Electronic Journal of Plant Breeding (2009) 1: 12-17

Research Article

Genetic diversity as assessed by ISSR markers in Blackgram (*Vigna mungo* (L.) Hepper)

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Abstract

An investigation was carried out on a collection of 23 blackgram genotypes involving 16 released varieties, six pre release cultures and one wild species *Vigna mungo* var. *silvestris* to study the genetic diversity using twelve ISSR primers. The number of alleles produced by different ISSR primers ranged from eight to 17 with an average of 11.5 per primer and the level of polymorphism was found to be 82.05 percent. Similarity measures and clustering analyses were made using ISSR data. The resulting dendrogram distributed the 23 blackgram genotypes into five main clusters. The highest genetic similarity coefficient was measured between genotypes CBG 671 and CBG 632. The results of PCoA were comparable to that of grouping based on UPGMA and 23 genotypes were grouped into four groups. Genotype *Vigna mungo* var. *silvestris* was placed separately from rest of the genotypes in both the analyses. Grouping of varieties using ISSR markers did not show any relevance to their pedigree. All the pre release cultures in one group revealed that only a portion of genetic variation has been exploited. The results revealed that, genetic diversity is low among the varieties released from the respective institute and hence genotypes were grouped according to the research institutes from which they released. It suggests that the research institutes have to enlarge the genetic base for variety development.

Key words : Blackgram - ***Vigna mungo*** - ISSR - Genetic diversity

Introduction

Blackgram, *Vigna mungo* (L.) Hepper, popularly known as urdbean or Mash in India is a grain legume domesticated from *V.mungo* var. *silvestris*. It is a good source of phosphoric acid, proteins, carbohydrates and calcium. It contains a wide variety of nutrients and is popular for its fermenting action and thus it is largely used in making fermented foods. Major constraints in achieving higher yield of this crop are lack of genetic variability, thermo sensitivity, absence of suitable ideotypes for different cropping systems, poor harvest index and susceptibility to pests and diseases. Research on this crop has lagged behind that of cereals and other legumes. Therefore, improvement of this crop is needed through utilization of available genetic diversity.

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Genetic diversity is an important factor and also a prerequisite in any hybridization programme. Evaluation of genetic diversity would promote the efficient use of genetic variations in the breeding programme (Paterson *et al*. 1991). The accurate estimation of genetic diversity can be invaluable in the selection of diverse parental combinations to generate segregating progenies with maximum genetic variability. The study of genetic diversity can be particularly useful for precise identification of purelines or cultivars with respect to plant varietal protection and germplasm maintenance by removing the duplicity and misidentity in the core accessions. Furthermore, monitoring the genetic variability within the gene pool of elite breeding material could make crop improvement more efficient by the directed accumulation of favoured alleles.

DNA markers provide an opportunity to characterize genotypes and to measure genetic relationships more precisely than other markers (Soller and Beckmann 1983). A large number of polymorphic markers are required to measure genetic

relationships and genetic diversity in a reliable manner (Santella *et al.,* 1998). The level of polymorphism is considerably low with morphological and isozyme markers making them inadequate to study the genetic diversity in blackgram. ISSR markers have been successfully utilized for assessing the genetic diversity in the genus *Vigna* (Ajibade *et al*. 2000), blackgram (Souframanien and Gopalakrishna 2004) and greengram (Sreethi Reddy *et al.* 2008). The present study was aimed to evaluate and compare the genetic diversity in the elite genotypes of blackgram using ISSR markers.

Materials and methods

Plant material and DNA extraction

Twenty three blackgram genotypes including released varieties, pre release cultures and wild species collected from different regions of the state were used in the study. Blackgram genotypes along with pedigree, place and year of release are listed in Table 1. DNA was isolated from young seedlings using modified CTAB method (Murray and Thomson, 1980).

