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Original Article

Study of the pattern of variation for microsatellite markers in Black gram, *Vigna mungo* (L.) Hepper germplasm

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Abstract:
In this study we present the pattern of variation in *Vigna mungo* (black gram) using sequence tagged microsatellite markers. The variability of the specific microsatellite regions was assessed in a set of 85 accessions of black gram collected from different regions of India. Eighty primer pairs were tested, out of which 30 primers gave satisfactory amplification. Some variation in the size of amplification products was observed,. Out of the 30 primers, 10 primers were found to be polymorphic. Primers VM27 and AB128079 were identified as the most efficient primers in detecting the variation among black gram accessions. The result indicates that all the characters were in general highly heritable and stable over all the locations studied. The pattern of clustering revealed that the clusters were in general predominantly represented by the accessions originating from the same state and the accessions involving common parentage also remained together between the two parental clusters. All of the primers used for this study were generated from other legume crops, so we also checked for the transferability of the primers. Out of 80 primer pairs 30 (37.5 %) were found to be transferable and out of the 37.5% transferable primers only 30% were found to be polymorphic. The results of the study help in better understanding of similarities in the genetic makeup of *Vigna* and related pulse crops. This work can further be useful for genetic diversity studies, mapping of agriculturally important traits, and this will also facilitate marker assisted breeding in black gram.

Key words *Genetic diversity, Black gram, STMS, Transferability, Microsatellites.*

Introduction

Black gram [*Vigna mungo* (L.) Hepper] is one of the Asiatic species of the pantropical genus *Vigna*. It is one of the most economically important legume crops of South-East Asia and it is widely adapted to both semi-arid and sub-tropical areas. It has a long history of cultivation in the Indian subcontinent as the carbonized grains. Black gram has been recorded from the chalcolithic sites at Navdotoli and Maheshwar dated to 1660-1440 BC [De Candolle 1883]. It is considered to have originated in the Indian subcontinent [De Candolle 1883, Vavilov 1926] with huge diversity in the Western Ghats. Its secondary centre of origin is thought to be Central Asia. In addition to being a source of protein, black gram is the richest among various

pulses in phosphoric acid (Girigowda et.al. 2005), it is also an important N-fixing crop its grains and crop residues can be used as animal feed.

Assessment of genetic diversity of pulses is an important step in a program aimed at improving crop yields. Among the molecular marker techniques, STMS (Sequence tagged microsatellites) offers a simple, efficient and economic means for cultivar identification and diversity analysis (Williams et al., 1990). Considering the importance of black gram, for supplying the much-needed proteins for the predominantly vegetarian population of the country, adaptation to shorter growth duration, lesser water supply and minimal soil fertility conditions the present study was undertaken with a view to study the genetic diversity of accessions of black gram from different regions of India.

Genetic diversity in black gram like in other living organisms largely originates and propagates due to the primary mechanism of mutation, migration and genetic drift resulting in speciation and adaptation [Maestri et al., 2002]. The loss of genetic diversity is the major threat for the maintenance and adaptive potential of the species. The elucidation of the genetic diversity has become vital for many applied fields such as plant breeding, linkage map construction and dissection of quantitative traits [Mazur and Tingey 1995]. Characterization of the genepool is an important aspect for the management of germplasm collected for the future use. Many social and ethical issues are also associated with the characterization of accessions for their proper management [Plucknett et al., 1987], as it is one of the ways to stop bio-piracy. The only way to preserve the genepool that is being lost because of the high selection pressure for the desired traits such as yield and ignoring the other economically less important traits. Over the years the genetic diversity assessment has historically progressed from a functional level to the structural level with the advent of DNA based molecular markers.

Materials and Methods

Plant material and growth conditions

The experimental material consisted of 85 accessions of black gram [Table S1] representing a wide spectrum of variability, which were chosen randomly on the basis of variations in seed colour and size from black gram collections collected from various parts of India. Healthy seeds were germinated in rolled wet towels incubated at 27^{o}C. For each accession, 4-5 g of young tissue i.e., whole seedling after removing the seed coat, from 7-8 days old seedlings were taken for DNA extraction.

