Journal of Plant Breeding and Crop Science Vol. 3(3), pp. 53-59, March 2011
Available online http://www.academicjournals.org/jpbcs
ISSN 2006-9758 ©2011 Academic Journals

Full Length Research Paper

Genetic diversity analysis in different varieties of black gram using RAPD markers

Priya Srivastava[1], Anjana Pandey[1]* and Diamond Prakash Sinha[2]

[1]Nanotechnology and Molecular Biology Lab, Centre for Biotechnology, University of Allahabad, Allahabad, 211002, U.P., India.
[2]Indian Institutes of Vegetable Researches (ICAR), Jakhani, Varanasi, U.P. 221002, India.

Accepted 1 March, 2011

The molecular marker is a useful tool for assessing genetic variation and resolving cultivars identities. The objective of the present study was to investigate the genetic diversity and relationships among 8 black gram (*Vigna mungo*) varieties from diverse geographic locations of U.P. and Andhra Pradesh, (India) using random amplified polymorphic deoxyribonucleic acid (RAPD) markers. Forty decamer - primers could generate a total of 346 RAPD fragments, of which 338 or 97.68% were polymorphic. The size of amplified fragments was ranged from 50 to 3000 bp. The similarity coefficient was maximum between PLU-289 and PLU-456 (0.76) indicated the less divergence between them. Lower similarity was observed between LBG-20 and PLU-289 (0.4337) indicating more divergence.

Key words: Black gram, random amplified polymorphic deoxyribonucleic acid, genetic diversity, similarity coefficient, polymorphism.

INTRODUCTION

Black gram, (*Vigna mungo)* popularly known as urd bean or mash, is a grain legume domesticated from *V. mungo var. silvestris* (Lukoki et al., 1980). It is one of the important grain legumes and is an excellent source of easily digestible good quality protein. It belongs to the family Leguminaceae under the genus *Vigna* and subgenus *Ceratotropis*. Black gram is widely cultivated in the Indian subcontinent and to a lesser extent in Thailand, Australia and other Asian and South Pacific countries (Poehlman, 1991). It is cultivated as follow-up crop after rice cultivation in India. In recent years, there has been significant decline in the pulse production in India.

The major constraints in achieving higher yield of this crop are lack of genetic variability, absence of suitable ideotypes for different cropping system, poor harvest index and susceptibility to disease. Lack of suitable varieties and genotypes with adaptation to local condition is among the factors that affects the production. Black gram (*V. mungo)* forms one of the important con-stituents in the dietary practices of the local population. Identification of different genotypes of crop species is essential when diverse accessions of crop germplasm are to be characterized, newly developed cultivars are to be registered and purity of the variety is to be determined. There are numerous techniques available for assessing the genetic variability and relatedness among crop germplasm.

DNA based markers are effective and reliable tools for measuring genetic diversity in crop germplasm and studying evolutionary relationship. Molecular genetic techniques using DNA polymorphism is increasingly used to characterize and identify a novel germplasm for uses in the crop breeding process (O' Neill et al., 2003). The evaluation based on RAPD profiles would be suitable for providing such information due to its high level of polymorphism of this technique. In recent years, DNA fingerprinting system based on RAPD analysis have been increasingly utilized for detecting genetic polymorphisms in several plant genera. The evaluation of the genetic diversity would promote the efficient use of genetic variation in the breeding program (Paterson et al., 1991).

*Corresponding author. E-mail: apandey70@rediffmail.com. Tel: 923540807.

Abbreviations: RAPD, random amplified polymorphic DNA; **EDTA,** ethylene di amine tetra acetic acid.

Table 1. List of black gram genotypes used in the study.

Serial No.	Genotype	Pedigr source	Remarks	Morphology of seeds
1	LBG-17	Andhra Pradesh	Resistance to powdery mildew, photoperiod sensitive.	Small, rounded and light blackish and shiny seeds.
2	LBG – 20	Andhra Pradesh	Moderately resistance to powdery mildew	Dark green, small and oval seed with bright and smooth surface.
3	LBG-645	Andhra Pradesh	Susceptible to powdery mildew	Sap green, oval in shape, shiny and smooth.
4	LBG-623	Andhra Pradesh	Susceptible to powdery mildew	Sap green, drum/rectangular shaped and larger in size.
5	T-9	Uttar Pradesh	Resistance to powdery mildew, wider adaptability	Whitish gray, rounded and oval shape, non smooth surface.
6	U-19	Uttar Pradesh	Moderately Susceptible to powdery mildew.	Dark sap green, oval shaped, larger, non smooth seed.
7	PLU-839	Uttar Pradesh	Susceptible to powdery mildew.	Greenish black, rectangular /oval, dry seed with non smooth surface.
8	PLU-456	Uttar Pradesh	Susceptible to powdery mildew	Greenish black, oval, small seed with smooth and bright surface

