Theor Appl Genet (2004) 109: 1687–1693
DOI 10.1007/s00122-004-1797-3

ORIGINAL PAPER

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A comparative analysis of genetic diversity in blackgram genotypes using RAPD and ISSR markers

Received: 14 May 2004 / Accepted: 12 August 2004 / Published online: 11 September 2004

Abstract Random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) markers were used to study the DNA polymorphism in elite blackgram genotypes. A total of 25 random and 16 ISSR primers were used. Amplification of genomic DNA of the 18 genotypes, using RAPD analysis, yielded 104 fragments that could be scored, of which 44 were polymorphic, with an average of 1.8 polymorphic fragments per primer. Number of amplified fragments with random primers ranged from two (OPA-13) to nine (OPK-4) and varied in size from 200 bp to 2,500 bp. Percentage polymorphism ranged from 16.6% (OPK-7) to a maximum of 66.6% (OPE-5, OPH-2, and OPK-8), with an average of 42.7%. The 16 ISSR primers used in the study produced 101 bands across 18 genotypes, of which 55 were polymorphic. The number of amplified bands varied from two (ISSR 858) to ten (ISSR 810), with a size range of 200–2,200 bp. The average numbers of bands per primer and polymorphic bands per primer were 6.3 and 3.4, respectively. Percentage polymorphism ranged from 25% (ISSR 885) to 100% (ISSR 858), with an average percentage polymorphism of 57.5% across all the genotypes. The 3′-anchored primers based on poly(GA) and poly(AG) motifs produced high average polymorphisms of 54.98% and 58.32%, respectively. ISSR markers were more efficient than the RAPD assay, as they detected 57.4% polymorphic DNA markers in *Vigna mungo* as compared to 42.7% for RAPD markers. The Mantel test between the two Jaccard's similarity matrices gave r =0.32, showing low correlation between RAPD- and ISSR-based similarities. Clustering of genotypes within groups was not similar when RAPD and ISSR derived dendrogram were compared, whereas the pattern of clustering of the genotypes remained more or less the same in ISSR and combined data of RAPD and ISSR.

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Introduction

Blackgram, *Vigna mungo* (L.) Hepper, popularly known as urdbean or mash, is a grain legume domesticated from *V. mungo* var. *silvestris* (Lukoki et al. 1980). Blackgram is widely cultivated in the Indian subcontinent and to a lesser extent in Thailand, Australia, and other Asian and South Pacific countries (Poehlman 1991). The major constraints in achieving higher yield of this crop are lack of genetic variability, absence of suitable ideotypes for different cropping systems, poor harvest index and susceptibility to diseases. Research on this species has lagged behind that of cereals and other legumes. Therefore, improvement of this crop is needed through utilization of available genetic diversity.

The evaluation of genetic diversity and construction of linkage maps would promote the efficient use of genetic variations in the breeding program (Paterson et al. 1991). DNA markers provide an opportunity to characterize genotypes and to measure genetic relationships more precisely than other markers (Soller and Beckmann 1983). RAPD markers have been used for the identification of cultivars and for assessing the genetic diversity among cultivars of several crops like bean (Skroch et al. 1992), cowpea (Mignouna et al. 1998), pea (Hoey et al. 1996), soybean (Brown-Guedira et al. 2000), *Ceratotropis* (Kaga et al. 1996), mungbean (Santalla et al. 1998; Lakhanpaul et al. 2000), and *Vigna angularis* (Yee et al. 1999).

Inter simple sequence repeat (ISSR) analysis involves the polymerase chain reaction (PCR) amplification of regions between adjacent, inversely oriented microsatellites, using a single simple sequence repeat (SSR) motifs (di-, tri-, tetra-, or pentanucleotides) containing primers anchored at the 3′ or 5′ end by two to four arbitrary, often degenerate nucleotides (Zietkiewicz et al. 1994). The primers used in our analysis were anchored at 3′ end to ensure that perfect annealing of the primer occurs at the 3′ end of the microsatellite motif, thus obviating internal priming and smear formation. The anchor also allows only a subset of the targeted inter-repeat regions to be amplified, thereby reducing the high number of PCR

products expected from the priming of dinucleotide inter-repeat region (Zietkiewicz et al. 1994).

