academicJournals

Vol. 12(51), pp. 7031-7037, 18 December, 2013
DOI: 10.5897/AJB2013.13115
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Genetic diversity studies in common bean (*Phaseolus vulgaris* L.) using molecular markers

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Accepted 25 November, 2013

Molecular characterization of thirteen common bean genotypes was done with random amplified polymorphic DNA (RAPD) markers. Initially, 15 primers were screened out of which only seven were selected which generated a total of 65 amplification products out of which 63 bands (96.62%) were polymorphic indicating fair amount of polymorphism. The genotypes shared 43% genetic similarity among themselves. Cluster analysis delineated the genotypes into three groups with seven, five and one genotype in cluster-I, II and III, respectively. The maximum similarity index (82.35) based dice similarity coefficient was obtained between SKUA-R-21 and SKUA-R-19, while it was minimum (27.72) between genotypes PBG-29 and SKUA-R-01.

Key words: Genetic divergence, common bean, random amplified polymorphic DNA (RAPD).

INTRODUCTION

Common bean is regarded as “Grain of hope” as it is an important component of subsistence agriculture and feeds about 300 million people in tropics and 100 million people in Africa alone. Besides, it is emerging as an important income generation especially in Central America where beans are No. 1 income generators among field crops. Globally, with 21 million tons produced from about 26 million hectares, it accounts for about half of the total pulse production. In India, common bean is grown over an area of about 6 million hectares with a production of about 2.5 million tons (FAO, 2010). Among the pulses, *Phaseolus* genus contains approximately 70 species and within this genus, common bean (*Phaseolus vulgaris* L.) is an excellent food choice with its nutritional composition includes carbohydrates, proteins, vitamins, minerals and antioxidants (Svetleva et al., 2006). Common bean is a diploid ($2n = 2x = 22$) and predominantly self crossing species although 3% or more out crossing ratio has also been observed (Ibarra-Perez et al., 1997). Studies using molecular, physiological and morphological analyses in common bean strongly support the existence of two distinct centers of genetic diversity known as the Mesoamerican or small-seeded type and Andean or large seeded type gene pools (Blair et al., 2007; Burle et al., 2010). Today common beans are grown in many countries but widely cultivated in the tropics, subtropics, and temperate regions (Burle et al., 2010).

A complete understanding of the genetic diversity and population structure of the common bean is essential for its conservation and management, but limited germplasm characterization is a major challenge for systematic use of common bean diversity in genetic breeding programs.

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Abbreviations: RFLP, Restriction fragment length polymorphism; **RAPD,** random amplified polymorphic DNA; **CTAB,** cetyl trimethyl ammonium bromide; **PCR,** polymerase chain reaction.

Table 1. List of germplasm lines used in the study.

Genotype	Status	Place of collection
SKUA-R-01	Breeding line	PRS, Habbak
SKUA-R-11	Landrace	-do-
SKUA-R-19	Landrace	-do-
SKUA-R-21	Landrace	-do-
SKUA-R-28	Landrace	-do-
SKUA-R-34	Landrace	-do-
PBG-01	Landrace	Assar Doda
PBG-03	Traditional cultivar	Gool-Gulab Gad Doda
PBG-09	Traditional cultivar	Kishtward
PBG-16	Traditional cultivar	Beejic Poonch
PBG-29	Traditional cultivar	Khangund Pulwama
PBG-30	Traditional cultivar	Shangund Pulwama
Canadian Red	Breeding line	PRS, Habbak

Classical methods for characterizing genetic diversity in plants include the use of morpho-agronomic traits to establish genetic relationships among commercial cultivars, landraces and wild. Several types of DNA markers, developed to study genetic diversity and crop evolution, are now considered to be better for documenting the organization of diversity, when compared to former methods, such as morphologic markers (Blair et al., 2009; Kwak and Gepts, 2009; Burle et al., 2010). Human-directed selection of common-bean populations has influenced crop evolution, with cultivars originating through domestication of adjacent areas now being conceived as showing higher mutual similarity than germplasm from distant regions. Molecular characterization is required, not only to corroborate previous findings based on morpho-agronomic characterization, but also to increase the efficient use of germplasm for crop breeding. Molecular markers would also be beneficial towards improving representation in the core collection, by using a reduced number of cultivars.