RAPD markers have been used for the identification of cultivars and for assessing the genetic diversity among cultivars of several crops like beans (Skroch et al., 1992), cowpea *(*Mignouna et al., 1998), pea (Hoey et al., 1996), soybean (Brown et al., 2000), Ceratotropis (Kaga et al., 1996), mung bean (Santalla et al., 1998), and *Vigna angularis* (Yee et al., 1999). Genetic diversity studies in various species of the genus *Vigna* using DNA – based marker system are limited to a larger extent. The objective of the present study was to investigate and compare genetic diversity among 8 elite genotypes of known origin, using random amplified polymorphic DNA (RAPD). This would help in the identification and differentiation of various cultivars being cultivated/for export.

The information generated from this study will be used to identify effective strategies for the sustainable management of the genetic resources of black gram.

MATERIALS AND METHODS

Plant materials

Eight different varieties of black gram were used in the present study. All genotypes were collected from Indian Institute of Pulse Researches (IIPR), Kanpur, U.P. and Andhra Pradesh in the present study. Seeds of each accession were sown in plastic pots and grown under controlled conditions. The different accession, details of their geographical area of collection, seed characteristics and name of variety are given in Table 1. Young and healthy leaves were pooled from 20 to 25 days old plant, washed and quickly frozen and powdered using liquid nitrogen.

Isolation of DNA

Younger leaves from all plants of each accession grown under controlled condition were harvested, bulked and immediately frozen in liquid nitrogen, crushed and grounded to fine powder. DNA was extracted from young leaves by using a modified CTAB (cetyl tri methyl ammonium bromide) method described by Saghai -Maroof et al. (1984) with certain modifications. 0.3 to 0.5 g of leaves were crushed with liquid nitrogen and then suspended in 700 µl of extraction buffer (20 mM (EDTA) in ethylene di amine tetra acetic acid at pH - 8.0, 100 mM Tris-HCl at pH- 8.0, 1.5 M NaCl, 2% CTAB and 1% β mercapto ethanol). The suspension was mixed well and incubated for 60 min at 60°C. The solution was extracted twice with an equal volume of a mixture of chloroform – iso amyl alcohol (24:1). After centrifugation, the supernatant was transferred to another tube and incubated at room temp for 1 h in presence of 0.1 mg/ml RNase (Sigma Aldrich, USA). After incubation, 15% CTAB was added, mixed gently and then incubated for 15 min at 60°C. Solution was extracted with chloroform: isoamyl alcohol solution. After centrifugation DNA was precipitated in presence of equal volume of iso - propyl alcohol.

The DNA was pelleted down by centrifugation at 12000 rpm for 10 min and was then suspended in TE buffer (10 mM Tris HCl and 1 mM EDTA-pH- 8.0). The quality of genomic DNA was checked by using 0.8% agarose gel in presence of EtBr. The DNA samples were stored at -20°C until further analysis.

Polymerase chain reaction (PCR)

