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Full Length Research Paper

Assessing Genetic Variation in Vigna radiata Landraces Through RAPD Analysis

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Green gram [*Vigna radiata* (L.)] landraces were collected from various localities of Southern Tamil Nadu, India, to determine the extent of genetic diversity at DNA level by random amplified polymorphic DNA (RAPD) analysis using 20 decamer primers. All the primers produced polymorphic amplification products with some extent of variation. A total of 200 bands were generated with an average of 10 per primer and exhibited 83.0% polymorphism. Jaccard's similarity coefficient ranged from 0.64 to 0.93 and concentrated mostly between 0.76 to 0.93. This indicated a rather narrow genetic base of tested green gram landraces. Clustering of green gram landraces into two groups showed reasonable variability that may be exploited for selecting parents for breeding purposes. Generally distinct phenotypes identified using RAPD markers could be potential sources of germplasm for green gram improvement.

Key words: genetic diversity, Jaccard's similarity, RAPD marker, *Vigna radiata*.

INTRODUCTION

Green gram [*Vigna radiata* (L.)] is an essential crop in developing countries of Africa, Asia and Latin America where it is consumed as dry seeds, fresh green pods or leaves. Because of its high proteins, vitamins and minerals contain, green gram plays an important role in human consumption and animal feeding. It is cultivated in rotation with cereals and therefore important as a nitrogen fixer for maintaining soil fertility. However, the average yield of 384 kg ha^{-1} worldwide is very low (Jaiwal and Gulati, 1995). Many biotic and abiotic stresses such as disease, insects, drought, high temperature, salinity and heavy metals limit green gram yields. Despite the efforts of plant breeders during the past few decades, the yield of green gram has not increased substantially due to lack of sufficient genetic diversity for desirable traits in the germplasm used for improvement (Skrotch and Nienhuis, 1995). It is imperative to further explore the genetic diversity available in this crop for better utilization of genetic resources in yield improvement. Several markers may be used to identify and assess the genetic diversity and phylogenetic relationships in plant genetic resources. The traditional method based on morphological traits requires extensive observation of mature plants but cannot serve as unambiguous markers because of environmental influences (Wrigley et al., 1987).

Taxonomic uncertainties exist because phylogenetic relationships among taxa have been established considering mainly the morphological features, crossability and F_1 fertility. Establishing genetic affinities on such parameters are insufficient, as green gram

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Table 1. Morphological characteristics of green gram landraces used in the diversity analysis.

Localities		Seed size (mg)	Seed coat color	Surface luster	Hypocotyl color
Kanniyakumari	L1	42.2	Green	Shiny	Purple
Tirunelveli	L2	38.3	Green	Shiny	Green
Tuticorin	L3	56.1	Green	Dull	Purple
Ramanathapuram	L4	39.1	Green	Dull	Green
Virudhunagar	L5	52.4	Green	Shiny	Purple
Teni	L6	46.2	Brown	Shiny	Purple
Madurai	L7	43.2	Green	Shiny	Purple
Sivagangai	L8	52.1	Green	Shiny	Green
Pudukottai	L9	38.2	Green	Dull	Green
Dindigul	L10	48.2	Green	Shiny	Purple
Coimbatore	L11	52.1	Green	Dull	Green
Thanjavur	L12	47.1	Green	Shiny	Purple
Tiruchirappalli	L13	39.6	Brown	Dull	Green
Karur	L14	50.2	Green	Dull	Purple
Thiruvarur	L15	47.4	Brown	Shiny	Purple