Since restriction fragment length polymorphism (RFLP) were abundant and were more informative due to their co-dominant nature. Their application to breeders, however, was restricted by the costly and sophisticated techniques required. Conversely, the advantage of random amplified polymorphic DNA (RAPD) markers as a rapid, cost effective tool for the indirect selection of economic traits was immediately recognized by breeders, despite initial problems in reproducibility between laboratories. In addition to their value in genetic mapping (Grisi et al., 2007) and 'gene tagging studies', RAPD have been deployed extensively in different plant species for germplasm classification and have proved to be more useful in detecting genetic variation and classification of germplasm accessions (Ender et al., 2008; Tiwari et al., 2005). In recent years, molecular techniques including RAPD analysis have been used to characterize variability in *Phaseolous* spp. (Martins et al., 2006; Marotti et al., 2007). In view of this, the present study was undertaken to estimate the genetic diversity in common bean genotypes using molecular markers (RADP).

MATERIALS AND METHODS

Plant material

The experimental material for the present study comprised of 13 genotypes of common bean including one check viz., Canadian Red. All the genotypes were local landraces/traditional cultivars collected from different common bean growing areas of state of Jammu and Kashmir (India). The list of the genotypes is presented in Table 1. The experiment was laid out during *kharif* 2008 and 2009 in a randomized complete block design with three replications. The experimental materials were provided the cropping geometry of 30 cm between the rows and plant to plant spacing of 10 cm. The experimental fields were well prepared and standard recommended package of practices were followed to raise a good crop.

DNA extraction

Plant DNA was isolated using cetyl trimethyl ammonium bromide (CTAB) method as modified by Saghai-Maroof et al. (1984). In this method, young leaves at trifoliate stage were harvested from 5-8 plants from each row of individual genotypes (approximately 5-7 g of fresh weight). The leaves were ground to fine powder using pre-chilled pestle and mortar after adding liquid nitrogen to make leaves brittle as well as to stop DNase activity. The powder was transferred immediately to a 50 ml autoclaved polypropylene centrifuge tube containing 15 ml of pre-warmed (65°C) 2% CTAB extraction buffer. The powder was suspended in the buffer by inverting and rotating the tubes properly. The tubes were incubated at 65°C for 30-40 min in a water bath. The samples were mixed occasionally while maintaining at 65°C. After incubation, 15 ml of chloroform: isoamyl alcohol (24:1) was added and tubes were swirled, till it made a dark green emulsion. The tubes were placed on a rotary shaker for 30 min and then centrifuged at 13,000 rpm for 5 min at room temperature. The supernatant was transferred to a clean sterile 50 ml falcon tube. Four microliter (4 μl) RNase (10 mg/ml) was added to each tube and incubated at 37°C in water bath for 1 h.

Chloroform: isoamyl alcohol extraction and centrifugation step was repeated after RNase treatment if required. Following centri-

Table 2. Base sequence of primers used for DNA fingerprinting.

Primer	Sequence (5′ to 3′)
OPA-01	CAG GCC CTT C
OPA-02	TGC CGA GCT G
OPA-03	ATG CAG CCA C
OPA-04	AAT CGG GCT G
OPA-05	AGG GGT CTT G
OPA-06	GGT CCC TGA C
OPA-07	GAA ACG GGT G
OPA-08	GTG ACG TAG G
OPA-09	GGG TAA CGC C
OPA-10	GTG ATC GCG T
OPA-11	CAA TCG CCG T
OPA-12	TCG GCG ATA G
OPA-13	CAG CAC CCA C
OPA-14	TCT GTG CTG G
OPA-15	TTC CGA ACC C

Table 3. Temperature profile used in PCR.