PCR amplification reactions (Williams et al., 1990) were performed

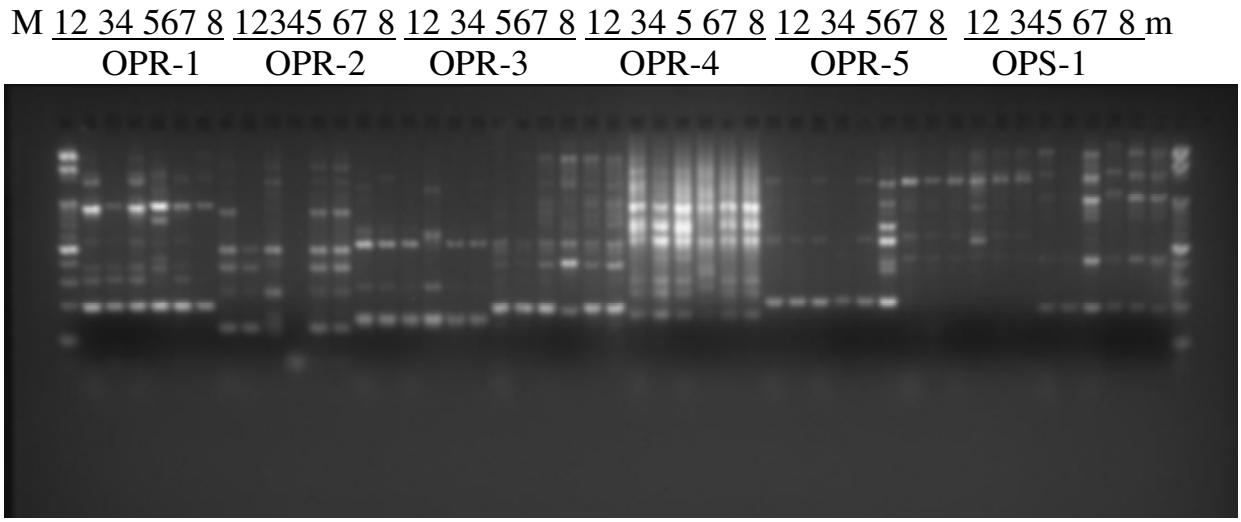

Figure 1. RAPD banding profile of 8 black gram accession using primer OPR 1 to 5. Lane-M, 100 bp markers, 1-8 Lane with different primers represents RAPD profile of DNA from 8 Black gram cultivars.

with random decamer primers obtained from Operon Technology (Almeda, Calif., USA). Total of forty eight 10-mer oligonucleotides primers from RAPD primers kits were subsequently used for PCR amplification. For RAPD analysis, PCR reaction was performed in 25 µl reaction mixture consisting 3U Taq DNA polymerase, 1X Taq DNA polymerase buffer, 1.5 µl of 50 mM $MgCl_2$, 10 µM dNTP, 70 ng sample DNA and 10 µM of primer with the following cycle repeated 40 times: pre denaturing at 94°C for 4 min, denaturing at 94°C for 1 min, annealing at 33°C elongation at 72°C for 1 min. Final elongation segment was held for 10 min at 72°C in thermal cycler PCR (thermo).

DNA electrophoresis

Amplified fragments were separated electrophoretically on 1.2% (w/v) agarose (Sigma Aldrich, USA) gels with 1X TAE (Tris- Acetic acid – EDTA) buffer and stained with ethidium bromide (EtBr). The 1 Kb DNA ladder plus molecular weight marker (Fermentas) was used to compare the molecular weight of amplified products. The gel was then visualized under UV light and photographs analyzed by using gel – documentation system (Alpha InfoTech, Alpha Imager).

Data analysis

RAPD profile was scored visually, based on presence (1) or absence (0) of band for each genotype. The binary matrix was used to estimate genetic similarity coefficients using Nei and Li (1979) equation. The similarity coefficients were subjected to UPGMA (Unweighed Pair-group Method of Arithmetic averages) method of clustering in order to generate the dendrogram. Analysis was performed using NTSYS-PC software (version 2.02).

RESULT AND DISCUSSION

The objective of the present study was to assess the extent of genetic diversity and relationships among the 8 black gram varieties which has been collected from Indian Institute of Pulse Researches (IIPR), Kanpur, U.P. and Andhra Pradesh possessing entirely different soil pattern. DNA extraction yielded 50 to 250 µg DNA from every gram of fresh sample and DNA quantified by UV absorption and agarose gel analysis. Amplification of genomic DNA of the 8 genotypes using 40 primers for RAPD analysis yielded 346 fragments. All the selected primers amplified successfully; 3 to 14 bands/loci which varied in size from 50 bps to 3000 bps. A total of 336 polymorphic RAPD fragments were generated. In this study 50 decamer primers were screened using the pooled DNA, prepared by mixing equal volume of diluted DNA from each of the 8 black gram species. 40 primers were identified, which yielded higher number of clear bands and these primers were subsequently used for PCR reactions with DNA from each of the 8 black gram cultivars separately. A representative profile of RAPD products (amplified with primer OPR-1 to OPR-6) from all 8 black gram is shown in Figure 1. The black gram landraces were analyzed by using 40 random primers.