makes successful cross with putative progenitors as well as distantly related species. To overcome these problems, isozymes were used as tool but met with little success. The use of molecular techniques in genetic diversity studies is supported by the finding that evolutionary forces such as natural selection and genetic drift produce divergent phylogenetic branchings which can be recognized because the molecular sequences on which they are based share a common ancestor. Genetic fingerprinting has been accomplished traditionally through the use of isozymes, total seed protein and more recently through various types of molecular markers. However, DNA-based markers provide powerful tools for discerning variations within crop cultivars and for studying evolutionary relationships (Skrotch and Nienhuis, 1995). Molecular markers based on the DNA sequence are more varied and reliable. In fact, the restriction fragment length polymorphism (RFLP) approach has been used successfully to identify genetic markers in plants, including green gram. However, the RFLP technique needs specific probes for the target DNA sequences, and use of radioactive elements makes it more costly and tedious. The development of PCR technique has offered a good alternative to the RFLP analysis (Welsh and McClelland, 1990). The PCR-based RAPD approach using arbitrary primers requires much less DNA, and is technically simple and cheaper compared to the RFLP (Williams et al., 1990; Welsh and McClelland, 1990). It can provide assessment of genetic distances, seed purity and resolution of uncertain parentage.

Most variability studies in green gram have focused mainly on morphology, crossability, anatomy, isozyme and chloroplast DNA diversity. Limited work has been done so far with nuclear DNA diversity. RAPD markers have been already successfully used on many other crops (Gepts, 1993). Therefore the present study was undertaken for evaluating the genetic diversity among 15 green gram landraces in various localities of Southern Tamil Nadu, India, using RAPD markers.

MATERIALS AND METHODS

Materials for RAPD study

The plant material for the study comprised of 15 green gram landraces (morphologically and geographically distinct, Table 1). The leaf samples were collected from various localities of Southern Tamil Nadu, India (Figure 1). Leaf samples were pooled from 15 plants of 20-day-old of each population used in the morphological study. Additional leaf samples were collected from another five plants selected randomly from each population. Twenty leaf samples from each population were kept in an ice chest before being brought back to the laboratory and then stored in -70°C freezer before DNA extraction.

Isolation of DNA

DNA isolation was followed by the CTAB method of Doyle and Doyle (1987). For each sample, 500 mg of fresh leaf sample was ground with liquid nitrogen in a mortar and pestle, then transferred to a 1.5 ml centrifuge tube (preheated in 60°C water) containing 700 µl of urea buffer (8.0 M urea, 0.05 M NaCl, 0.05 M Tris-HCl (pH 7.5), 0.02 M EDTA, 1 % sarcosyl), mixed thoroughly and incubated in water bath at 60°C for 10 min. The tube was inverted periodically. To this was added 700 µl of phenol : chloroform (1:1; v/v, Tris pH 8.0 saturated), and the tube was gently inverted repeatedly. The

Figure 1. Geographic distribution of sampling localities of wild green gram in Southern Tamil Nadu, India.

tube was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was transferred to a new centrifuge tube, and 0.7 volume of iso-propanol and 1/10 volume of 4.4 M NH_4OAc were added. The tube was centrifuged at 10,000 rpm 10 min at 4°C to collect precipitated DNA. The DNA pellet was resuspended with 400 µl of 10 mM TE (Tris-HCl (pH 8.0), 1 mM EDTA) and incubated with 5 µg DNase-free RNase (SigmaAldrich, USA) for 10 min at 65°C. The RNase and the remaining protein were extracted with an equal volume of phenol : chloroform (1:1; v/v, Tris pH 8.0 saturated) and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was transferred to a new tube, and the DNA was precipitated by the addition of a 1/10 volume of 4.4 M NH_4OAc and three volumes of 95% ethanol. Precipitated DNA was collected by centrifugation at 10,000 rpm for 10 min at 4°C, washed with 70% ice cold ethanol twice, and dried before redissolving in 200 µl of 10 mM TE (Tris-HCl (pH 8.0), 1 mM EDTA). Approximate DNA yields were calculated by a spectrophotometer (Hitachi U-2000, Japan), and DNA samples were stored at -20°C until further analysis.