ISSR analysis

ISSR amplification reactions were carried out in 15 ml volume containing 25 ng template DNA, 0.5 units of Taq DNA polymerase, 0.1 mM dNTP each, 10mM primer, 1X reaction buffer and distilled de- ionized water. The PCR amplification was done using the Thermalcycler (Biorad, USA) with an initial denaturation step of 5 min at 94 °C, followed by 45 cycles at 94^0C for 1 min, 48 ^{0}C- 53 ^{0}C (depending upon the primer pair) for 2 min, 72 ^{0}C for 1 min and final extension 1 cycle of 72^0C for 10 minutes. PCR amplified products were subjected to electrophoresis in a 3% agarose gel in 1x TBE buffer at 80 v for 3 hours. Ethidium bromide stained gels were documented using Alpha Imager ^{TM} 1200. (Alpha Innotech Corporation, California, USA).

Statistical analysis

Each ISSR marker was scored as 1 and 0 for its presence and absence, respectively across the genotypes. The binary data of marker – genotype matrix was used for analysis using NTSYS –pc (Numerical taxonomy system, version 2.02 (Rohlf, 2000). The SIMQUAL programme was used to calculate the Dice co efficient (Nei and Li, 1979), a common estimate of genetic identity. Similarity matrix was utilized to construct the UPGMA (unweighted pair group method with arithmetic average) dendrogram. The marker data was then standardized for principal co- ordinate analysis (PCoA) using NTSYS – pc software to highlight the resolving power of the ordination.

Shannon index

The efficiency of primers to bring out the genetic diversity was estimated by Shannon index (Lewontin 1972). The Shannon index was calculated as $H= - \sum Pi \ lnPi$, in which p_i is the frequency of a given ISSR fragment.

Results and Discussion

ISSR band pattern

The PCR amplification using ten, 5'-anchored dinucleotide repeat primers gave rise to reproducible amplification products. ISSR primers produced varying number of DNA fragments, depending on their SSR motifs. Both minimum and maximum number of markers produced by poly (GA) primers UBC 809 (8 bands) and UBC 808 (17 bands), respectively. The primers that were based on the poly (AC) motif produced more polymorphism on average (13.5) than the primers based on the poly (AG) motifs (9.5) and poly (GA) motifs (9.3) (Table 2.).

All 10 ISSR primers studied were polymorphic and a total of 115 scorable markers were generated, of which 96 were polymorphic among the genotypes. Among the primers, UBC808 was found to amplify the highest number of ISSR fragments followed by UBC825 and UBC826 while UBC809 produced the least number of ISSR fragments. The highest amount of polymorphism was exhibited by UBC811 and UBC826 (100 per cent). Alleles produced by different primers ranged from eight to 17 with an average of 11.5 per primer and the level of polymorphism was found to be 82.05 per cent. The Shannon indices varied from 1.29 (UBC820) to 3.56 (UBC826) with an average value of 2.19 indicating high resolving power of the ISSR markers. In the present study, ISSR markers detected 82% polymorphism. High level of polymorphism was observed in blackgram by Souframanien and Gopalakrishna (2004).

ISSR markers have been successfully utilized for analysis of repeat motifs in greengram (Singh et al. 2000), genetic relationships in the genus *Vigna* (Ajibade *et al.* 2000), *Oryza* (Joshi et al. 2000), Peanut (Raina et al. 2001), varietal identification in blackgram (Ranade and

Gopalakrishna 2001) and Potato cultivars (Prevost and Wilkinson 1999). The number of potential ISSR markers depends on the variety and frequency of microsatellites, which changes with species and the SSR motifs that are targeted (Despeiger *et al.*1995). The Potential for integrating ISSR – PCR into plant improvement programme is enormous. Their applications in different crop species are sufficiently reviewed by Reddy et al (2002).

Grouping of genotypes

ISSR data generated was subjected to cluster analysis. The 23 genotypes were grouped into five groups with a Dice similarity co – efficient ranging from 0.62 to 0.92. The dendrogram comprising of the genotypes grouped into different clusters is shown in Figure 1. Among the five groups, group II was the largest with 13 genotypes and group IV had four genotypes (VBN 2, VBN(Bg) 4, VBN(Bg) 5 and T9), group I had three genotypes (Co 2, Co 4 and VBN 1), group III had two genotypes (KM 2 and VBN 3) and group V had one genotype (*Vigna mungo var. silvestris*). High similarity co efficient (0.92) was observed between CBG 671 and CBG 632. T9 was the most distinct among the genotypes.

