2015; Chen *et al*., 2015; Pratap *et al.,* 2016). The low polymorphism may be due to the low diversity of samples used, given that the 12 genotypes were all from India. A study of SSR and other molecular markers in the Indian mungbean similarly showed limited diversity (Singh *et al*., 2014; Gupta *et al*., 2014; Kaur *et al.,* 2018). The low diversity in *Vigna* germplasm has been reported for several *Vigna* species, including mungbean, urdbean, ricebean and adzuki bean, except when the tested samples were selected wide geographical region or wild genotypes were included. In summary, the novel genomic SSR markers generated in the present study are a valuable genomic resource for the mungbean research community. These newly developed SSR markers will help assess genetic diversity, fill the gaps of current maps, QTL fine mapping and map-based cloning of genes of interest in mungbean.

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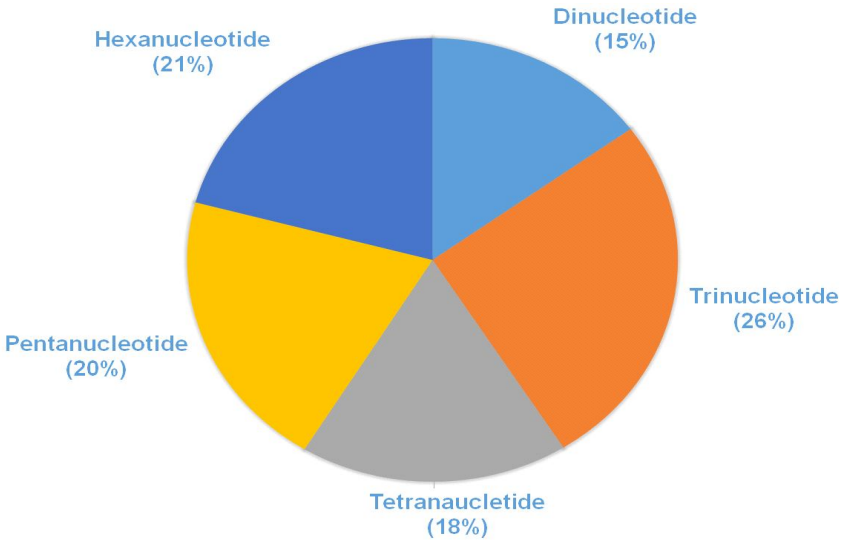

Fig 2. Distribution of different nucleotide repeats in the mungbean genome.

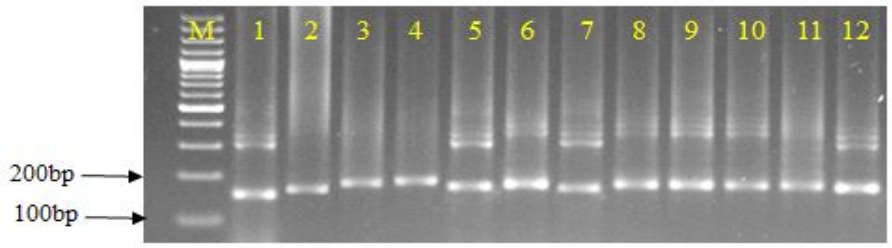

Fig 3. PCR amplification of SSR marker Vr_SSR_03_ 07 on mungbean genotypes (M – Marker 100bp; 1-12: VRM (Gg) 1, HG 22, CO (Gg) 6, CO (Gg) 7, CO (Gg) 8, VBN (Gg) 2, VBN (Gg) 3, ADT3, Tenkasi, Paiyur-1, Srivilliputhur and Kovilpatti.

Table 2. Details of perfect SSR repeats in the mungbean genome.

Chromosome	1	2	3	4	5	6	7	8	9	10	11
Dinucleotide	19462	14203	8308	11907	21957	19363	31657	24851	11300	12041	11790
Trinucleotide	27266	19406	10609	16124	98683	28094	42939	35108	15971	16610	15422
Tetranucleotide	23089	16611	9403	13920	25493	23857	37363	29829	13611	14218	13528
Pentanucleotide	26582	19281	11122	15750	29726	27110	43326	34700	15701	16508	15738
Hexanucleotide	27203	19334	11025	16351	30037	28299	44166	35047	16305	16663	15807

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