Step	Temperature	Time (minutes)	Number of cycles
Initial denaturation	94	5	1
Denaturation	94	1	40
Annealing	37	2	
Elongation	72	2	
Final extension	94	5	
Hold	4	5	1

fugation, the upper aqueous phase was transferred to a clean sterile 50 ml falcon tube. About 0.8 volume of chilled isopropyl alcohol was added and the tubes were inverted gently several times. The DNA formed white cotton like precipitate and good quality DNA floated atop. The floating DNA was hocked out using a sterile hocked Pasteur pipette, if the DNA was not hockable, it was pelleted by centrifugation. The hocked or pelleted DNA was transferred into a clean sterile 2.0 ml microfuge tubes and was rinsed with 70% ethanol for 5 min so as to remove any residual salts followed by re-centrifugation. Pellet was collected and the left over ethanol was dried up completely by turning down microfuge tubes on a blotting paper and was allowed to air dry (at room temperature) for 1 h. Then 50-80 µl volume of 1x TE (Tris EDTA buffer 10 mM tris HCl, 1mM EDTA, pH 8.0) was added. The tubes were left for few hours at room temperature to allow DNA to dissolve. The quality and quantity of DNA was checked by agarose gel electrophoresis.

RAPD analysis

Primer selection

Fifteen 10-mer oligonucleotides primers (Operon Technologies Inc., CA. USA) were used for characterization of genotypes. The selected RAPD primers along with their base sequence are presented in Table 2.

Polymerase chain reaction (PCR) amplification

In vitro amplification using PCR was performed in a 96 well Biometra Mode-II T-gradient thermoblock using 50 ng of genomic DNA of each genotype in a final volume of 20 µl per reaction. Amplification was performed using temperature profile mentioned in Table 3.

Visualization of PCR products

To 20 µl of the amplified product, 3.33 µl of 6x loading dye was added so as to make the final concentration of the loading dye in the reaction samples to 1x. The PCR products were resolved on 1.55 super fine resolution agarose gel. The gel was prepared in 0.5x TBE buffer. Ethidium bromide was added at concentration of 0.4 mg/µl. The gel was run at 10 v/cm^2, visualized under UV light and photographed using utra cam digital imaging (A6 rc canon camera).

Scoring of RAPD fragments

The size of the DNA fragments was estimated by comparing the DNA bands with a 1 Kb DNA ladder. The amplified bands were scored as present (1) or abscent (0) and were assembled in a data matrix table.

Table 4. Primers, total number of bands, polymorphic band and percentage of polymorphism obtained by PCR amplification of DNA of *Phaseolus vulgaris* L. genotypes.

Primer	Total Number of bands	Number of polymorphic bands	Percentage of polymorphism
OPA_1	12	12	100
OPA_2	11	11	100
OPA_3	8	6	75
OPA_{10}	12	12	100
OPA_{11}	11	11	100
OPA_{13}	6	6	100
OPA_{15}	5	5	100
Total	**65**	**63**	**96.92**
Mean per primer	**9.28**	**9.00**	

Cluster analysis

Cluster analysis of the germplasm, using binary data generated by RAPD markers was conducted using computer software programme numerical taxonomic and multivariate analysis system (NTSYS-PC) version 2.02e (Rohlf, 1997). RAPD marker amplification profile for all the genotypes under study was compared to each other and DNA fragments were scored as present (scored as 1) or absent (scored as 0). Data from all the 15 primers were used to estimate the similarity based on the number of shared amplified bands. Similarity was estimated using SIMQUAL function of NTSYS, which computes a variety of similarity coefficient for quantitative data (nominal data). Similarity matrix value based on Nei and Li (1979) coefficient of similarity (D_{ij}) were calculated as

$$D_{ij} = 2a/(2a + b + c)$$

Where, 'a' represents matched fragments b and c are unmatched fragments. The 2a + b + c are the total number of fragments amplified in a particular set. The similarity matrix was then generated and dendrogram was constructed using unweighted pair group method using arithmetic averages (UPGMA) available in NTSYS.