The sequence of repeats and anchored nucleotides were randomly selected and had the advantage of analyzing multiple loci in a single reaction. ISSR markers have been successfully utilized for the analysis of repeat motifs in mungbean (Singh et al. 2000), genetic relationships in the genus *Vigna* (Ajibade et al. 2000), and varietal identification in blackgram (Ranade et al. 2000). The potential supply of ISSR marker depends on the variety and frequency of microsatellites, which changes with species and the SSR motifs that are targeted (Depeiges et al. 1995). ISSR primers with a given microsatellite repeat should reflect the relative frequency of that motif in a given genome and would provide an estimate of the motif's abundance.

A large number of polymorphic markers are required to measure genetic relationships and genetic diversity in a reliable manner (Santalla et al. 1998). This limits the use of morphological characters and isozymes, which are few or lack adequate levels in blackgram. Molecular genetic markers have developed into a powerful tool to analyze genetic relationships and genetic diversity.

The objective of the present study was to investigate and compare genetic diversity among 18 elite genotypes of known origin, using random amplified polymorphic DNA (RAPD) and ISSR markers. This would aid the long-term objective of identifying diverse parental lines to generate segregating populations for tagging important traits, such as bruchid and yellow mosaic virus resistance, with molecular markers.

Materials and methods

Plant materials and DNA extraction

Table 1 lists the blackgram genotypes collected from different parts of India that were used in the present study. DNA was extracted from young leaves using the method described by Dellaporta et al. (1983). The concentration of the DNA samples was determined in a Hoefer DNA Fluorometer using Hoechst dye, and the DNA samples were diluted to 25 ng μl^{-1} for PCR amplification.

RAPD amplification

PCR amplification (Williams et al. 1990) was performed with random decamer primers obtained from Operon Technologies (Almeda, Calif., USA). Amplifications were performed in a 25-µl reaction volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each dNTP, 20 pmol RAPD primer, 50 ng genomic DNA, and 0.5 U *Taq* DNA polymerase (Bangalore Genei, Bangalore, India). Amplifications were performed in an Eppendorf Master cycler gradient (Eppendorf Netheler-Hinz, Hamburg, Germany). Amplification conditions were an initial denaturation at 94°C for 4 min and 45 cycles at 94°C for 1 min, 37°C for 1 min, 72°C for 2 min, followed by 5 min at 72°C. Amplified products were separated on 1.5% agarose gel in 1× TBE buffer (100 mM Tris-HCl, pH 8.3, 83 mM boric acid, 1 mM EDTA) at 50 V. The gels were stained with 0.5 µg/ml ethidium bromide solution and visualized by illumination under UV light. The sizes of the amplification products were determined by comparison to λDNA digested with *Eco*RI and *Hind*III.

Table 1 List of blackgram genotypes used in the study

Serial no.	Genotype	Pedigree	Remarks
1	AKU-4	Local selection	Tolerant to stress
2	CO-5	Pure line selection Musiri local	Moderate resistance to powdery mildew, leaf crinkle, pod borer, and yellow mosaic virus (YMV)
3	TU 94-2	TPU-4 × TAU-5 (Mutant of EC-168200)	YMV resistant
4	TAU-1	T-9 × UM-196 (Mutant of No. 55)	Large seed size
5	TPU-4	UM-201 (Mutant of No. 55) × T-9	Large seed size
6	Trombay wild	*Vigna mungo* var. *silvestris*	Indeterminate and resistant to bruchids
7	LBG-17	Netiminumu × Chikkuduminumu	Photoperiod sensitive and resistant to powdery mildew
8	LBG-402	Selection from PLU-91	Tall erect plant type
9	Nayagarh	Local selection	Photoperiod sensitive, resistant to YMV
10	TAU-5	Mutant of EC-168200	Resistant to YMV and early maturity
11	T-9	Local selection from Bareilly U.P.	Wider adaptability
12	No. 55	Local selection from Satpur Plateau	Large seed size
13	M-1-1	Local selection from Punjab	Tolerant to YMV
14	Shindekheda	Selection from Jalgaon	Suitable for black soil
15	S-76	Local selection	Light green seed coat
16	EC-168200	Exotic collection from AVRDC, Taiwan	Exotic, late maturity, resistant to YMV
17	Pusa-3	L-151 × T-9	Resistant to YMV
18	Pant-19	UPU-1 × UPU-2	Resistant to YMV, bacterial blight and *Cercospora* leaf spot

