*International Journal of Plant Breeding* ©2009 Global Science Books

# Development and Characterization of Trinucleotide Microsatellite *Loci* from *Vigna radiata*

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## ABSTRACT

Microsatellites are very useful as co-dominant markers having a large number of alleles. We report here the development and characterization of 12 microsatellite loci from *Vigna radiata* L. Wilczek. These sequenced tagged microsatellite loci (STMS) were evaluated using collection of 87 mungbean genotypes. Six STMS were polymorphic with 3 to 10 alleles per locus with an average of 6.2. The PIC values for the six STMS ranged from 0.25 to 0.98 with an average of 0.71. These STMS will be useful in assessing genetic variation within *V. radiata*.

**Keywords:** mungbean, PIC, polymorphism, STMS

**Abbreviations: ISSR**, Inter Simple Sequence Repeats; **PCR**, Polymerase Chain Reaction; **PIC**, Polymorphic Information Content; **RAHM**, Random Amplified Hybridizing Microsatellite; **RAPD**, Random Amplified Polymorphic DNA; **STMS**, Sequence Tagged Microsatellite Site

## INTRODUCTION

For most Asians legumes are a major dietary source for protein. A number of legumes that are very popular in Southeast Asian countries belong to the subgenus *Ceratotropis* of the genus *Vigna* which is also known as "Asian Vigna" as the species within this subgenus have originated from Asia (Saini *et al*. 2008). There are eight cultivated species under Asian Vigna, of that *Vigna radiata* (mungbean) is one of the major pulse crops of India (Sathyamoorthi *et al*. 2008). Being a centre for diversity, a large collection of mungbean germplasm is available in the country. For the maintenance, characterization and for the efficient use in breeding, diversity analyses of the germplasm plays an important role. With the availability of new techniques genetic diversity and relationships among the species from subgenus *Ceratotropis* has been analyzed by variety of markers. The interspecies and intraspecies relationships among species belonging to *Ceratotropis* have been analyzed by RFLP (Fatokun *et al.* 1993), RAPD (Kaga *et al.* 1996; Santalla *et al.* 1998), ITS sequence analysis (Doi *et al.* 2002; Goel *et al.* 2002; Saini *et al.* 2008) and recently by 5S rRNA IGS sequence analysis (Saini and Jawali 2009). A few linkage maps of mungbean based on RAPD and RFLP markers are presently available (Menacia-Hautea *et al.* 1992, 1993; Lambrides *et al.* 2000; Humphry *et al.* 2002). Genetic diversity among the varying number of mungbean accessions has been analyzed by RAPD alone (Afzal *et al*. 2004; Saini *et al*. 2004; Lakhanpaul *et al*. 2000) or in combination with ISSR (Ajibade *et al*. 2000; Chattopadhyay *et al*. 2005). Use of RAHM, another microsatellite-based marker (Cifarelli *et al.* 1995), for mungbean analysis is also reported (Prasad *et al.* 1999). Microsatellite loci for *V. radiata* (Kumar *et al.* 2002) as well as *V. unguiculata*, a closely related species (Li *et al.* 2001) have been reported.

Microsatellites, tandem repeated units of 1 to 6 nucleotides, are abundant and ubiquitous in the eukaryotic genomes (Powel *et al.* 1996; Kubis *et al.* 1998; Varshney *et al.* 2002; Cuc *et al*. 2008). A number of markers have been developed to exploit polymorphism in and around microsatellites (Powel *et al.* 1996; Gupta *et al.* 2000; Varshney *et al.* 2005). Microsatellite based semi-arbitrary, multi-*loci* fingerprinting methods involves inter-repeat amplification using single or double primers. The markers using single primer amplifies region between two adjacent, inversely oriented repeats. Among the methods that use multiple primers one of the primers will always have a microsatellite sequence and amplifies region between a repeat and a non-repeat priming site. These methods are based on the following properties. Microsatellite loci are abundant and well dispersed in the genome. Frequently adjacent copies of a particular repeat will be in inverse orientation and within PCR amplification distance. Any insertions or deletions between conserved regions or sequence variation at a priming site will appear as PCR polymorphisms when a repeat specific primer is used.