RAPD reaction

Twenty decamer primers (Operon Technologies Inc., Alameda, CA, USA) were screened by polymerase chain reaction (PCR). PCR amplifications were performed in a 25 µl reaction volume containing, 10X PCR buffer (10 mM Tris-HCl (pH 8.0), 50 mM KCl, 0.01% gelatin) with 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 units of *Taq* polymerase (Fermentas GmbH, Lithuania), 0.2 µM of random primer and 50 ng of DNA. Amplification was performed using Eppendorf Master Cycle gradient (Eppendorf, Germany), programmed for initial denaturation at 94°C for 2 min and 45 cycles of 94 C for 1 min, 36°C for 1 min and 72°C for 2 min. The amplification was completed with a 5 min final extension at 72°C. The amplification products were separated by electrophoresis in 1.5% (w/v) agarose (SigmaAldrich) gels with 0.5X TAE buffer, stained by 1 µg/ml of ethidium bromide (EtBr) and photographed under exposure to UV light (Biorad, Model 2000, USA).

Data analysis

PCR-RAPD analysis was repeated at least three times and only the primers producing strong and reproducible bands were considered for further analysis. The RAPD data were analyzed using NTSYS-PC (version 2.02) (Numerical Taxonomy and Multivariate Analysis System) computer package (Rohlf, 1990). Each RAPD fragments was treated as a unit character and was scored as 1 (present) or 0 (absent). The 1/0 matrix was prepared for all fragments scored and

Table 2. Decamer primers selected for RAPD analysis of 15 green gram landraces including number of bands for each primer and number of polymorphic bands produced.

Primers	Sequence (5'-3')	Nb	Np	% Polymorphism
A-01	CAGGCCCTTC	10	10	100.0
A-02	TGCCGAGCTG	10	7	70.0
A-03	AGTCAGCCAA	11	8	72.7
A-04	AATCGGGCTG	6	6	100.0
A-05	AGGGGTCTTG	10	6	60.0
A-06	GGTCCCTGAC	7	5	71.4
A-07	GAAACGGGTG	9	6	66.6
A-08	GTGACGTAGG	10	9	90.0
A-09	GGGTAACGCC	11	9	81.8
A-10	GTGATCGCAG	10	10	100.0
OPA-01	GTGTCTCAGG	9	8	88.8
OPA-02	GTGGGCTGAC	10	8	80.0
OPA-03	GTCCATGCCA	13	13	100.0
OPA-04	ACATCGCCCA	10	7	70.0
OPA-05	GTGGTCCGCA	10	8	80.0
OPA-06	TCCCGCCTCA	10	8	80.0
OPA-07	AACGCGTCGG	12	11	91.6
OPA-08	AAGGGCGAGT	11	10	90.9
OPA-09	GGAAGCCAAC	9	9	100.0
OPA-10	GGCTTGGCCT	12	8	66.6
Total		200	166	83.0

Nb: Number of total bands.
Np: Number of polymorphic bands.

the data were used to generate Jaccard's similarity coefficients for RAPD bands showed in Table 3 (Jaccard, 1908). The Jaccard's coefficients were used to construct a dendrogram using the unweighted pair group method with arithmatic averages (UPGMA).

RESULTS AND DISCUSSION

The present investigation identifies the degree of genetic diversity based on DNA bands data in green gram from various localities. This is perhaps, as far as the authors are aware of, the first attempt to study genetic variation in green gram in various localities of Southern Tamil Nadu, India. Morphological characteristics provide the basic information about the magnitude of genetic variability in seed coat color, surface luster and hypocotyl color in green gram of various locations of Southern Tamil Nadu, India. Table 1 reveals a wide variability in seed size; seed is influenced by natural and artificial selection, socio economic conditions and consumers preferences within localities. Breeders take such local demand into account when developing improved varieties of green gram. Twenty decamer primers were randomly used to detect RAPD markers among the 15 green gram landraces (Table 2). A total of 200 bands were scored of which 83.0% exhibited polymorphism. The number of bands ranged between 2 to 13 with an average of 10 per primer. Out of 20 primers 11 were showed more than 80% polymorphism. The primer OPA-03 produced the maximum number of polymorphic bands. RAPD patterns of green gram landraces produced by primer OPA-03 are shown in Figure 3.