Sample preparation

Total genomic DNA was extracted using the CTAB method [Saghai-Maroof et al., 1984]. DNA concentration was estimated with a DNA fluorometer (Hoeffer Scientific, San Francisco, USA) using Hoechst 33258 as the DNA intercalating dye and calf thymus DNA as the standard [Brunk et al., 1979]. These estimates were confirmed by staining DNA with ethidium bromide after electrophoresis on 0.8 per cent agarose gel at 100V for 1h in TAE buffer (0.4 M Tris-acetate, 0.001 M EDTA, pH 8.0) using known DNA concentration standards (λ DNA, uncut).

PCR optimization and primer survey

Varying concentrations of template DNA (10-40 ng), *Taq* DNA polymerase (0.5-1.5U), Mg^{2+} salt (0-2.5 mM) and annealing temperature (40-55^{0}C) were used to optimize the reaction conditions of the polymerase chain reaction, using the PCR thermo-cycler (BIOER^{TM}). Three randomly selected accessions, viz., IPU-99-219, Symp-2 and JV-469 were chosen for the primer

survey. Eighty primers from the VM [Li et al., 2001], AB [Wang et al., 2004], MB [Gwang et al., 2006], VJ [Kumar et al., 2002], AY [Basak et al., 2005, Pal et al., 2005], DQ (Gene bank) and Gi (Gene bank), series (Table S2) were surveyed with the three accessions to identify primers that were reproducible and generated the most polymorphic patterns. The properties of the STMS primers used in the study including the sequences, repeat motif and annealing temperature are present in Table S2. The annealing temperature for optimum amplification with different STMS primer pairs, ranged from 44^{O}C to 60^{O}C (Table S2).

PCR and gel electrophoresis

PCR reactions were carried out in a DNA Thermal Cycler. Each 25 µl reaction mixture contained 1X reaction buffer (10 mM Tris-HCl, pH 8.3 and 50 mM KCl), 2 mM $MgCl_2$, 0.2 mM of dNTP mix, 0.6 µM of primer mix, 1U of *Taq* DNA polymerase (MBI Fermentas, UK) and 20 ng/µl of genomic DNA. The PCR amplification conditions were as follows. Initial extended step of denaturation at 94^{0}C for 1 min followed by 30 cycles of denaturation at 94^{0}C for 1 min, primer annealing at 40^{0}C-60^{0}C (according to the primer pair used) for 1 min. and primer elongation at 72^{0}C for 1 min, followed by an extended elongation step at 72^{0}C for 10 min. Reaction products were mixed with 2.5 µl of 10X loading dye (0.25% bromophenol blue, 0.25% xylene cyanol and 40% sucrose (w/v) and spun briefly in a microfuge before loading [Sambrook et al., 1989]. The amplification products were electrophoresed on 1.8 % agarose gel at 120 volts. Gels were stained with ethidium bromide and photographed using a computerized gel documentation system under ultra-violet light.

Scoring and data analysis

Each amplification product was considered a DNA marker and was scored across all the samples. Bands were recorded either as present (1) or absent (0). Molecular weights of the bands were estimated by using Gene Ruler 100 bp DNA ladder (MBI Fermentas, UK) as standard. All amplifications were repeated at least twice and only reproducible bands were considered for analysis. The data set of accessions and reproducible bands were used to calculate pair-wise similarity coefficients following Jaccard (1908) and Nei & Li's (1973) genetic distance. This matrix of similarity coefficients and genetic distance coefficient were subjected to unweighted pair-group method analysis (UPGMA) to generate a dendrogram using the average linkage procedure (Figure S1 and S2). All the numerical taxonomic analyses were conducted using the NTSYS-PC, version 2.11 [Rohlf 2002].

Results

The characteristics of PCR product such as the product size and number of alleles per locus are presented in Table 1. The number of alleles detected with the primer pair varied from one (in 20 primers) to 5 (in 1 primer), with an average of 3 alleles per primer. The primer pairs selected, generated a total of 50 unique STMS fragments and the size of the amplicons ranged from 120 bp with AB128093 to 470 bp with VM15. The amplification profiles for primer Vm27 is given in figures 1.

With the 10 loci a total of 30 alleles were identified (allele sizes ranging from 150 bp to 320 bp). The locus VM27 detected the maximum (5) number of alleles. Primer discrimination power indices were calculated to identify a set of most useful loci for distinguishing even closely related black gram accessions (Table 1). In order to determine the most efficient primer (s), resolving power (Rp) marker indices (MI_{DI} & MI_{Ib}) and Polymorphism

information content (PIC) were calculated. Resolving Power (Rp) of the 10 primers ranged from 0.082 (MB122A) to 0.85 (AB128079). PIC value ranged from 0.002 (MB122A) to 0.92 (AB128079). The value of marker index based on diversity index (MI_{DI}) ranged from 1.00 (MB122A) to 4.26 (VM27) with the mean value 2.43, and the value of marker index based on band informativeness (MI_{Ib}) ranged from 0.08 (MB122A) to 0.85 (AB1280179) with the mean value 0.43.

