Utilization of molecular and morphometric tools for assessment of genetic diversity of Rice bean [*Vigna umbellata* (Thunb.) Ohwi and Ohashi]

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A B S T R A C T

Rice bean [*Vigna umbellata* (Thunb.) Ohwi and Ohashi] is an orphan grain legume, grown in several parts of the country and has a pivotal role as a pulse in supporting the food security of the rural poor people, particularly in hill areas. Multipurpose uses and high nutritional quality of the crop gains its importance in the future food security needs of the country. Despite many useful characteristics, it has not been subjected to systematic breeding, hence a study was undertaken to study the diversity of the available germplasm collected from Himachal Pradesh. In the present study, RAPD markers were employed to study the genetic diversity, a RAPD profile of 64 rice bean genotypes generated 83 bands, of which 47 were polymorphic (56.62% of polymorphism) with Jacard's similarity coefficient matrices variation from 0.48 to 1.00. Based on the polymorphism exhibited by RAPD markers, 64 rice bean genotypes were grouped into 2 main clusters which again sub divided into several sub-clusters. PCA analysis gave the 12 principal components; among them first two were accounted for 61.65% of the total variability. A biplot plotted, showed a positive correlation among all the traits except crude protein with days to fifty percent flowering and days to fifty percent maturity. Results revealed from the study were preliminary and can be utilized in rice bean improvement programmes and further advanced studies.

Introduction

Rice bean [*Vigna umbellata* (Thunb.) Ohwi and Ohashi] is an underutilized grain legume with chromosome number (2n=2x=22). Its presumed wild progenitor is *V. umbellata* var. gracilis with which it is cross-fertile (Tomooka *et al.,* 1991). The center of genetic diversity and domestication of rice bean is in Southeast Asia. The wild forms of rice bean are distributed in North-Eastern India, Burma, Thailand, Laos and Vietnam (Ohashi *et al.,* 1988). This non-traditional and underutilized legume has gained attention as supplementary food crop (Gruere *et al.,* 2006). Rice bean seed contains 25% protein, 0.49% fat and 5% fiber. It is also rich in methionine and tryptophan as well as vitamins (thiamine,

niacin and riboflavin) and restores soil fertility through biological nitrogen fixation (Lohani 1979). It is an important food legume in niche environments and has a pivotal role as a pulse in supporting the food security of the rural poor people, particularly in hill areas. This crop possesses immense potential due to its high nutritional quality, high grain yield and multipurpose usage as food, animal feed, cover crop, green manure and as soil enricher by fixing atmospheric nitrogen (Chaterjee and Dana 1977; Tomooka *et al.,* 2002; Doanh and Tuan 2004). Despite many useful characteristics, it has not been subjected to systematic breeding including disease resistance and the highest potential grain yield among Ceratotropis species (Smartt 1990), and hence little exploited. Thus, there is substantial dearth of scientific studies to assess diversity, use value and marketability.

Knowledge of genetic diversity in a crop species is fundamental to its improvement. It has the evolutionary significance for the survival and adaptation of species in different environments. Traditionally, a variety of morphological and biochemical markers were used to assess diversity based on phenotypic traits, isozymes and protein analysis. Moreover, with the advent of molecular biology techniques, DNA based markers viz. RFLPs, SSRs, RAPDs and AFLPs have played a significant role in species identification and characterization of germplasm. Molecular markers offer plant breeders a set of genetic tools that is abundant, non-deleterious and reliable. Molecular marker system has been successfully used to construct genetic maps, assess genetic diversity and locate genes of interest in a number of agriculturally important crops for the desired traits (Garcia *et al.,* 2005). Among various molecular markers, RAPD is a multi-locus, highly reproducible and rapid method because no prior DNA sequence information is needed

for designing PCR primers. Besides being dominant, convenient and allows a larger number of marker to be assayed in short time.

To diversify the primary gene pool of Rice bean accessions, promising genotypes of rice bean were collected from the diverse regions of Himachal Pradesh, Punjab and Uttarakhand. It is expected that the rice bean genotypes from these regions may have economically important adaptive traits that can potentially be incorporated into the rice bean breeding programme for improving disease and pest resistance, nutritional quality and other traits of interest. Therefore, the present study was carried out to assess the level of genetic diversity among sixty-four rice bean genotypes by using morphological and RAPD markers to aid in the selection and efficient utilization of diverse genotypes in improvement of rice bean.