Information on the levels and distribution of genetic diversity of any plant species may contribute to the knowledge of their evolutionary history and potential, and is critical to their conservation and management (Schaal et al., 1991; Hamrick and Godt, 1996). Although, a wide array of isozyme and protein based procedures introduced in the last two decades allow genetic diversity to be estimated with greater precision, RAPD studies still have numerous advantages like wider applicability, accuracy and speed of estimation. In conclusion it can be stated that the availability of RAPD technique has substantially increased our knowledge of the genetics of plant populations. These results have important implications for the conservation strategy. Information on the levels and distribution of genetic diversity of any plant species may contribute to the knowledge of their evolutionary history and potential, and is critical to their conservation and management (Schaal et al., 1991; Hamrick and Godt, 1996).

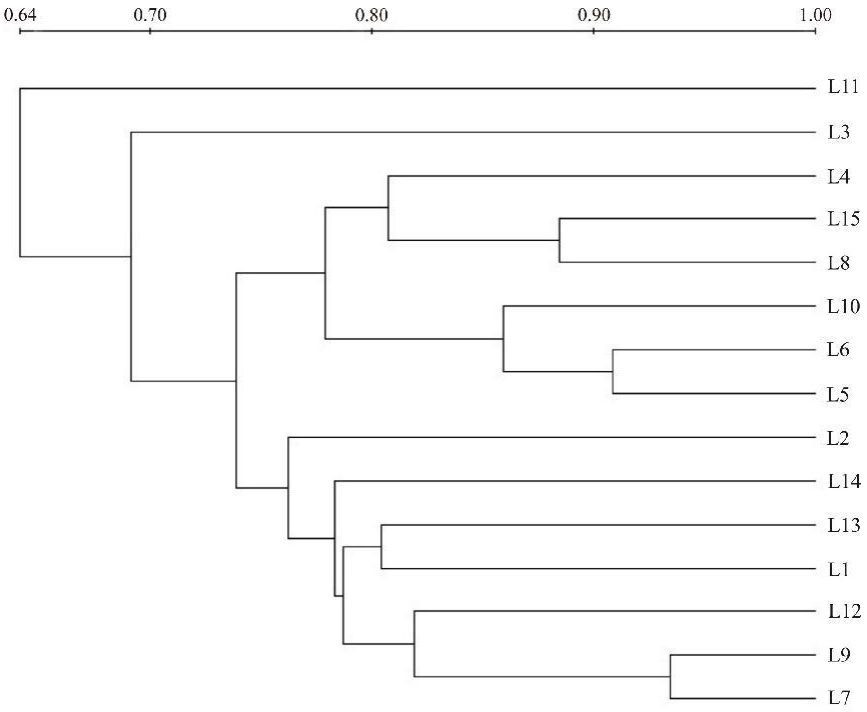

Figure 2. Dendrogram of the clustering of various localities of 15 green gram using UPGMA method based on 200 bands amplified by 20 arbitrary primers. The bar on the top represents similarity index based on Jaccard's coefficients.

Table 3. Similarity matrix for Jaccard's coefficients for 15 green gram landraces based on the 200 bands obtained with 20 arbitrary primers.