Dice similarity coefficients ranged from 0.62 to 0.92, indicative of average level of variation among these genotypes. By the application of heirarchial cluster analysis on ISSR data, the 23 genotypes were grouped into five clusters. *Vigna mungo var. silvestris* formed into a separate group. From the dendrogram it is clear that CBG 671 and CBG 632 were closely related. The level of observed polymorphism is very high, and the ability of the ISSR technique to effectively distinguish species in the genus *Vigna* was reported by Ajibade *et al.* (2000). A narrow genetic base has been reported for the released Indian mungbean cultivars using RAPD (Santalla et al. 1998, Lakhanpaul et al. 2000) and AFLP (Bhat et al 2005). *Vigna mungo var. silvestris* with the most distinct DNA profiles are likely to contain the greatest number of novel alleles. Wild blackgram *Vigna mungo var. silvestris* carries many useful traits including the resistance to bruchid beetles *Callasobruchus chinensis* and *C. maculatus*, the most destructive pests to grain legume storage. Therefore, this subspecies should be exploited in breeding programmes to broaden the genetic base and further increase the genetic variability in blackgram breeding lines and cultivars.

The result of PCoA analysis was comparable to the cluster analysis. Twenty three genotypes formed four main clusters in PCoA analysis (Fig 2.). The genotype VBN(Bg) 5 formed the separate cluster. *Vigna mungo var. silvestris* appeared to be distinct from other genotypes as in the cluster analysis. In UPGMA, individuals with similar descriptions are gathered into the same cluster and are having high internal (within cluster) homogeneity and high external (between clusters) heterogeneity. PCoA can be utilized to derive a 2-or3-dimensional scatter plot of individuals, to highlight the resolving power the ordination in order to visualize the difference between the individual, such that the geometrical distances among individuals in the plot reflect the genetic distances among them with minimal distortion.

PCoA analysis showed that all pre release cultures and varieties released from TRRI, Aduthurai have been grouped in group I. All the varieties released from NPRC, Vamban were grouped in group II except VBN (Bg) 5, which formed a separate group (Group III). Almost all the varieties grouped according to the research institute from which they were released except VBN (Bg) 5 and Co 5. All these showed that the research institutes have to enlarge the genetic base for blackgram improvement. Grouping of all the advanced breeding lines in one group revealed that only a portion of genetic variation has been exploited for blackgram improvement. The very low differentiation observed among advanced cultures shows us to enlarge breeder's genetic base. The results of PCoA were comparable to that of grouping based on UPGMA. Group II of UPGMA and group I of PCoA had the same 13 genotypes. Genotypes of group I, III and IV of UPGMA were present in group II of PCoA except VBN (Bg) 5. *Vigna mungo var. silvestris* formed a separate group in both analyses.

The variety VBN (Bg) 4 has Co 4 as one of the parent. KM 2 has T9 as one of the parent. These varieties were grouped in group II in PCoA analysis. VBN (Bg) 5 has VBN 1 as one of the parent. Both the varieties are resistant to YMV and have lanceolate leaves, which is different from other released varieties but the two varieties grouped separately. The variety ADT 4 has T9 as one of the parent but they both grouped separately. Hence, grouping of varieties did not show any relevance to pedigree. This may be explained with conservation and high similarity of DNA sequences among the

cultivated accessions evaluated. More number of markers could be used to detect genetic variation among *V.mungo* accessions.

In conclusion, ISSR markers are useful in the assessment of blackgram diversity. The diversity present in the elite genotypes would be useful in the selection of suitable parents for breeding purposes. The results revealed that, genetic diversity is low among the varieties released from the respective institute and hence genotypes were grouped according to the research institutes from which they released. It suggests that the research institutes have to enlarge the genetic base for variety development.