RESULTS AND DISCUSSION

The present investigation revealed a high level of polymorphism in the tested genotypes. Initially 15 decamer oligonucleoticle primers were screened out of which 8 showed unclear or non-reproducible behaviour and did not agree with the criteria of Lynch and Milligan (1994) and hence were rejected. These criteria were considered because it is an objective way to limit the bias resulting from the dominant biallelic properties of RAPD's. However, the rest of the 7 primers were selected based on robustness of amplification, reproducibility, scorability of banding patterns and were used for diversity analysis in all the 13 genotypes. The seven selected decamer oligonucleotide primers generated 65 amplification products, out of which 63 bands (96.92%) were polymorphic, which is slightly of higher percentage than the value obtained by Maciel et al. (2001) in common beans. These high values could be due to the nature of RAPD markers. Total number of bands obtained per primer in 13 genotypes ranged from 5 to 12 with an average of 9.28 bands per primers (Table 4). The maximum number of scorable bands 12 was obtained from primers (OPA_1 and OPA_{10}), followed by 11 bands from (OPA_2 and OPA_{11}). The present study is well supported by Jose et al. (2009) who investigated 20 common bean landraces using RAPD markers with respect to their genetic diversity. After initial screening of 72 primers, only thirteen primers were selected which generated a total of 102 amplicous with (63.55) polymorphism. Zhang et al. (2008) carried out genetic diversity study of 229 landraces with 30 micro-satellite markers and reported detection of 166 alleles with an average of 5.5 alleles per locus for all markers. Similarly, Sharma et al. (2006) investigated 46 common bean accessions with RAPD markers and reported a generations of 43 amplicons with (70%) polymorphism.

Banding pattern of genotypes were obtained after PCR amplification with primer (OPA_2) (Figure 1). The scorable bands were subjected to cluster analysis, which generated a dendrogram (Figure 2). The results revealed that the high level of genetic diversity was obtained among the 13 genotypes of common bean. The dendrogram was constructed using the tree plot option available an NTSYS-pc software. The genotypes were divided into three clusters in which cluster-I contained maximum of 7 genotypes (PBG-03, PBG-16, PBG-01, PBG-09, SKUA-R-01, SKUA-R-21 and SKUA-R-19), cluster-II contained 5 genotypes (SKUA-R-34, Canadian Red, SKUA-R-28, PBG-30 and SKUA-R-11) and cluster-III contained only one genotype, that is, PBG-29 (Table 5). All the three major clusters viz., cluster-I, cluster-II and cluster-III merged into a single cluster at 43 per cent similarity.

The phonetic representation of similarity coefficient among 13 genotypes of common bean is presented in Table 6. It is evident from the study that the similarity index, based on Dice similarity coefficient, ranged from 27.72 to 82.35 and the maximum similarity index was obtained among genotypes SKUA-R-2 with SKUA-R-19 and SKUA-R-11 (82.35) followed by genotypes PBG-30 and SKUA-R-28 (78.37). The minimum similarity index was obtained between genotypes PBG-29 and SKUA-R-01 (27.72). The

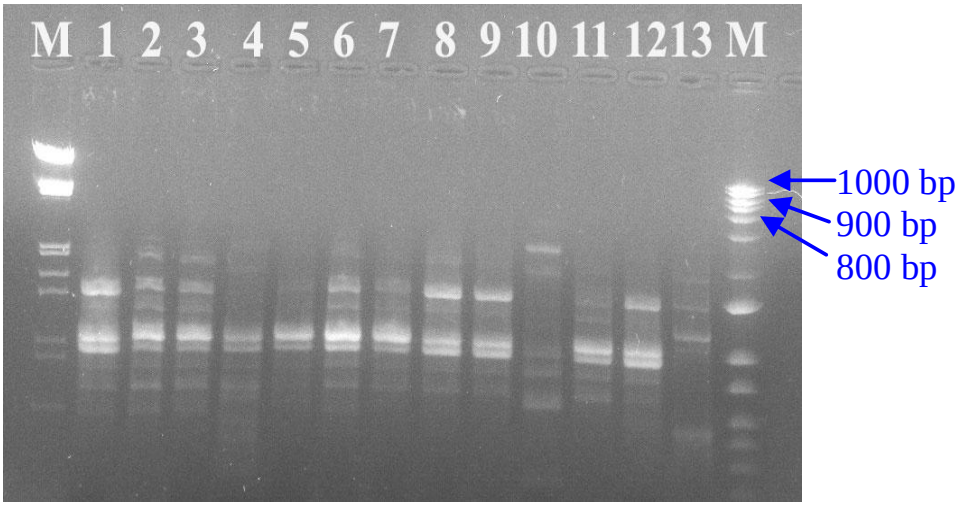

Figure 1. A typical RAPD banding pattern amplified with primer (OPA_2). Lane 1, PBG-03; lane 2, PBG-01; lane 3, PBG-09; lane 4, PBG-16; lane 5, SKUA-R-01; lane 6, SKUA-R-19; lane 7, SKUA-R-11; lane 8, SKUA-R-21; lane 9, SKUA-R-34; lane 10, Canadian Red; lane 11, SKUA-R-28; lane 12, PBG-30; lane 13, PBG-29; M, marker.