Localities	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15
L1	100.0														
L2	78.7	100.0													
L3	71.2	78.1	100.0												
L4	76.9	86.8	75.1	100.0											
L5	83.2	79.9	65.2	75.9	100.0										
L6	84.8	69.9	68.5	69.3	82.1	100.0									
L7	78.5	74.0	67.9	77.8	71.4	70.5	100.0								
L8	82.5	89.2	71.8	90.6	81.6	77.5	81.5	100.0							
L9	84.1	81.8	75.2	86.9	77.2	75.7	77.5	85.0	100.0						
L10	77.8	76.8	66.9	78.0	72.1	69.9	93.7	80.9	79.3	100.0					
L11	80.3	76.0	61.9	73.6	69.2	70.8	79.2	75.0	78.2	84.5	100.0				
L12	78.8	76.1	74.9	78.8	80.3	88.9	77.6	86.6	79.1	75.2	68.3	100.0			
L13	80.1	80.3	75.0	82.9	75.2	69.2	76.1	78.6	86.2	79.6	75.4	72.2	100.0		
L14	83.4	76.8	76.8	84.4	72.9	77.8	76.2	80.1	83.4	77.2	78.4	77.7	77.9	100.0	
L15	75.3	66.7	81.2	69.2	69.2	81.9	60.2	66.1	69.6	59.4	64.9	76.3	67.3	78.4	100.0

RAPD technique is a simpler and quicker method for characterization and analysis of genetic diversity among green gram landraces. Analysis of the relationship based on number of the DNA fragments. RAPD markers revealed the genetic diversity among the green gram landraces, which ranged from 0.64 to 0.93, but mostly concentrated between 0.76 and 0.93. The RAPD cluster pattern is presented in Figure 2. It showed two main clusters, but L11 and L3 each took an independent position. Such clustering of cultivars of different locations

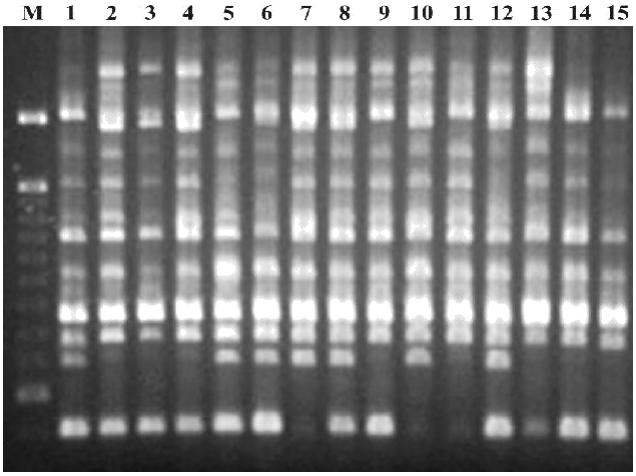

Figure 3. RAPD profile of 15 green gram landraces obtained with OPA-03 primer. Lane M: Molecular marker (1 kb); Lane 1 to 15 corresponds to green gram landraces listed in Table 1.

ignored the influence of geographic variations within the genetic diversity of green gram landraces. Bisht et al. (1998) also reported no correlation between geographic diversity and genetic diversity in green gram landraces. Manivannan et al. (1998) ascribed the fact that genetic drift and selection in different environments could cause greater genetic diversity than geographical diversity. In contrast, another small cluster formed by L4, L5, L6, L8, L10 and L15 in Southern Tamil Nadu, India, indicated the effect of geographical importance in the genetic similarity of green gram landraces. Paredes et al. (2002) also observed that genotypes originating from a single locality tended to fall within a single branch of the dendrogram, with roughly equal genetic distance occurring among them. The last cluster formed by L1, L2, L7, L9, L12, L13 and L14 were very distinct from the others, although L7, L9 and L12 appear to be close to each other with far distance from L1, L2, L13 and L14.

Genetic diversity is the resultant of natural selection, spontaneous mutation and artificial selection. These may aid in genetic diversity in crops over geographic locations. The greater variation among them showed the importance of locations in genetic diversity. The results revealed that the genetic base among these green gram landraces is rather narrow. Collection of diverse germplasm from centers of diversity and acquired from other sources may broaden the genetic base (Zhang et al., 1999). The genetic base could be broadened through the use of radiation, which is a drastic method of mutagenesis resulting in a major reshuffle of the genome (Guestine et al., 1999). Nevertheless, the reasonable diversity observed in this study may be exploited for further yield improvement.

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