Materials and Methods

Plant Genetic Materials

The experimental material for the present study comprised of 64 rice bean genotypes including 4 checks (RBL-1, RBL-6, PRR-1 and PRR-2) collected from the diverse regions of Himachal Pradesh, Punjab and Uttarakhand. These genotypes along with checks were evaluated in alpha lattice design consisting of three replications, eight blocks per replication and eight entries per block. The crop was raised by following standard agronomic practices. Five representative plants in each accession were tagged for recording qualitative and quantitative traits. The present investigation was carried out at Department of Crop Improvement, College of Agriculture, CSK HPKV, Palampur during kharif 2010-11. Different analysis were performed on mean data for each trait using different statistical software viz., principal component analysis, biplot drawing (XLSTAT) and cluster analysis.

Isolation of Genomic DNA

Total genomic DNA was extracted from young leaf tissues (0.5-1g) collected from randomly selected healthy plants per genotype using CTAB (Cetyl Tri Methyl Ammonium Bromide) method developed by Murray and Thompson (1980) with suitable modifications. The leaf tissues were powdered in liquid nitrogen in autoclaved pre-cooled pestles and mortars. The powder was then transferred to a separate 2 ml centrifuge tubes containing 800 µl of extraction buffer (2% CTAB, 100 mM Tris, 20 mM EDTA, 1.4 mM NaCl and 1% PVP, pH 8.0) maintained at 60oC in water bath and mixed gently. The mixture was incubated at 60oC for 1 hr with intermittent mixing. An equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) was added to the tubes followed by gentle mixing. The mixture was centrifuged at 10,000 rpm for 10 minutes at 4oC. The aqueous phase was transferred to fresh tube, followed by addition of 500 µl of pre-chilled isopropanol. The contents of the tubes were mixed gently and the mixture was incubated at -20oC for 1 hr. DNA was precipitated by centrifugation at 10,000 rpm for 10 minutes using centrifuge. The supernatant was drained and the resulting pellet was washed twice with 1 ml of 70% chilled ethanol. The pellet was dried in a stream of sterile air in a laminar air flow cabinet. Dried DNA pellet was dissolved in 1 ml TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). The dissolved DNA was treated with 1 µl of RNase (10 mg/ ml). The quality and concentration of DNA was estimated through electrophoresis using 0.8 per cent agarose gel with known concentration of uncut lambda (λ) DNA.

PCR amplification and Gel Electrophoresis

A total of 15 RAPD primers, each of 10 nucleotides (decamers) primers obtained from M/s Operon Technologies, Alameda, CA,

USA were used for the present study based on their polymorphism, easy to score and distinct fragments (Table-1). For amplification of genomic DNA, a reaction mixture of 12.5 µl volume was prepared using 7.15 µl of sterilized distilled water, 1.0 µl template DNA (25 ng/ µl), 1.0 µl primer, 1.0 µl MgCl2 (25 mM), 1.25 µl 10x PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.0 µl dNTP mix (0.2 mM each of dATP, dGTP, dCTP and dTTP) and 0.1 µl Taq polymerase (3U/ µl). The amplification was carried out in an Eppendorf Master cycler with an initial denaturation at 94oC for 5 minutes, followed by 39 cycles of amplification: 1-minute denaturation at 94oC; 1-minute annealing at 37oC; 2-minutes extension at 72oC. Final extension step was programmed at 72oC for 5 minutes. The PCR products obtained were stored at 4oC until the gel electrophoresis was done. PCR products mixed with 2µl of gel loading dye (0.25% bromophenol blue and 40% sucrose), were electrophoretically separated on a 1.8 % agarose gel containing ethidium bromide (0.5 µg/ ml) at 100 V for 90 minutes in 1x Tris acetate-EDTA (TAE) buffer (40 mM Tris, 40 mM Acetic acid Glacial, 1 mM EDTA, pH 8.0). The amplified products were visualized and photographed under UV light source using the Gel-Documentation Unit (Biovis). The size of amplicons was determined by comparing with DNA ladder (100 bp) with known molecular weight fragments.