Amplification of genomic DNA of the 8 genotypes using 40 primers for RAPD analysis yielded a total of 346 fragments that were scored. All the selected primers amplified fragments across the 8 genotypes studied, with the number of amplified fragment ranging from three (OPG-3) to fourteen (OPT-2) and which varies in size from 50 to 3000 bp. Of the 40 primers tested, the number of RAPD loci generated was higher for the primer OPT-2 which amplified 14 fragments followed by OPP- 4 (13 fragments) and OPP-3 (12 fragments). The lowest number of fragments was generated by the primer OPG-3 (3 fragments). The average number of markers produced per primer was 8.6. 34 primers showed 100% polymorphism. The primer OPT-4 showed lowest polymorphism (75%) among the accession tested (Table 2). The banding pattern of the black gram has been

Table 2. List of primers with their sequences used for the RAPD analysis and corresponding number of RAPD DNA markers generated.

Primer	Primer sequences	Total no. of bands	No. of poly morphic bands
OPG-01	CTACGGAGGA	06	06
OPG-02	GGCACTGAGS	06	06
OPG-03	GAGCCCTCCA	03	03
OPG-04	AGCGTGTCTG	11	11
OPG-05	CTGAGACGCA	11	11
OPH-01	GGTCGGAGAA	10	10
OPH-02	TCGGACGTGA	12	12
OPH-03	AGACGTCCAC	04	04
OPH-04	GGAAGTCGCC	11	11
OPH-05	AGTCCTCCCC	11	11
OPI- 01	ACCTGGACAC	09	09
OPI-02	GGAGGAGAGG	04	04
OPI-03	CAGAAGCCCA	09	09
OPI-04	CCGCCTAGTC	10	10
OPI-05	TGTTCCACGG	08	08
OPP-01	GTAGCACTCC	09	08
OPP-02	TCGGCACGCA	09	09
OPP-03	CTGATACGCC	12	12
OPP-04	GTGTCTCAGG	13	13
OPP-05	CCCCGGTAAC	08	07
OPQ-01	GGGACGATGG	11	10
OPQ-02	TCTGTCGGTC	09	09
OPQ-03	GGTCACCTCA	09	08
OPQ-04	AGTGCGCTGA	11	11
OPQ-05	CCGCGTCTTG	10	10
OPR-01	TGCGGGTCCT	09	08
OPR-02	CACAGCTGCC	10	10
OPR-03	ACACAGAGGG	08	08
OPR-04	CCCGTAGCAC	06	06
OPR-05	GACCTAGCAC	07	07
OPS-01	CTACTGCGCT	06	06
OPS-02	CCTCTGACTG	03	03
OPS-03	CACAGGTCCC	03	03
OPS-04	CACCCCCTTG	10	10
OPS-05	TTTGGGGCCT	06	06
OPT-01	GGGCCACTCA	10	10
OPT-02	GGAGAGACTC	14	14
OPT-03	TCCACTCCTG	04	03
OPT-04	CACAGAGGGA	12	09
OPT-05	GGGTTTGGCA	12	12

shown for the primer OPR-1 to OPR-6 (Figure 1). These results indicated the ability of RAPD to discriminate among black gram cultivars studied. RAPD technique is considered as a very sensitive method of screening for nucleotide sequence polymorphisms randomly distributed throughout the genome (Subudhi and Huang, 1999).

OPR-02_{1300} and OPR-02_{50} are the specific primers for generating polymorphic bands of 1300 and 50 bp respectively for cultivar LBG-20. OPR-01_{500} and OPR-05_{100} are the specific primers for generating polymorphic bands of 500 and 100 bp respectively for cultivar LBG-645. OPR-01_{2500}, OPR-01_{2000} and OPR-04_{100} are the specific primers for generating polymorphic bands of 2500, 2000 and 100 bp respectively for cultivar T-9. OPR-05_{1200} and OPR-05_{300} are the specific primers for generating polymorphic bands of 1200 and 300 bp

Table 3. Simple matching coefficient among 8 black gram sample based on the RAPD data.