ISSR amplification

ISSR amplification reactions were carried out in 25-μl volume containing 50 ng template DNA, 0.5 U *Taq* DNA polymerase, 10 mM dNTP, 10 μM primer (The University of British Columbia, Vancouver, Canada) in 1× reaction buffer that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, and 0.01% gelatin. Amplification was performed in an Eppendorf Master cycler gradient. Amplification conditions were one cycle at 94°C for 4 min, and 94°C for 30 s, 55°C for 45 s, followed by stepwise reduction of 1°C for the first five cycles, and 72°C for 2 min. In subsequent 35 cycles, annealing temperature was maintained at 50°C, followed by one cycle of 7 min at 72°C. Amplified products were loaded on 2% agarose gel and separated in 1× TBE buffer at 75 V. The gels were visualized under UV after staining with ethidium bromide and documented using a gel documentation and image analysis system (Syngene, UK).

Data analysis

The RAPD and ISSR bands were scored as present (1) or absent (0), each of which was treated as an independent character regardless of its intensity. Data analyses were performed using the NTSYS-pc (Numerical Taxonomy System, version 2.0, Rohlf 1990). The SIMQUAL program was used to calculate the Jaccard's coefficient, a common estimator of genetic identity and was calculated as follows:

$$\text{Jaccard}'\text{s coefficient} = N_{AB}/(N_{AB} + N_A + N_B)$$

where N_{AB} is the number of bands shared by samples, N_A represents amplified fragments in sample A, and N_B represents fragments in sample B. Similarity matrices based on these indices were calculated. Correlation between the two matrices obtained with two marker types was estimated by means of the Mantel matrix correspondence test (Mantel 1967). This test yields a product moment correlation (r) that is one measure of the relatedness between the two matrices. In this instance, the matrix correlation corresponds to two independently derived dendrograms. For matrix correlation of this type, a correlation value (r) greater than 0.5 will be statistically significant at 0.01 probability level if the number of observed taxonomic units exceeds 15 (Lapointe and Legendre 1992).

Similarity matrices were utilized to construct the UPGMA (unweighted pair group method with arithmetic average) dendrograms. Statistical stability of the branches in the cluster was estimated by bootstrap analysis with 2,000 replicates, using the Winboot software program (Yap and Nelson 1996). In order to estimate the congruence among dendrograms, cophenetic matrices for each marker and index type were computed and compared using the Mantle test. Finally, a principal coordinate analysis was performed in order to highlight the resolving power of the ordination.

Results

Amplification of genomic DNA of the 18 genotypes, using 25 primers for RAPD analysis, yielded 104 fragments that could be scored. All the chosen primers amplified fragments across the 18 genotypes studied, with the number of amplified fragments ranging from two (OPA-13) to nine (OPK-4) and which varied in size from 200 bp to 2,500 bp. Of the 104 amplified bands, 44 were polymorphic, with an average of 1.8 polymorphic fragments per primer. Percentage polymorphism ranged from 16.6% (OPK-7) to a maximum of 66.6% (OPE-5, OPH-2, and OPK-8), with an average of 44.9% polymorphism. Only three out of 25 primers showed more than 60% polymorphism. Figure 1 is the representative of the extent of polymorphism observed among the blackgram genotypes as revealed by OPK-6.

A dendrogram based on UPGMA analysis grouped the 18 genotypes into four main clusters, with Jaccard's similarity coefficient ranging from 0.83 to 0.95 (Fig. 3a). Two genotypes (AKU-4 and Pant-19) grouped in one cluster and appeared to be distinct from all others. Cluster II comprised 14 genotypes, four of them developed at BARC (Bhabha Atomic Research Centre, Mumbai, India); mutant No. 55, which was derived from EC-168200; land races; and one EC culture. Genotypes within cluster II are further grouped into four subclusters. The first subcluster IIa comprised TU94-2, TPU-4, No. 55, and LBG-17. Subcluster IIb comprised TAU-5, T-9, and Shindekheda. LBG-402 and M-1-1 formed subcluster IIc. Subcluster IId consisted of Nayagarh, S-76, and EC-168200. Within cluster II, TAU-5 and T-9 appeared to be closer to each other, with a 0.95 similarity coefficient. Pusa-3 and TW formed a separate OTUs III and IV, respectively showing less similarity with other genotypes studied.