STMS markers are also known as SSR markers, Simple Sequence Length Polymorphisms (SSLP) or microsatellites (Powel *et al.* 1996). In contrast to the above, STMS markers are essentially single locus markers specific for a species of interest. As the name indicates, PCR amplification using a STMS primer pair amplifies a specific region carrying a microsatellite. The STMS polymorphism is due to variations in the number of core repeat units and hence the number of alleles for a locus can be large. Although the development of STMS is tedious and expensive, the following attributes make them more attractive markers than other markers and have gained importance as a marker of choice for variety of applications (Gupta *et al.* 2000; Varshney *et al.* 2005). Firstly they are highly informative as the number of alleles for a locus is large. Secondly, STMS markers are co-dominant and are abundant and dispersed throughout the genome. STMS have also the flexibility to be used as STS to provide anchors between genetic linkage maps and physical chromosomal location. STMS markers can also be applied to related species and sometimes are also transferable across genera (Choumane *et al.* 2000; Phansak *et al.* 2005). We report here the development of STMS for 12 loci and their characterization using 87 mungbean genotypes, including genotypes from institutes within and outside India.

Received: 6 February, 2008. Accepted: 26 April, 2009.

Original Research Paper

## MATERIALS AND METHODS

### Material

The mungbean accessions used in this study are listed in **Table 1**. The restriction enzymes, agarose, high resolution agarose, 50 bp DNA ladder and other molecular biology reagents were from Sigma-Aldrich Corporation, St. Louis, Missouri, USA. The *Taq* DNA polymerase, Polynucleotide kinase and DNA ligase were from Bangalore Genei Ltd. Bangalore, India. The chemicals such as $CsCl_2$, EDTA, TRIS, etc. were molecular biology grade chemicals from Sigma-Aldrich Corporation, St. Louis, Missouri, USA.

### DNA extraction

DNA was isolated from young leaves according to Krishna and Jawali (1997). DNA preparation was treated with RNAse and further purified. DNA concentration was estimated as detailed in Prasad *et al*. (1999). The DNA isolated from mungbean cv. 'TARM18' was further purified by $CsCl_2$ gradient centrifugation (Sambrook *et al*. 1989).

The microsatellites were isolated by cloning small sized DNA fragments and identifying by hybridization followed by sequencing. Total DNA isolated from mungbean cv. 'TARM18' was digested with *Alu*I and size fractionated by sucrose density centrifugation. The fraction with an average size of 500 bp was ligated to *Eco*RV-digested pBluescript $SK^+$II (Stratagene, La Jolla, California, USA). The ligation product was transformed into *E. coli* DH5α. A library of recombinants was obtained by growing the transformants on Bi-Bond nylon membrane (Sigma-Aldrich Corp., St. Louis, Missouri, USA) and the master plates were preserved at -20°C on glycerol LB plates (Sambrook *et al.* 1989). Two replica libraries on nylon membranes were lysed, denatured and neutralized and probed with oligonucleotides $(AAC)_5$ and $(ACC)_5$ labeled with $^{32}P$ using $\gamma^{32}P$-ATP (BRIT, Mumbai, India) and polynucleotide kinase. Prehybridization, hybridization and washing were performed according to Church and Gilbert (1984). Hybridization was carried out at temperatures calculated for individual probe according to Sharma *et al.* (1995). Insert DNA from the clones giving a hybridization signal were sequenced. The plasmids were prepared by a maxiprep method (Sambrook *et al.* 1989). The insert DNA was sequenced from both ends using M13 Rev (5′-CAGGAAACAGCTATGAC-3′) and M13 For (5′-GTAAAACGA CGGCCAGT-3′) primers using an ABI PRISM Big Dye Terminator Ready Reaction kit (Applied Biosystems Inc, Foster City, CA USA). After purification, the PCR products were applied to an ABI 377 automated DNA sequencer (Applied Biosystems Inc, Foster City, CA USA). Sequence information was extracted and edited by Sequence Analysis software and used for designing primers by Primer Designer Version 1.01 (Scientific and Educational Software, http://www.bioinformatik.de/cgi-bin/browse/Catalog/PCR/Primer_Design/). Primer pairs were designed for 12 putative microsatellite loci and were custom synthesized by BRIT, Mumbai, India. These microsatellite loci were characterized by analyzing them among a set of 87 mungbean genotypes (**Table 1**). Amplifications were performed in a volume of 25 µl containing 50 ng genomic DNA, 2.5 units of *Taq* DNA polymerase, 0.2 mM dNTPs, 01% gelatin, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 9.0, 50 mM KCl and 0.5 µM of each primer. PCR was performed on an Amplitron II Thermolyne cycler. Thermal cycling included initially 5 min denaturation at 94°C followed by 35 cycles consisting of denaturation at 94°C for 1 min, 45 sec at annealing temperature of the primer pair (**Table 3**) and 30 sec extension at 73°C, and ending with a final step of 10 min at 73°C for extension. The allele analysis was carried out by electrophoresis run at 300 V on a horizontal gel (20 cm × 15 cm) using 1X TBE and a 2-4% high-resolution agarose. The gel was stained with ethidium bromide, visualized under UV transilluminator (Sambrook *et al.* 1989) and photographed using Asahi Pentex MX camera with a UV filter attachment (Hoya Corp., Tokyo, Japan). The sizes of the alleles were determined using a 50 bp DNA ladder as marker.