	LBG-17	LBG-20	LBG-645	LBG-623	T-9	U-19	PLU-839	PLU-456
	1	2	3	4	5	6	7	8
1.	1.0000000							
2.	0.7319277	1.0000000						
4.	0.5963855	0.5331325	0.6807229	1.0000000				
5.	0.5873494	0.5361446	0.6174699	0.6054217	1.0000000			
6.	0.5843373	0.5572289	0.5722892	0.5481928	0.7560241	1.0000000		
7.	0.4728916	0.4337349	0.5210843	0.5030120	0.6746988	0.6656627	1.0000000	
8.	0.4457831	0.4427711	0.4879518	0.5421687	0.6054217	0.6144578	0.7620482	1.0000000

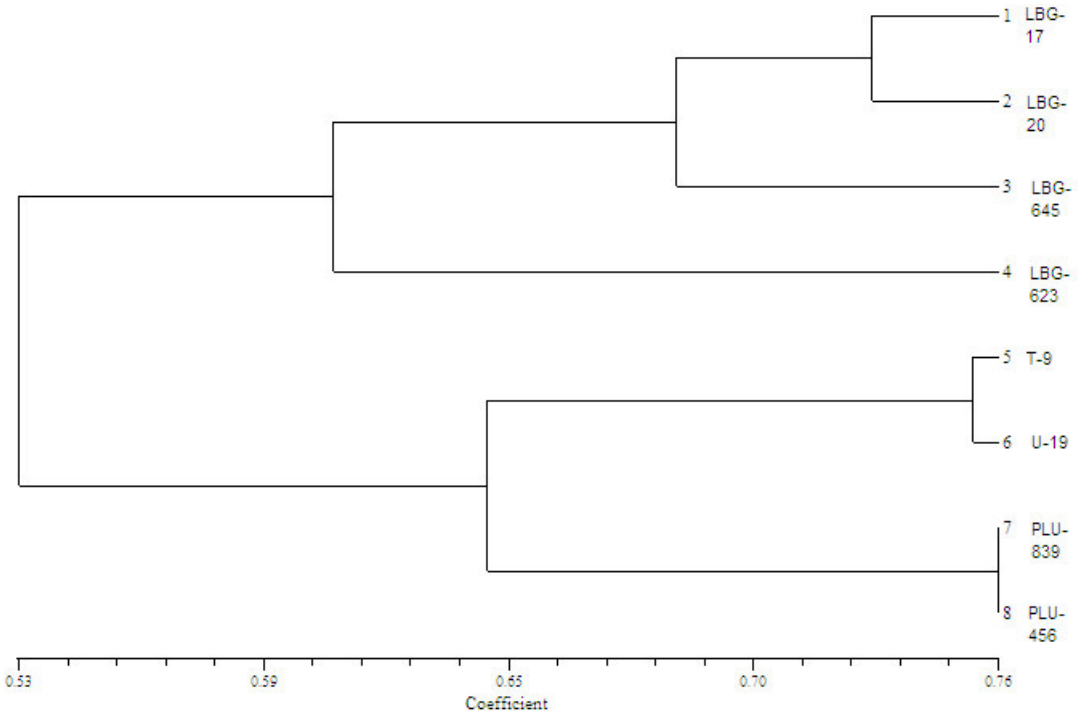

Figure 2. Dendrogram showing the relationships among the 8 accessions of black gram landraces drawn on simple matching coefficient.

respectively for cultivar PLU-839. The ability to resolve genetic variation may be more directly related to the number of polymorphism detected by the marker techniques and the percentage of polymorphic RAPDs. However, it does not correlate with the influence of rare and common alleles on the genetic diversity as a fragment of the lowest frequency has the same importance as a fragment with the highest frequency across the genome (Welsh and McClelland, 1990; Gepts, 1993; Ehlers and Hall, 1997). Simple matching coefficient was used to assess the genetic similarities, divergence and relationship among 8 black grams species. A dendogram based on simple matching coefficient analysis grouped the 8 genotype in to 2 clusters, with similarity coefficient ranging from 0.59 to 0.65 (Figure 1). In the present study, Simple matching coefficient was used to assess the genetic distance, variability and relationship among 8 black gram cultivars (Table 3) by using NTSYS-pc software 2007 version.