The results of PCA analysis were comparable to the cluster analysis (Fig. 4a). The first three most informative

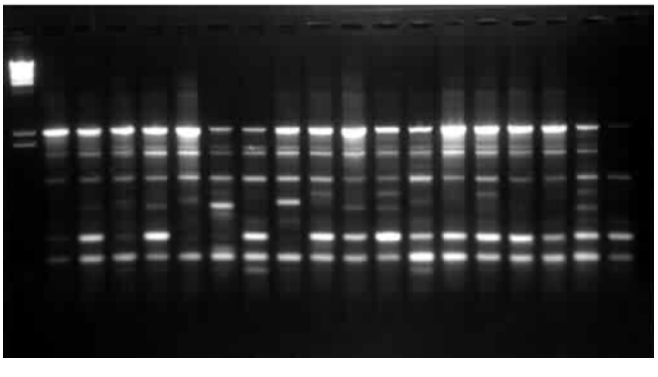

Fig. 1 The random amplified polymorphic DNA (RAPD) profile of blackgram genotypes, using the random primer OPK-6. *Lane M* λDNA *Eco*RI and *Hind*III double-digest marker. *Lanes 1–18* Blackgram genotypes as listed in Table 1

PC components explained 45.2% of the total variation. Four genotypes TW, Pusa-3, Pant-19, and AKU-4 appear to be distinct from other genotypes in the PCA.

ISSR band patterns

The PCR amplification using 14 3′-anchored dinucleotide repeat primers and two 5′-anchored dinucleotide repeat primers gave rise to reproducible amplification products. ISSR primers produced varying numbers of DNA fragments, depending on their SSR motifs (Fig. 2). The number of bands produced with different selective nucleotide (CG) varied more with the poly(GA) primers (7.7) than with the poly(AG) primers (5.0). The primers that were based on the poly (GA) motif produced more polymorphism on average (4.7) than the primers based on the poly(AG) motifs (3.0). Amplifications using the two 5′-anchored dinucleotide repeat ISSR primers (ISSR 885 and ISSR 887) produced an average of 7.5 bands over all the genotypes.

The 16 primers on an average produced 101 bands across 18 genotypes, of which 55 were polymorphic, accounting for 54.5%. Number of bands varied from two (ISSR 858) to ten (ISSR 810), and sizes ranged from 200 bp to 2,200 bp. Average numbers of bands and polymorphic bands per primer were 6.3 and 3.4, respectively. Percentage of polymorphism ranged from 25.0% (ISSR 885) to 100% (ISSR 858), with an average polymorphism of 57.4% across all the genotypes. The 3′-anchored primers based on (GA) and (AG) motifs produced high average polymorphism rates of 55% and 58.3%, respectively.

Between the two 5′-anchored primers, ISSR 887 showed 42.8% polymorphism. The ISSR bands were scored for presence or absence among the genotypes and used for the UPGMA cluster analysis. The complete data was based on a total of 1,769 bands. A dendrogram based on UPGMA analysis with ISSR data is shown in Fig. 3b. Jaccard's similarity coefficient ranged from 0.58 to 0.87. The 18 genotypes were clustered into five clusters. Cluster I comprised AKU-4 and Co-5. Cluster II consisted of TU-94-2, Shindekheda, TPU-4, TAU-1, No. 55, LBG-17, and Nayagarh. Cluster III comprised M-1-1, Pusa-3, S-76, EC-168200, and Pant-19. TAU-5 and T-9 formed a separate cluster. TW formed a separate OTU in cluster showing less similarity coefficient (0.58) with other genotypes studied.