**Table 1** Mungbean genotypes used for genetic diversity analysis.

| Genotypes of Mungbean | Pedigree/source | Characteristics |
|---|---|---|
| TARM-1 | RUM 5 X TPM1 Trombay, BARC | |
| TARM-2 | idem. | |
| TARM-13 | idem. | |
| TARM-18 | PDM54 X TARM2 Trombay, BARC | Late flowering |
| TARM-21 | RUM 5 X TPM1 Trombay, BARC | Powdery mildew |
| TARM-22 | idem. | resistant |
| TARM-26 | idem. | |
| TARM-32 | idem. | |
| TARM-35 | idem. | |
| TPM-1 | idem. | |
| E-11-18 | PDM 54 X TARM2 Trombay, BARC | |
| E-11-24 | idem. | |
| E-24-26 | idem. | |
| E-26 | idem. | Early maturity |
| E-28 | idem. | Powdery mildew |
| E-29 | idem. | resistant |
| E-30 | idem. | |
| E-31-5 | idem. | |
| E-48 | idem. | |
| E-65 | idem. | |
| E-92-3 | idem. | |
| PUSA-22 | IARI, Delhi | |
| PUSA-SR-22 | idem. | |
| PUSA-71 | idem. | |
| PUSA-102 | idem. | |
| PUSA-103 | S-8 mutant X Cj-4 IARI, Delhi | |
| PUSA-104 | Ps-16 X V3476 IARI, Delhi | |
| PUSA-105 | Tainan X ML6 X EG-MG-16 X ML3 IARI, Delhi | Tolerance to YMV, powdery mildew, Macrophomia blight, aphids, white flies |
| PUSA-116 | IARI, Delhi | |
| PUSA-117 | idem. | |
| PUSA-168 | idem. | |
| PUSA-90-11 | idem. | |
| PUSA-90-31 | idem. | |
| PUSA-90-71 | idem. | |
| PUSA-91-31 | idem. | |
| | idem. | |
| PUSA-93-71 | idem. | |
| PUSA-93-72 | idem. | |
| PUSA-94-72 | idem. | |
| PUSA-95-31 | Selection from NM-9473 IARI, Delhi | YMV resistant |
| PUSA-95-71 | idem. | |
| PUSA-96-31 | idem. | |
| PUSA-96-32 | idem. | |
| PUSA-96-72 | idem. | |
| PDM1 | Selection of germplasm collected | Early maturity, YMV |
| PDM-54 | from Kundawa IIPR, Kanpur | resistant |
| PDM-84-131 | idem. | |
| PDM-116 | idem. | |
| ML-3 | PAU, Ludhiana | Tolerant to powdery mildew |
| ML-5 | No; 54 X Hyb 45 PAU, Ludhiana | Tolerant to YMV |
| Ml-6 | PAU, Ludhiana | Early maturity |
| ML-127 | idem. | |
| ML-337 | Ml1 X ML 987 PAU, Ludhiana | |
| CO-5 | KM2 X Mg-50-10 TNAU, Coimbatore | Tolerant to YMV |
| WGG-13 | Warangal, Andhra Pradesh | |
| WGG-35 | idem. | |
| WGG-48 | idem. | |
| WGG-320 | idem. | |
| LGG-410 | LAM. Andhra Pradesh | |
| LGG-444 | idem. | |
| LGG-450 | idem. | |
| LGG-458 | idem. | |
| MMG-316 | - | |
| UPM-92-3 | - | |