Analysis of the relationship based on 346 RAPD markers revealed genetic diversity among the cultivars which ranged from 0.4337 to 0.7620, and was mostly concentrated within 0.68 to 0.76. A dendrogram (Figure 2) was prepared based on simple matching coefficient taking into account the presence (1) or absence (0) of the bands. These coefficients have divided into two major clusters or there are two major clusters formed at the 0.53. The first cluster (A) consisted the 4 black gram cultivars-namely LBG-17, LBG-20, LBG-645, LBG-623 (kharif and rabi) whereas the second cluster included 4 cultivars of black gram namely, T-9, U-19, PLU-839 and PLU-456 (kharif). The dendogram and simple matching

coefficient values give an idea about the nature of the individual sample in the whole sample set. All black gram samples could be distinguished from one another based on these polymorphic bands. Similarities among the test samples was calculated with Simple matching coefficient which, indicated highest similarity index (0.7620) observed between PLU-839 (susceptible to powdery mildew disease) and PLU-456 (also susceptible to disease) while the lowest similarity index (0.4337) was observed between LBG-20 (moderately resistance to powdery mildew disease) and PLU-839 (susceptible to powdery mildew disease). These genotypes could be useful in breeding programmes. The present study has represented the high level of polymorphism (97.68 %).

Dendogram revealed that cultivar 7 (PLU-839) and 8 (PLU-456) had equal similarities thus, could not be used in hybridization process. These results are in accordance to the studies of phylogenetic diversity and relationship in cowpea (*Vigna unguiculata* L. walp.) by using RAPD polymorphic DNA marker (Karuppanapandian et al., 2006). Genetic diversity in black gram (*V. mungo* L. Hepper) has been studied by using multivariate techniques on the basis of agronomic characters and identified the best genotypes for breeding (Abdul and Moh. Arshad, 2008). Genetic relationships between wild and cultivated *Vigna* species were studied by cluster analysis and genetic distance determination by using RAPD polymorphic markers (Samarajeewa et al., 2002). The evolution of varieties in distinct agro – climatic area demonstrates significant levels of variation in response to the selection pressure in the zones (Singh et al., 1998). The RAPD technique has been applied to assess molecular polymorphism in *Vigna* (Kaga et al., 1996), mung bean (Santalla et al., 1998; Lakhanpaul et al., 2000), chick pea (Sonnante et al., 1997), pea (Simioniuc et al., 2002) and pigeon pea (Ratnaparkhe et al., 1995). In addition, the studies on morphological markers, molecular markers are quite useful in analyzing the genetic differences in plant population at DNA level (Yoon et al., 2000). The evaluation of the pattern and the extent of genetic variability and relatedness among traditional varieties to improved cultivars of rice were studied by using RAPD markers (Rabbani et al., 2008). This result indicates the presence of great genetic variability among elite genotypes of black gram.

The PLU-839 variety and PLU-456 showed very minimum differences between them both at phenotypic and genotypic levels. This study could identify diverse genotypes like LBG-20 and PLU-839 for their use in hybridization program of black gram. RAPD markers are useful in the assessment of black gram diversity, through detection of duplicate samples in germplasm collection, and the selection of a core collection to enhance the efficacy of germplasm management for use in black gram breeding and conservation programs. The genetic diversity obtained in this study might be useful in future strategies for evolution of desired genotypes.

ACKNOWLEDGEMENTS

The author is grateful to Department of Science and Technology- Integrated Long Term Project, Government of India, New Delhi for providing funding facility and IIPR, Kanpur, U.P., Andhra Pradesh for providing the seed used for this work.

REFERENCES

Abdul Ghafoor, Muhammad Arshad (2008). Multivariate Analysis for Quantitative Traits to Determine Genetic Diversity of Black Gram (*Vigna mungo* L. Hepper). Pak. J. Bot., 40(6): 2307-2313.

Brown- Guedira GA, Thompson JA, Nelson RL, Warburton ML (2000). Evaluation of genetic diversity of soybean introductions and North American ancestors using RAPD and SSR markers. Crop Sci., 40: 815-823.

Ehlers JD, Hall AE (1997). Cow pea (*Vigna unguiculata* L. Walp.). Field Crops Res., 53: 187- 204.

Gepts P (1993). The use of molecular and biochemical markers in crop evolution studies. Evol. Biol., 27: 51-94.

Hoey BK, Crowe KR, Jones VM (1996). A phylogenetic analysis of Pisum sativum based on morphological characters and allozyme and RAPD markers. Theor Appl Genet., 92: 92–100.