The results of PCA analysis were comparable to the cluster analysis (Fig. 4b). The first three most informative PC components explained 44.2% of the total variation. Three genotypes TW, T-9, and TAU-5 appear to be distinct from other genotypes in the PCA.

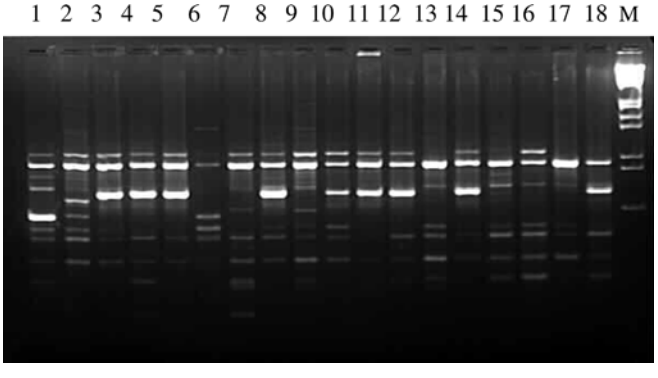

Fig. 2 The inter-simple sequence repeat (ISSR) polymorphism in blackgram genotypes, using the ISSR primer UBC 832. *Lane M* λDNA *Eco*RI and *Hind*III double-digest marker. *Lanes 1–18* Blackgram genotypes as listed in Table 1

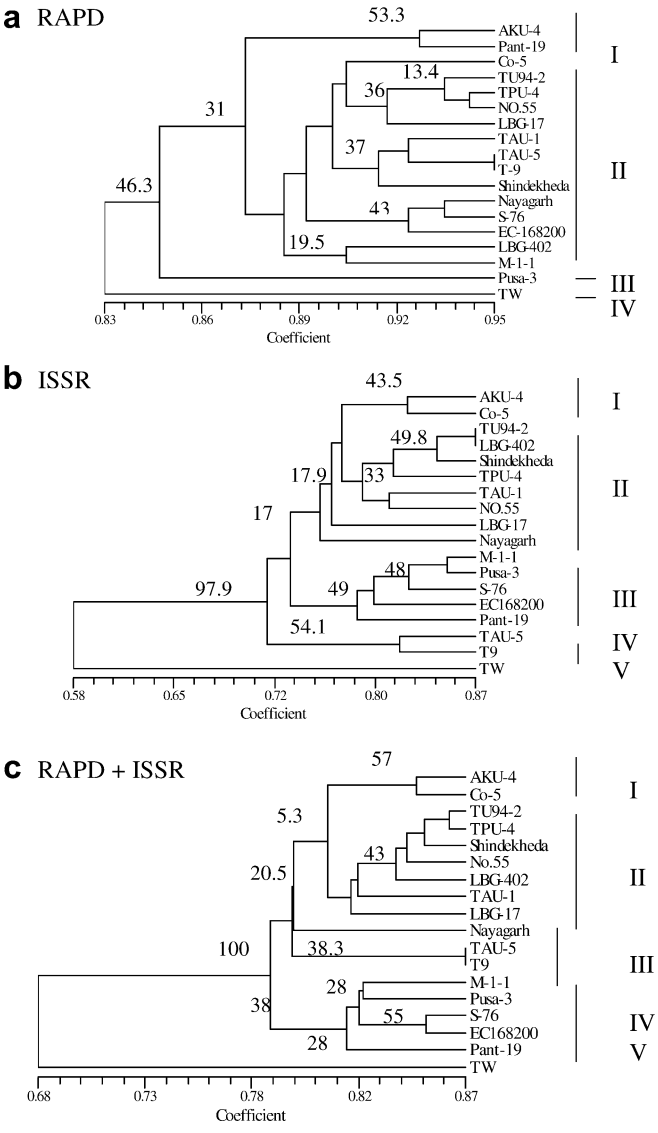

Fig. 3 Dendrograms generated using unweighted pair group method with arithmetic average analysis, showing relationships between blackgram genotypes, using RAPD, ISSR, and combining both RAPD and ISSR data. The *numbers at the forks* indicate the confidence limits for the grouping of those species in a branch occurred, based on 2,000 cycles in bootstrap analysis, using the Winboot program