Band Scoring and RAPD Analysis

To analyze the amplified DNA profile of sixty-four rice bean genotypes, the presence or absence of each RAPD band of a particular molecular weight was scored manually. A binary data matrix with '1' indicating the presence of particular molecular weight and '0' indicating its absence, was generated separately for each primer. The binary data were used to generate a similarity matrix

using Jaccard's similarity coefficient (Jaccard 1908) values, $J_{ij} = C_{ij}/ (n_i + n_j - c_{ij})$, where 'Cij' is the number of positive matches between two genotypes, while ni and nj is the total number of band in genotype i and j respectively in SIMQUAL programme of NTSYS–PC package (version 2.02) (Rohlf 1993). Genetic distances (GD) were calculated as $GD = 1 − [C_{ij}/ (n_i+n_j- C_{ij})]$. The data were subsequently used to construct a dendrogram using the Unweighted Pair Group Method with Arithmetical Averages (UPGMA) employing sequential agglomerative hierarchic and non-overlapping (SAHN) clustering program of NTSYS − PC. Different analysis were performed on mean data for each trait using different statistical software viz., principal component analysis, biplot drawing (XLSTAT).

Results and Discussion

The present study was undertaken to assess the genetic diversity among the rice bean germplasm lines collected from Himachal Pradesh, Punjab and Uttarakhand, understanding the patterns of variation and relatedness among them, and to explore their utilization in the breeding programme for rice bean improvement. Molecular markers have proven to be powerful tools in the assessment of genetic variation and also in the elucidation of genetic relationships within and among the species.

RAPD has been standardized and employed successfully by different workers (Tomooka et. al. 1995; Kaga et.al. 1996; Xu et. al. 2000; Choudhury et. al.; Bora et. al.) to analyze samples of various crops including Vigna species. RAPD profile of 64 rice bean genotypes generated 83 bands, of which 47 were polymorphic (56.62% of polymorphism). Each primer gave 3-9 amplification products with an average of 5.53 bands. The RAPD primer OPQ-6 and OPA-14 generated maximum and minimum number of bands which was found to be 9 and 3 respectively. RAPD primer OPA-19, showed maximum polymorphism (75.00%) followed by OPI-04 (66.67%) and OPQ-6 (66.67%). The polymorphic band pattern given by primer OPA-19 and OPB-10 is presented in Fig-1 and 2, respectively.

The Jacard's similarity coefficient matrices showed variation from 0.48 to 1.00 (Fig-3). The average similarity across 64 genotypes was found to be substaintially high (0.74). Among the genotypes tested in the study, the rice bean accession PRR-2 was most divergent from rest of the genotypes, which may be due to its different geographical distribution from other genotypes which are mostly from hilly areas. Further, RBL-1 and RBL-6 showed very low dissimilarity as revealed from their inter se similarity coefficient values, they are from the same locations and probably originated from related parents.

Cluster analysis

Based on the polymorphism exhibited by RAPD markers, 64 rice bean genotypes were grouped into 2 main clusters and cluster A is further divided in several sub clusters. The genotypes included in different clusters are shown in Table-2 and Fig-3. The genetic similarity between the second clusters was merely 48 per cent.

Cluster A consisted of 63 genotypes whereas cluster B had only one genotype i.e. PRR-2 due to its highly divergent character. Within 6 sub-clusters of A, maximum genotypes were found in sub-cluster AII (41) followed by sub-cluster AI (7), sub cluster AIV and AVI (5 each), sub-cluster AIII and AV (3, 2) respectively.

Table.1 Number of scorable and polymorphic RAPD bands obtained in the PCR amplified
DNA of rice bean genotypes generated by 15 primers