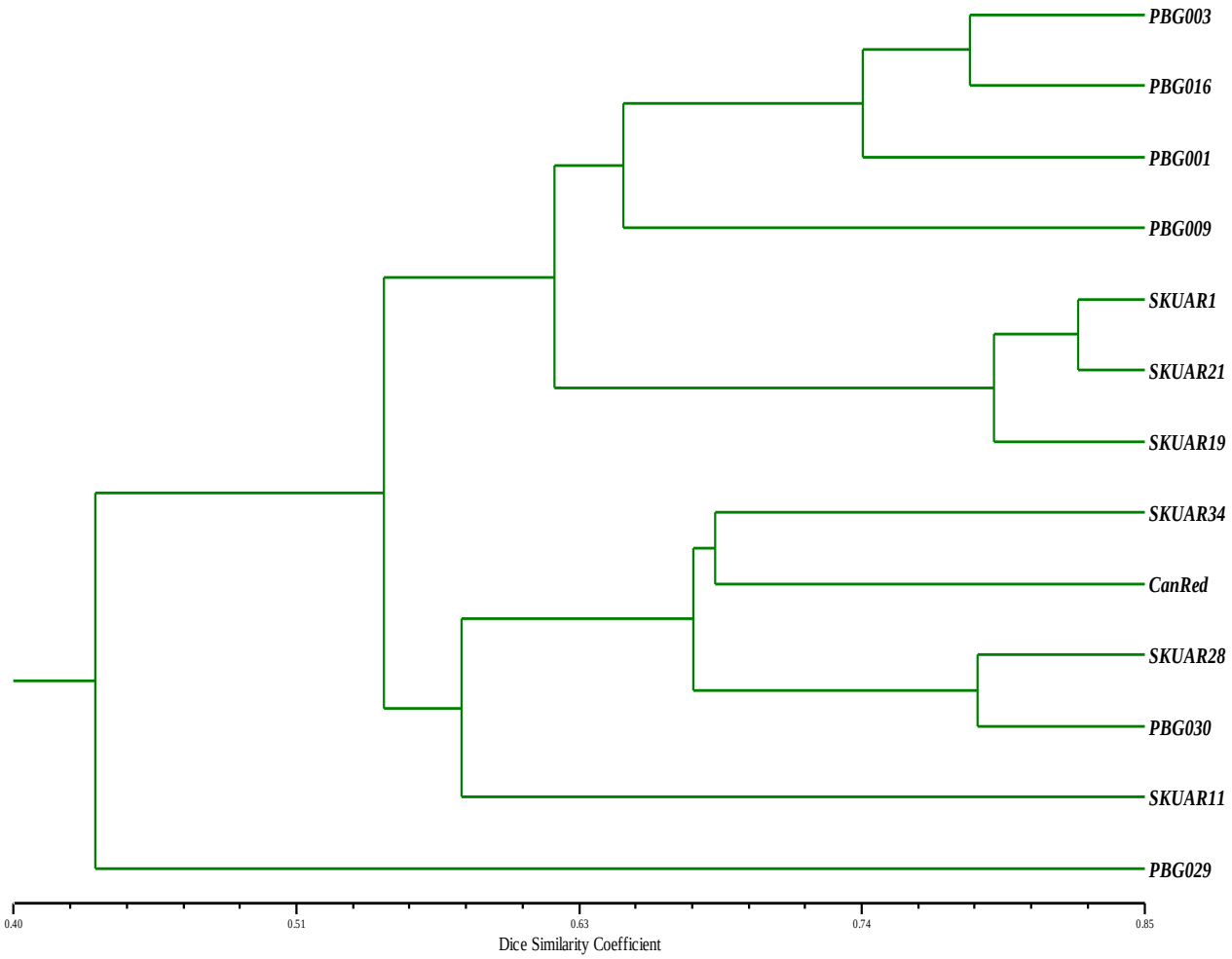

Figure 2. Dendrogrm depicting genetic relationship among 13 genotyeps of common bean (*Phaseolus vulgaris* L.) based on RAPD data using UPGMA (Dice coefficient).