Dendrograms constructed based on Nei & Li's genetic distance and Jaccard's similarity coefficients (Figure S1, Figure S2) showed a high genetic identity among the 85 accessions. The maximum Jaccard's similarity coefficient of 1 was found among many samples, minimum of 0.827 among 37 samples with an average of 0.954. Nei & Li's genetic distance corresponded to the Jaccard's values, the minimum being 0, maximum being 0.09804 and an average of 2.455091. In the UPGMA analysis, The dendrogram based on Nei & Li's genetic distance analysis revealed that the accessions were separated into 4 main clusters (Figure S1), at about 25 % similarity (average genetic distance coefficient). Jaccard's similarity coefficient analysis of 85 accessions of *Vigna mungo* revealed a 95% similarity between the accessions. The dendogram generated on the basis of similarity coefficient was divided into 3 main clusters. The pattern of clustering revealed that the clusters were not separating on the basis of origin of the place but the accessions involving common parentage remained together in between the two parental clusters (Figure S2).

Transferability of SSR primers

Intergeneric and interspecific transferability of the 80 microsatellite primers developed from cowpea *(Vigna unguiculata* (L.) Walp), mungbean *(Vigna radiata* (L.) Wilczek), and azuki bean *(Vigna angularis* (Wild) Ohwi & Ohashi), was tested for their ability to amplify microsatellite loci in black gram. Out of the 80 selected primers only 30 primer pairs showed amplification in black gram i.e. a total of 37.5% transferability was observed from all the different series of primers. Among the 30 transferable markers, only 10 (33.3%) primer pairs showed polymorphism (Table 2). Out of a total of 18 mungbean primers, 14 primers (77.8%) showed transferability to black gram accessions.

Discussion

Molecular characterization of black gram germplasm is essential for a scientific assessment of the variability and diversity for its better utilization in breeding programs. STMS markers are highly reproducible molecular markers and are found to be the most effective marker system for analysis of the genetic diversity of *Vigna mungo*. To the best of our knowledge, there are very few reports of DNA fingerprinting of *Vigna mungo* accessions using STMS markers. Till date only RAPD and ISSR markers have been used for fingerprinting black gram [Dikshit et al., 2007, Dikshit et al., 2009]. The ability to distinguish closely related accessions is a function of the high heterozygosity values of STMS markers. Their robustness enables them to be routinely employed for DNA fingerprinting. In the present study microsatellites were found to be very informative, exhibiting the high effective number of alleles, higher Rp, marker indices (MIDI & MIIB), PIC and discrimination power; thus identified as most suitable for fingerprinting purpose.

The resolving power for the 10 primers ranged from 3.6 (VM22) to 8.4 (AB128079) with an average of 4.58. The present study reaffirms the higher discrimination power of hypervariable, multiallelic SSR markers in comparison to

previous studies based on RAPDs in *Vigna mungo* by Dikshit his team in 2009. A study of relative frequencies of all amplified alleles in the 85 black gram accessions showed the most frequent allele to be $AB128113_{100b}$ (frequency of 0.858). Common alleles, having frequency of >0.50 were only 15 of the 30 alleles. A majority of the amplified alleles had frequency between 0.05–0.50. Primer VM27 was useful in amplifying many 'very rare alleles' (frequency of less than 0.01) so is the most efficient primer among the set of primers we used in the present study.

STMS markers have a higher discrimination power, and showed considerable transferability among the related crop species. Genetic diversity analysis in the present study showed low divergence. The reasons for this could be attributed mainly to the self pollinated nature of the crop, as well as use of similar or closely related parents in crossing programs, thus giving rise to the narrow genetic base or the reason could be the different origin of primers, as all of the primer pairs used in this study were generated from different crops. The present study also revealed a high level of transferability of STMS primers among related pulse crops, but lack of sufficient number of polymorphic STMS in this crop also limits the characterization of the germplasm. Mung bean primers showed maximum transferability (77.8%) of primers in blackgram accessions, this also indicates maximum genomic similarities between mung bean and black gram in comparison to other pulses.