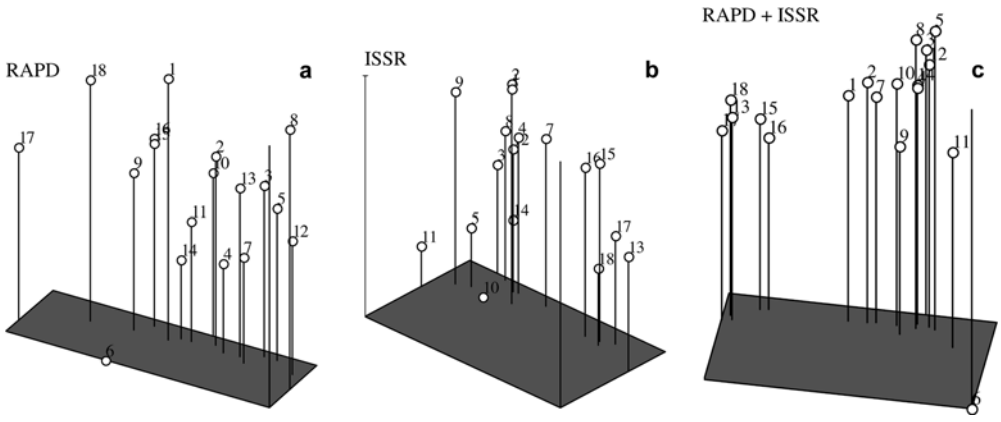

Fig. 4 Three-dimensional plot of principal component analysis of using elite blackgram genotypes RAPD, ISSR, and both combined analysis. The *numbers plotted* represents individual cultivars and corresponds to the ones listed in Table 1

RAPD and ISSR data

The RAPD and ISSR data were combined for UPGMA cluster analysis. Five clusters were formed similar to ISSR cluster. The UPGMA dendrogram obtained from the cluster analysis of RAPD and ISSR data gave similar clustering pattern, with Jaccard's similarity coefficient ranging from 0.68 to 0.87. The three-dimensional ordination confirms the cluster analysis results showing that TW (No. 6), T9 (No. 11), and TAU-5 (No. 10) were separated.

Three dendrograms based on RAPD, ISSR, and combined data are shown in Fig. 3c. TW (*V. mungo* var. *sylvestris*) was used as an outgroup species. The pattern of clustering of the genotypes remained more or less the same in ISSR and ISSR and RAPD data, whereas the dendrogram based on RAPD showed some variation in the clustering of genotypes. The matrices for RAPD and ISSR markers were also compared using Mantel's test (Mantel 1967) for matrix correspondence. The correlation between the matrices of cophenetic correlation values for the dendrogram based on RAPD and ISSR data was low (r=0.32). Eighteen genotypes were grouped in five clusters in ISSR and ISSR and RAPD data, whereas a dendrogram based on RAPD data showed only four clusters. In all the three dendrograms, TW was represented as an OTU. The results of PCA analysis were comparable to the cluster analysis (Fig. 4c). The first three most informative PC components explained 44.2% of the total variation. Two genotypes TW, and T-9 appear to be distinct from other genotypes in the PCA.

Discussion

The evolution of varieties in distinct agro-climatic zones demonstrates significant levels of variation in response to the selection pressure in the zones (Singh et al. 1998). It is, therefore, not surprising to find significant levels of polymorphism among the 18 genotypes of blackgram in RAPD (42.7%) and ISSR (57.4%) markers. The RAPD technique has been applied to assess molecular polymorphism in *Vigna* (Kaga et al. 1996), mung bean (Santalla et al. 1998; Lakhanpaul 2000), chickpea (Sonnante et al. 1997), pea (Simioniuc et al. 2002), pigeonpea (Ratnaparkhe et al. 1995), and in cowpea (Mignouna et al. 1998). The success of our study in identifying polymorphism is due to the use of a number of randomly selected prescreened highly informative primers.