Kaga AN, Tomooka N, Egawa Y, Hosaka K, Kamijima O (1996). Species relationships in subgenus Ceratotropis (genus *Vigna*) as revealed by RAPD analysis. Euphytica, 88:17–24.

Lakhanpaul S, Sonia Chadha, Bhat KV (2000). Random amplified polymorphic DNA (RAPD) analysis in Indian mung bean (*Vigna radiat*a (L.) Wilczek) cultivars. J. Genetica, 109, (3): 227-234.

Lukoki L, Marechal R, Otoul E (1980**)**. Les ancetres sauvages des haricots cultives: *Vigna radiata* (L.)Wilczek et *V. mungo* (L.) Hepper. Bull. Jard. Bot. Nat. Belgique., 50: 385-391.

Mignouna HD, Ng NQ, Ikea J, Thottapilly G (1998). Genetic diversity in cowpea as revealed by Random amplified polymorphic DNA. Genet. Breed, 53: 151-159.

O'Neill R, Snowdon RJ, Kohler, W (2003). Population genetics aspects of biodiversity. Progress Bot., 64: 115-137.

Paterson AH, Damon S, Hewitt JD, Zamir D, Rabinowitch HD (1991). Mendelian factors underlying quantitative traits in tomato: comparison across species, generations, environments. Genet., 127: 181–97.

Samarajeewa PK, Nanayakkara HLV, Ekanayake EMD SN, Sumanasinghe VA (2002). RAPD analysis of genetic relationship of wild and cultivated *Vigna* species. Annals of Sri Lanka Depart. Agric., 4: 215-226.

Poehlman JM (1991). History, description, classification and origin. In: Poehlman JM (ed) The mungbean, West View, Boulder. 6–21.

Ratnaparkhe MB, Gupta VS, Venmurthy MR (1995). Genetic fingerprinting of pigeon pea [Cajanus cajan (L.) Millsp.] and wild relatives using RAPD markers. Theor. Appl. Genet. 91:893–898.

Saghai- Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984). Proc. Natl. Acad. Sci. USA, 83: 1757–1761.

Santalla M, Power JB, Davey MR (1998). Genetic diversity in mungbean germplasm revealed by RAPD markers. Plant Breed, 117: 473–478.

Simioniuc D, Uptmoor R, Friedt W, Ordon F (2002). Genetic diversity and relationships among pea cultivars revealed by RAPDs and AFLPs. Plant Breed, 121: 429–435.

Singh AK, Smart J, Simpson CE, Raina SN (1998). Genetic variation vis-à-vis molecular polymorphism in groundnut, (*Arachis hypogaea*) L. Genet. Resour Crop Evol., 45: 119–126.

Skroch PW, dos Santos JB, Nienhuis J (1992). Genetic relationships among Phaseolus vulgaris genotypes based on RAPD marker data. Annu. Rep. Bean Improv. Coop, 35: 23–24.

Sonnante G, Marangi A, Venora G, Pignone D (1997). Using RAPD markers to investigate genetic variation in chickpea. J. Genet Breed., 51: 303–307.

Subudhi P K, Huang N **(**1999). RAPD mapping in a doubled haploid

population of rice (*Oryza sativa* L.) Hereditas, 130: 2-9.
Karuppanapandian T, Karuppudurai T, Sinha PB, Haniya AMK, Manoharan K (2006). Phylogenetic Diversity and relationships among cowpea (*Vigna unguiculata*, L. Walp.) landraces using Random amplified Polymorphic Marker. Gen. Appl. Plant Physiol., 32(34): 141-152.
Welsh J, McClelland M (1990). Finger printing genomes using PCR with arbitrary primers. Nucl. Acids Res., 18: 7213-7218.
Williams JGK, Kubelik AR, Livak KJ, Rafalaski JA, Tingey SV (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., 18: 6531–6535.
Yee E, Kidwell KK, Sills GR, Lumpkin TA (1999). Diversity among selected Vigna angularis (Azuki) accessions on the basis of RAPD and AFLP markers. Crop Sci., 39: 268–275.
Yoon MS, Doi K, Kaga A, Tomooka N, Vaughan DA (2000). Analysis of genetic diversity in the Vigna minima and related species in east. Asia J. Plant Res., 113: 375-386.