Table 5. Classification of common bean (*Phaseolus vulgaris* L.) genotypes on the basis of RAPD data.

Cluster	Genotype	Number of genotypes in the cluster
I		
Ia	PBG-03, PBG-16, PBG-01	7
Ib	PBG-09	
Ic	SKUA-R-01, SKUA-R-21, SKUA-R-19	
II		
IIa	SKUA-R-34, Canadian Red	5
IIb	SKUA-R-28, PBG-30	
IIc	SKUA-R-11	
III	PBG-29	1

Table 6. Similarity coefficient matrix for 13 genotypes of common bean (*Phaseolus vulagaris* L.) using UPGMA (dice coefficient).

Genotype	PBG-03	PBG-09	PBG-01	PBG-16	SKUA-R-01	SKUA-R-19	SKUA-R-11	SKUA-R-21	SKUA-R-34	Canadian Red	SKUA-R-28	PBG-30	PBG-29
PBG-03	1.00												
PBG-09	60.71	1.00											
PBG-01	77.55	62.74	1.00										
PBG-16	78.04	69.38	70.00	1.00									
SKUA-R-01	40.00	53.84	31.11	38.09	1.00								
SKUA-R-19	51.42	74.41	52.94	57.89	59.45	1.00							
SKUA-R-11	71.42	58.62	62.74	65.11	50.00	75.67	1.00						
SKUA-R-21	69.38	58.82	54.54	61.11	56.52	82.35	82.34	1.00					
SKUA-R-34	61.53	51.85	55.31	63.15	43.47	58.82	69.09	72.34	1.00				
Canadian Red	57.62	49.18	48.14	66.66	42.27	60.00	65.57	72.72	67.92	1.00			
SKUA-R-28	50.00	51.42	34.92	59.25	40.62	41.66	57.14	53.96	64.40	71.23	1.00		
PBG-30	53.00	54.83	43.63	70.83	35.71	57.14	61.29	54.54	67.85	64.61	78.37	1.00	
PBG-29	45.83	40.00	32.55	61.53	27.72	48.48	44.00	46.51	50.00	56.60	58.06	6.66	1.00

difference at molecular level can be explained on their long span of cultivation in different areas which differ on the basis of soil types, climatic conditions and cultivation practices. The recombination event in support of adaptation to the environment leads to the creation of distinct genotypes. Molecular markers, unlike morpho-agronomic traits, are not influenced by environmental conditions and, therefore, are more reliable tools not only to characterize genotypes, but also to measure genetic relationship more precisely. Present study established the utility of DNA fingerprinting in genotypes using RAPD markers which revealed the presence of genetic diversity among the genotypes studied. In spite of the fact that

common bean described as an autogamous plant, recent evidences raises the possibility that some variability exists in the reproductive system of domesticated and wild varieties (Santalla et al*.,* 2002). The study can be well supported by the study of Chiorato et al*.* (2007). They studied a set of 220 common bean genotypes and reported that theses genotypes made two groups with 47 and 60% genetic similarity and interpreted that both molecular and morpho-agronomical data sets are equally effective to quantify and organize the genetic diversity of common beans. Similarly, Jose et al*.* (2009) found that Jaccard's pair-wise similarity coefficient value of 0.5 to 0.95 indicated an intra-specific genetic variation prevalent in landraces of common bean.

Breeding strategies need to exploit the existing variation within and between wild beans and landraces. Hybridization programme can be initiated between the identified diverse genotypes in order to create variation and for incorporation of the desire trait. The molecular markers, especially SCAR can be utilized for transfer of the desired trait in short duration. These bean germplasm could broaden the genetic base of commercial beans to develop high yielding cultivars.

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