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Table 1. List of genotypes used for molecular studies and their pedigree

Genotype	Pedigree	Year of release	Place of release
T 9	Selection from Bareilly	1972	Kanpur
Co 2	Pureline selection from PLS 150	1973	Coimbatrore
Co 4	Induced mutant from Co 1 (MMS 0.02%)	1978	Coimbatrore
KM 2	T 9 x L 64	1978	Kudumianmalai
TMV 1	Midhi Ulundu x KM 1	1979	Tindivanam
Co 5	Pureline selection from Musiri type	1981	Coimbatrore
ADT 3	Pureline selection from Tirunelveli	1981	Aduthurai
ADT 4	T9 / ADT2 / PANT U- 14 cross derivative	1987	Aduthurai
VBN 1	KM 1 x H 76-1	1987	Vamban
ADT 5	Pureline selection from Kanpur variety	1988	Aduthurai
APK 1	ADT 2 x RU 1	1993	Aruppukottai
K1	Co 3 x VS 131	1994	Kovilpatti
VBN 2	Spontaneous mutant selection from Type 9	1996	Vamban
VBN 3	LBG 402 x LBG 17	2000	Vamban
VBN(Bg) 4	CO 4 x PDU 102	2003	Vamban
VBN(Bg) 5	VBN 1× UK 17	2007	Vamban
CBG 647	TMV 1 × CBG 282	-	Coimbatrore
CBG 662	CBG 282 × VBN (Bg) 4	-	Coimbatrore
CBG 653	DU 2 × VB 20	-	Coimbatrore
CBG 671	CoBG 10 × DU-2	-	Coimbatrore
CBG 632	(T 9 × VBN 1) × VBN 1	-	Coimbatrore
CBG 683	CoBG 671 × TU-94-2	-	Coimbatrore
Vigna mungo var. silvestris	Wild species and progenitor of blackgram	-	Coimbatrore

Table 2. List of ISSR primers used and the level of polymorphism detected

Primer name	Sequence 5'→3'	Annealing temperature (°C)	Product size (bp)	Total number of bands	Polymorphic bands	% of polymorphic bands	SHANNON INDEX
UBC 808	AG AG AG AG AG AG AG AGC	54	1000- 5000	17	14	82.35	3.13
UBC 809	AG AG AG AG AG AG AG AGG	50	1000-3000	8	5	62.5	1.44
UBC 810	GA GA GA GA GA GA GA GAT	52	1000-4500	11	8	72.73	1.66
UBC 811	GA GA GA GA GA GA GA GAC	50	1000-4000	11	11	100	1.79
UBC 812	GA GA GA GA GA GA GA GAA	50	1500-4500	11	9	81.82	2.33
UBC 815	CT CT CT CT CT CT CT CTG	52	1000-5000	9	7	77.78	1.59
UBC 820	GT GT GT GT GT GT GT GTC	52	1000-5000	9	7	77.78	1.29
UBC 825	AC AC AC AC AC AC AC ACT	50	1500-5000	14	13	92.86	3.33
UBC 826	AC AC AC AC AC AC AC ACC	52	2000-5000	14	14	100	3.56
UBC 828	TG TG TG TG TG TG TG TGA	52	1000-5000	11	8	72.73	1.77
Total score		-	-	115	96	820.55	21.89
Mean per primer		-	-	11.5	9.6	82.05	2.19