**Table 1** (Cont.)

| Genotypes of Mungbean | Pedigree/source | Characteristics |
|---|---|---|
| NARP-1 | - | |
| PIMS-1 | - | |
| NN-19-19 | Pakistan | Early maturing mutants and resistant to YMV |
| NN-94-73 | idem. | |
| NN-20-21 | idem. | |
| AKM-30 | Maharastra, India | |
| NAYAGARH | Local variety, India | |
| GHAGA-1 | Sriganganagar, India | |
| CHANTANPUR | - | |
| EC 337104 | AVRDC, Bangladesh | |
| EC 318991 | idem. | |
| PHULE M-1 | Maharastra, India | |
| VC-6144-B-10 | AVRDC, Bangladesh | Bold seed and tolerant to YMV |
| VC-6173-B-6 | idem. | |
| VC-6173-B-13 | idem. | |
| VC-3960-A-88 | idem. | |
| VC-6173-C | IARI, Delhi | |
| PUSABHARTI-IVTRII7 | idem. | |
| RUM-5 | Raipur uteramung from Raipur | Powdery mildew resistant |
| JL-781 | Selection from China moog | |
| KOPERGOAN | Local selection from Maharastra, India | Bold seed and high yielding variety |
| TAP-7 | Mutant of S- 8, BARC/Akola, India | Tolerant to powdery mildew |
| TM-97-25 | Kopergoan X TARM 2 Trombay, BARC | Tolerant to powdery mildew |

## RESULTS AND DISCUSSION

Twenty-six clones were found to carry unique sequences and among these a clone was considered as microsatellite if its sequence contained a minimum five repeats of dinucleotide or four repeats of trinucleotide. Twelve clones were identified to harbor microsatellite, of which 5 were perfect, 5 complex and 2 imperfect repeat structures (**Table 2**). Details of nucleotide sequence of primers for STMS loci and their names are given in the **Table 3**. Since the length and GC content of the primers for a locus were different, the PCR condition for each STMS was optimized using DNA from TARM 18 and the results including the expected allele length are given in **Table 3**. All the 12 STMS primer pairs yielded PCR product of expected size. Analyses of 87 mungbean genotypes that included genotypes from different institutions within and outside India (**Table 1**) revealed that six STMS were polymorphic and yielded 38 alleles. As an example the profile of ViRad 26 STMS profile from a few genotypes is shown in **Fig. 1**. The number of alleles per

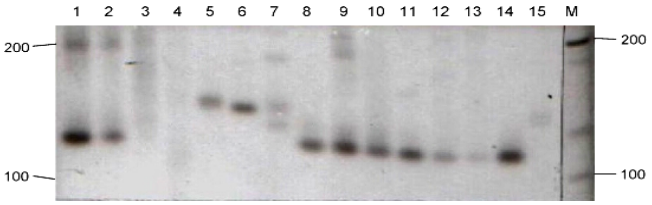


**Fig. 1 Polymorphism at the ViRad 26 *locus* obtained among the mungbean genotypes.** Lanes 1-15 corresponds to the mungbean genotypes. The lane M shows a DNA ladder in base pairs being used as marker. The PCR amplification was carried out as detailed in Materials and Methods except the annealing temperature used was 55°C. The PCR products were separated on 4% high resolution agarose gel and for the sake of clarity a negative of the photograph is shown.