The ISSR technique has been used in genetic relationships in genus *Vigna* (Ajibade et al. 2000) and in several other crops (Reddy et al. 2002). ISSR markers were more efficient than the RAPD assay, as they detected 57.4% polymorphic DNA markers in *V. mungo* as compared with 42.7% for RAPD markers. Similar results were obtained for several other plant species like wheat (Nagoaka and Ogihara 1997) and groundnut (Raina et al. 2001). The 25 RAPD and 16 ISSR primers in the present study yielded 99 polymorphic markers that unambiguously discriminated 18 genotypes into five clusters. The number of total polymorphic and discriminant fragments is higher for ISSRs than RAPD. Similar results were observed in *Nothofagus* spp. (Mattioni et al. 2002). In fact, the ISSRs have a high capacity to reveal polymorphism and offer great potential to determine intra- and intergenomic diversity as compared to other arbitrary primers like RAPDs (Zietkiewicz et al. 1994). Geographically isolated population accumulates genetic differences as they adapt to different environment. Genetic variation among elite genotypes of blackgram based on RAPD and ISSR analysis could be useful to select parents to be crossed for generating appropriate populations intended for both genome mapping and breeding purposes.

The correlation between RAPD and ISSR Jaccard's similarity coefficient value was low in magnitude. A possible explanation for the difference in resolution of RAPDs and ISSRs is that the two-marker techniques target different portions of the genome. The ability to resolve genetic variation among different genotype may be more directly related to the number of polymorphisms detected with each marker technique rather than a function of which technique is employed. Studying the sampling variance of heterozygosity and genetic distance estimates, Nei (1978) reported that a relatively reliable estimate of average heterozygosity can also be obtained from a small number of individuals if a large number of loci are examined. The number of polymorphisms detected among genotypes influences the standard errors of the genetic diversity estimates. The correlation between Jaccard's

similarity values generated from different marker techniques was low (r =0.32, P<0.01), and there was some consensus between the RAPD- and ISSR-based grouping of the 18 genotypes.

Clustering of genotypes within groups was not similar when RAPD- and ISSR-derived dendrograms were compared. These differences may be attributed to marker sampling error and/or the level of polymorphism detected, reinforcing again the importance of the number of loci and their coverage of the overall genome in obtaining reliable estimates of genetic relationships among cultivars (Loarce et al. 1996). The putatively similar bands originating for RAPDs in different individuals are not necessarily homologous, although they may share the same size in base pairs. This situation may lead to wrong results when calculating genetic relationships (Fernandez et al. 2002).

Dendrograms did not indicate any clear pattern of clustering according to the location in which they were collected. Similar results were obtained in Azukibean (Yee et al. 1999) and in groundnut (Dwivedi et al. 2001).

The genetic closeness among the cultivars can be explained by the high degree of commonness in their pedigree. Four genotypes grouped in cluster IIa along with No. 55, whose mutants were involved in the pedigree. LGB-402 and Shindekheda are the only exceptions that have been grouped in cluster IIa. Similar results were observed in blackgram (Gaffor et al. 2001) and mungbean (Lakhanpaul et al. 2000). In all the dendrograms, TW was represented as an OTU. The lowest genetic similarity of TW with other genotypes is probably associated with their difference in subspecies *V. mungo* var. *silvestris*, which is resistant to bruchids. 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). The primer containing the CT repeats was one of those, which did not give interpretable phenotype analyzed, while primers with GA and CA repeats revealed polymorphism (Ajibade et al. 2000).

Accessions with the most distinct DNA profiles are likely to contain the greatest number of novel alleles. It is these accessions that are likely to uncover the largest number of unique and potentially agronomic useful alleles. This strategy has resulted in a high proportion (≈50%) of new and useful quantitative trait loci alleles in rice and tomato (Tanksley and McCouch 1997). TU94-2 has been crossed with TW to develop recombinant inbred lines for identifying DNA markers linked to bruchid resistance and to transfer the character into yellow mosaic virus-resistant background to develop multiple biotic-resistant genotypes. Further studies are envisaged to quantify the genetic gain in populations derived from genotypes with distinct DNA profiles.

Our results indicate the presence of great genetic variability among elite genotypes of blackgram. ISSR markers are useful in the assessment of blackgram diversity, the detection of duplicate sample in germplasm collection, and the selection of a core collection to enhance the efficiency of germplasm management for use in blackgram breeding and conservation.

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