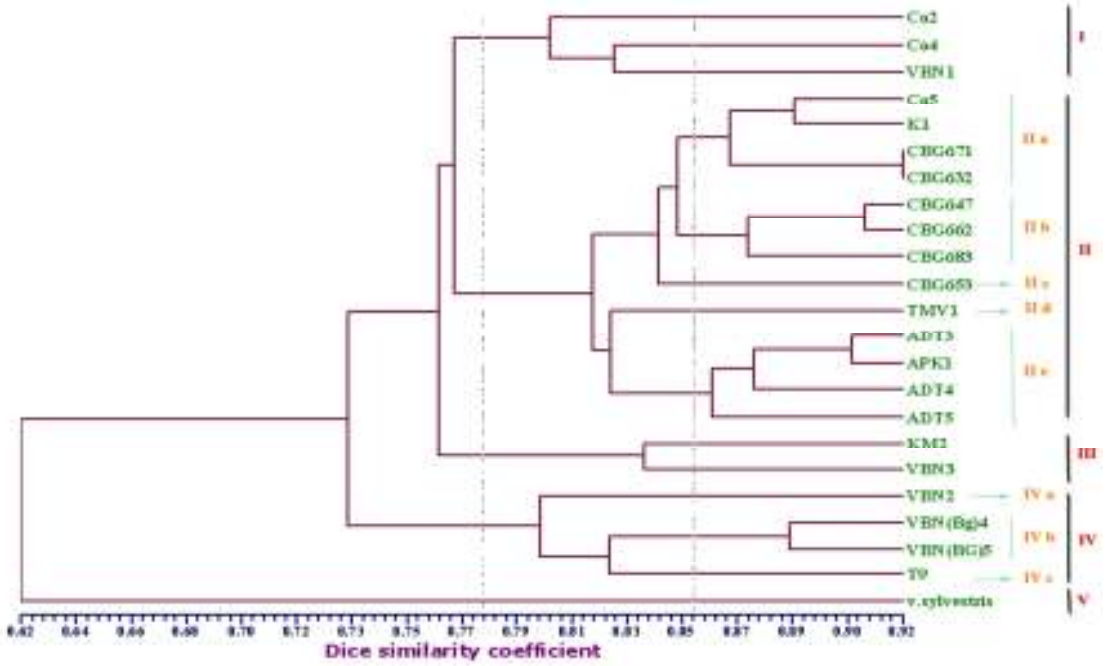

Fig.1 Dendrogram generated using UPGMA analysis showing relationships among blackgram genotypes obtained by ISSR data

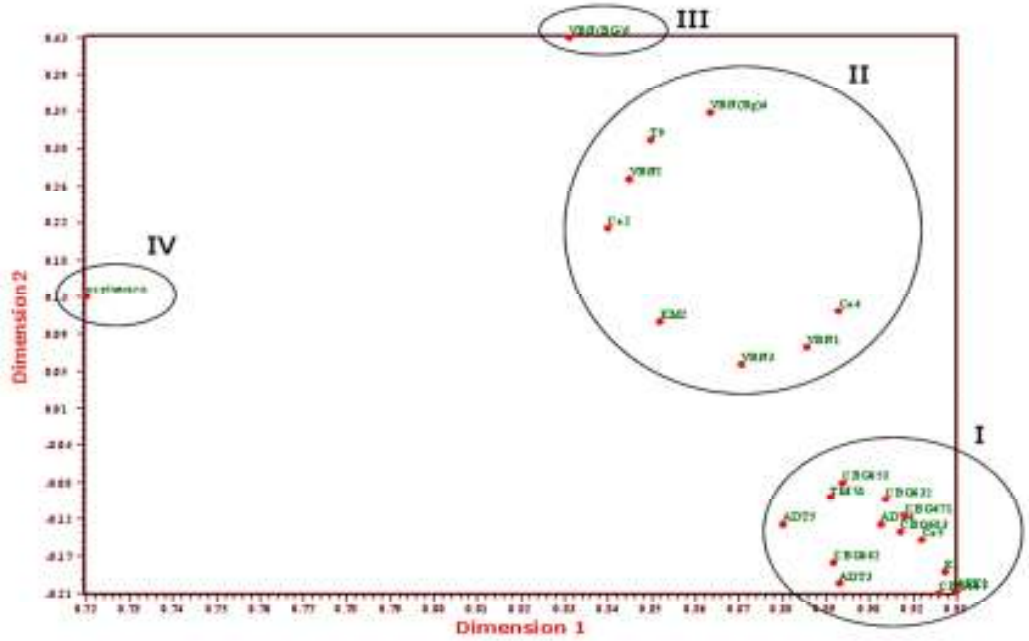

Fig. 2 Principal Co-ordinate Analysis of 23 blackgram genotypes using ISSR data