locus ranged between 3 and 10 with an average of 6.2. A maximum of 10 alleles was detected for the ViRad 9 locus. The allele frequencies ranged from 0.01 to 0.80 with a mean value of 0.10 (**Table 3**). Thirty alleles occurred at a frequency of ≤ 0.10. Null alleles were obtained at all the microsatellite loci. Maximum number of null alleles (in 65 genotypes) were at ViRad 26 and minimum (9 genotypes) at ViRad 13 locus. The PIC value for the STMS ranged from 0.25 to 0.98 with an average of 0.71 and the locus harboring TTC repeat exhibited the highest average PIC value of 0.98. Previously, Kumar *et al.* (2002) reported the characterization of seven polymorphic tetranucleotide microsatellites loci in *V. radiata*. The number of alleles per locus ranged from 2 to 6. Li *et al.* (2001) developed 46 microsatellite DNA markers for cowpea [*Vigna unguiculata* (L.) Walp] and evaluated genetic similarities among 90 cowpea lines. They found 27 STMS were polymorphic with 2 to 7 alleles per primer and a PIC varying from 0.02 to 0.73. Subsequently, Vijaykumar and Jawali (2007) used 22 of the STMS of Li *et al*. (2001) to analyze genetic relationship among 21 geographically diverse accessions of *V. unguiculata* ssp. and found 12 were polymorphic with number of alleles per locus ranging between 2 and 11. They also observed the presence of null alleles in six loci. Their results indicated an interplay of geographic isolation (founder effect) and inter sub-species hybridization among the *V. unguiculata* ssp. The characteristics of the STMS developed in this report are comparable with the STMS reported from mungbean (Kumar *et al.* 2002) and cowpea (Li *et al.* 2001). It needs to be evaluated as to how many of the cowpea STMS would be useful for mungbean.

The microsatellites reported here along with the ones reported in the literature will be useful in determining genetic diversity in the germplasm collection and in constructing a linkage map of mungbean.

## ACKNOWLEDGEMENTS

We thank Dr. S. K. Mahajan for his support and Dr. K. S. Reddy for providing the mungbean germplasm.

**Table 2** Characteristics of mungbean clones containing microsatellite repeats.

| Clone name | GenBank Accession number | Repeat sequence | No. of repeat units | Nature of repeat |
|---|---|---|---|---|
| SS1 | AY253195 | $(GTG)_5N_{23}(TGAATG)_6$ | 6 | Perfect |
| SS5 | AY253198 | $T(GTT)_5$ | 5 | Perfect |
| SS9 | AY253200 | $(CAC)_3(ACC)_2(TTC)_8$ | 8 | Complex |
| SS11 | AY253202 | $(GTT)_2(GCT)(GTT)_4$ | 7 | Imperfect |
| SS12 | AY253203 | $(TGG)ATA(TGG)_2T(GA)_5$ | 5 | Complex |
| SS13 | AY253204 | $(CAA)_8$ | 8 | Perfect |
| SS16 | AY253207 | $(TTG)_2N_9(TTG)_4$ | 4 | Imperfect |
| SS18 | AY253208 | $(CAA)_6$ | 6 | Perfect |
| SS19 | AY253209 | $(CAT)_2N_8(CAT)_3\ldots..(AC)_7$ | 7 | Complex |
| SS24 | AY253213 | $(CAC)_3AC(CAC)C(TTC)_8N_{27}(TC)_6$ | 8 | Complex |
| SS26 | AY253214 | $(GA)_{10}N_{15}(AGA)_7N_7(AGA)_2AAA(GTT)_1T(GTT)_4$ | 10 | Complex |
| SS30 | AY253216 | $(ACC)_6$ | 5 | Perfect |