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References

1) Basak J, Kundagrami S, Ghose TK and Pal A (2005) Partial resistance gene and predicted protein (VMYR1) of *Vigna mungo* conferring resistance against yellow mosaic virus. GenBank Accession Nos. AY297425 and AAQ63168.
2) Brunk CF, Jones KC and James TW, (1979) Assay for nanogram quantities of DNA in cellular homogenates. *Analytical Biochemistry*, 92: 497–500.
3) De Candolle A. (1883) Origine des Plantes Cultivées. (Baillière, Paris).
4) Dikshit HK, Jhang T, Singh NK, Koundal KR, Bansal KC, Chandra N, Tickoo JL and Sharma TR (2007) Genetic differentiation of *Vigna* species by RAPD, URP and SSR markers. *Biologia Plantarum*, 51: 451–457.
5) Dikshit HK, Sharma TR, Singh BB and Jyoti Kumari (2009) Molecular and morphological characterization of fixed lines from diverse cross in Mung bean (*Vigna radiata* (L.) Wilczek.). *Journal of Genetics*, 88: 341-344.
6) Girigowda, K., Prashanth, S.J. and Mulimani, V.H. 2005. Oligosaccharides of black gram (Vigna mungo.L.) as affected by processing methods. Plant. Foods. Hum. Nutr. 60: 173-180
7) Gwang JK, Chung JW and Chung HK (2006) Characterization of new microsatellite markers in mung bean, Vigna radiata (L.), *Molecular Ecology Notes,* 6:1132–1134.
8) Jaccard P (1908) Nouvelles recherches sur la distribution florale. Bul. Soc. Vaudoise. *Science Nature*, 44: 223–270.
9) Kumar SV, Tan SG, Quah SC, and Yusoff K (2002) Isolation and characterization of seven tetranucleotide microsatellite loci in mungbean, *Vigna radiata. Molecular Ecology Notes*, 2: 293-295.

10) Li C-D, Fatokun CA, Ubi B, Singh BB and Scoles GJ (2001) Determining genetic similarities and relationships among cowpea breeding lines and cultivars by microsatellite markers. *Crop Science*. 41:189-197.
11) Maestri E, Malcevschi A, Massari A and Marmiroli N (2002) Genomic analysis of cultivated barley (*Hordeum vulgare*) using sequence-tagged molecular markers. Estimates of divergence based on RFLP and PCR markers derived from stress-responsive genes, and simple-sequence repeats (SSRs). *Molecular Genetics & Genomics*, 267: 186–201.
12) Maniatis T, Fritsch EF, and Sambrook J (1989). Molecular Cloning: A Laboratory Manual. *Cold Spring Harbor Laboratory Press, New York*, Vol. 1, 2, 3
13) Mazur BJ and Tingey SV (1995) Genetic mapping and introgression of genes of agronomic importance. *Current Opinion in Biotechnol*ogy, 6: 175-182.
14) Nei, M. and W.-HUA. Li (1973) Linkage disequilibrium in subdivided populations. *Genetics,* 75: 213-219.
15) Pal A, Basak J and Ghose TK (2005) *Vigna mungo* yellow mosaic virus resistant gene candidate 2 and predicted protein, GenBank Accession Nos. AY301990 and AAQ75745.
16) Plucknett DL, Smith NJH, William JT and Anishetty NM (1987) Gene Banks and The World's Food. *Princetown University Press*, Princetown, New Jersey, USA.
17) Rohlf FJ (2002) NTSYS-pc version 2.11p numerical taxonomy and multivariate analysis system version 2.11p. *Exeter Software, Setauket*, New York.
18) Saghai-Maroof MA, Soliman KM, Jorgensen RA and Allard RW (1984) Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proceedings of the National Academy of Science. USA.* 81: 8014-8018.
19) Vavilov NI (1926) Studies on the origin of cultivated plants. *Bulletin of Applied Botany and Plant Breeding,* 16: 1-248.
20) Wang XW, Kaga A, Toomoka N and Vaughan DA (2004) The development of SSR markers by a new method in plants and their application to gene flow studies in azuki bean [*V. angularis* (Willd.) Ohwi and Ohashi]. *Theoretical and Applied Genetics,* 109: 352-360.
21) Williams JGK, Kubelik AR, Livak KJ, Rafalski JA and Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, 18: 6531-6535.