**Table 3** Characteristics of mungbean STMS loci.

| Clone name | STMS *locus* | Primer | Primer sequence | Length (bp) | Annealing Temp (°C) | Product size (bp) | | Number of Alleles | PIC |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Expected* | observed | | |
| SS1 | ViRad1 | SS1.1 | 5′ CTCGTCTGAGATCGGAGGAG 3′ | 20 | 60 | 312 | 312-333 | 4 | 0.84 |
| | | SS1.2 | 5′ GGCTCCAAGCACCACTATAC 3′ | 20 | | | | | |
| SS5 | ViRad5 | SS5.1 | 5′ GGAAGATGGTCATGGTGG 3′ | 18 | 50 | 120 | 120 | 1 | 0 |
| | | SS5.2 | 5′ TTCTCAGTTCAATGTTGTCC 3′ | 19 | | | | | |
| SS9 | ViRad9 | SS9.1 | 5′ TTAATATCACCACCACAC 3′ | 18 | 55 | 226 | 205-268 | 10 | 0.85 |
| | | SS9.2 | 5′ GTACAGGACAAGATGCTT 3′ | 18 | | | | | |
| SS11 | ViRad11 | SS11.1 | 5′ AGGTTGAGCAGTTGGTTGG 3′ | 19 | 54 | 136 | 136 | 1 | 0 |
| | | SS11.2 | 5′ CATTGCGCCACAACAAG 3′ | 17 | | | | | |
| SS12 | ViRad12 | SS12.1 | 5′ TGGTATTGTGCGTGTTGA 3′ | 18 | 50 | 97 | 97 | 1 | 0 |
| | | SS12.2 | 5′ TCTTCAGCCTCATTGTGC 3′ | 18 | | | | | |
| SS13 | ViRad13 | SS13.1 | 5′ TTGTGACCTCCACCTACTAGCA 3′ | 22 | 60 | 72 | 72-84 | 3 | 0.25 |
| | | SS13.2 | 5′ GAGGATGAGAGTTGGTTGGTAG 3′ | 22 | | | | | |
| SS16 | ViRad16 | SS16.1 | 5′ GAGAGAGTAGCAGGAAGTCC 3′ | 20 | 60 | 337 | 110-126 | 4 | 0.90 |
| | | SS16.2 | 5′ CTCATCGAGAGACACTCTTC 3′ | 20 | | | | | |
| SS18 | ViRad18 | SS18.1 | 5′ CACACACATACCATTCAGATAC 3′ | 22 | 55 | 103 | 103 | 1 | 0 |
| | | SS18.2 | 5′ CTAAGGTTGTGATTGTGATGAG 3′ | 22 | | | | | |
| SS19 | ViRad19 | SS19.1 | 5′ GACATCTCTAGTGCACACAT 3′ | 20 | 55 | 94 | 94 | 1 | 0 |
| | | SS19.2 | 5′ TGAGACACAGACACAACTCT 3′ | 20 | | | | | |
| SS24 | ViRad24 | SS24.1 | 5′ TTTAATATCACCACCACACC 3′ | 20 | 60 | 135 | 120-144 | 8 | 0.79 |
| | | SS24.2 | 5′ CTCAAATCCAACACCATAAC 3′ | 20 | | | | | |
| SS26 | ViRad26 | SS26.1 | 5′ GAAGGGTAATTCAGAGCCA 3′ | 19 | 55 | 138 | 108-150 | 9 | 0.98 |
| | | SS26.2 | 5′ CAGGCATAGTGTCACTCTT 3′ | 19 | | | | | |
| SS30 | ViRad30 | SS30.1 | 5′ CTTCTGCCTCCACCTAAACT 3′ | 20 | 55 | 109 | 109 | 1 | 0 |
| | | SS30.2 | 5′ GTGATAGGTCCGACAATACG 3′ | 20 | | | | | |

* The size of the PCR product on using TARM-18 DNA as template;

**PIC:** Polymorphic information content and was calculated using the formula: PIC = $1-\sum (P_i)^2$ where $P_i$ is the proportion of the population carrying the $i^{th}$ alleles, calculated for each of the microsatellite marker.

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