S.No.	Primer	Sequences (5'-3')	Nf	Np	Nm	Polymorphic bands (%)
1	OPI-04	5'-CCG CCT AGT C-3'	6	4	2	66.67
2	OPI-14	5'-TCT GTG CTG G-3'	4	2	2	50.00
3	OPA-05	5'-AGG GGT CTT G-3'	5	2	3	40.00
4	OPA-14	5'-TCT GTG CTG G-3'	3	1	2	33.33
5	OPA-19	5'-CAA ACG TCG G-3'	4	3	1	75.00
6	OPB-10	5'-CTG CTG GGA C-3'	8	5	3	62.50
7	OPO-08	5'-CCT CCA GTG T-3'	4	2	2	50.00
8	OPQ-06	5'-GAG CGC CTT G-3'	9	6	3	66.67
9	OPZ-20	5'-ACT TTG GCG G-3'	4	4	0	50.00
10	OPBA-15	5'-GAA GAC CTG G-3'	5	3	2	60.00
11	OPBB-06	5'-CTG AAG CTG G-3'	8	5	3	62.50
12	S-226	5'-ACG CCC AGG T-3'	8	3	5	37.50
13	S-228	5'-GGA CGG CGT T-3'	6	3	3	50.00
14	OPBD-09	5'-CCA CGG TCA G-3'	5	3	2	60.00
15	OPBD-10	5'-GAC GCT ATG G-3'	4	1	3	25.00
	Mean		5.53	3.1	2.4	56.05
	Total		83	47	36	56.62

N_f- number of fragments; N_p- number of polymorphic fragments; N_m- number of monomorphic fragment

Table.2 Distribution of rice bean genotypes among different clusters on the
Basis of RAPD data

Cluster	Sub cluster	Number of Genotypes in each cluster	Genotypes
A	A_I	7	RBHP-2, RBHP-11, RBHP-12, RBHP-13, RBHP-14, RBHP-16, RBHP-35
	A_{II}	41	PRR-1, RBL-1, RBL-6, RBHP-19, RBHP-20, RBHP-22, RBHP-26, RBHP-31, RBHP-32, RBHP-37, RBHP-39, RBHP-42, RBHP-43, RBHP-44A, RBHP-44C, RBHP-46, RBHP-47, RBHP-49, RBHP-53, RBHP-56, RBHP-62B, RBHP-65, RBHP-66, RBHP-70, RBHP-73, RBHP-74, RBHP-75, RBHP-76, RBHP-77, RBHP-81, RBHP-83, RBHP-84, RBHP-88, RBHP-89, RBHP-93, RBHP-94, RBHP-108, RBHP-100, RBHP-111, RBHP-112, RBHP-113
	A_{III}	3	BRS-2, RBHP-17, RBHP-45
	A_{IV}	5	RBHP-3, RBHP-7, RBHP-62A, RBHP-71, RBHP-72
	A_V	2	RBHP-23, RBH P-61
	A_{VI}	5	RBHP-9, RBHP-80, RBHP-86, RBHP-90, RBHP-110
B		1	PRR-2

Table.3 Estimation of Principal component, Eigen value, proportional and
Cumulative percent variation

Components	Eigen value	Variability (%)	Cumulative %
P1	5.42	45.13	45.13
P2	1.98	16.52	61.65
P3	1.55	12.89	74.54
P4	1.12	9.30	83.84
P5	0.67	5.61	89.46
P6	0.55	4.62	94.07
P7	0.31	2.58	96.65
P8	0.17	1.45	98.10
P9	0.15	1.21	99.31
P10	0.08	0.65	99.96
P11	0.00	0.03	99.99
P12	0.00	0.01	100.00

Table.4 Estimation of percent contribution of traits for principal component

	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Days to 50% flowering	2.054	39.573	5.434	0.435	1.944	0.261	0.056
Days to 75% Maturity	2.079	39.527	5.437	0.447	1.873	0.202	0.106
Plant height (cm)	9.445	0.453	1.587	11.746	28.618	1.248	34.690
Branches/plant	12.022	0.158	7.848	3.424	0.004	0.002	43.565
Pods/ plant	10.621	1.461	19.508	0.073	0.674	0.669	0.183
Pod length (cm)	9.555	6.061	11.025	0.353	3.298	12.909	2.064
Seeds /pod	9.497	2.892	12.842	2.160	1.142	24.199	0.114
Seed yield /plant (g)	16.645	0.434	0.958	1.905	3.744	0.923	1.090
100-Seed weight (g)	2.079	7.479	25.946	1.979	0.187	53.288	4.556
Crude protein (%)	0.908	1.526	4.705	53.565	32.797	3.894	2.226
Biological yield/ plant	16.236	0.414	3.962	0.631	0.116	0.159	0.002
Harvest Index (%)	8.859	0.021	0.747	23.282	25.601	2.246	11.349

Fig.1 RAPD profile in 64 rice bean genotypes using primer OPA-19, M = 100 bp DNA ladder
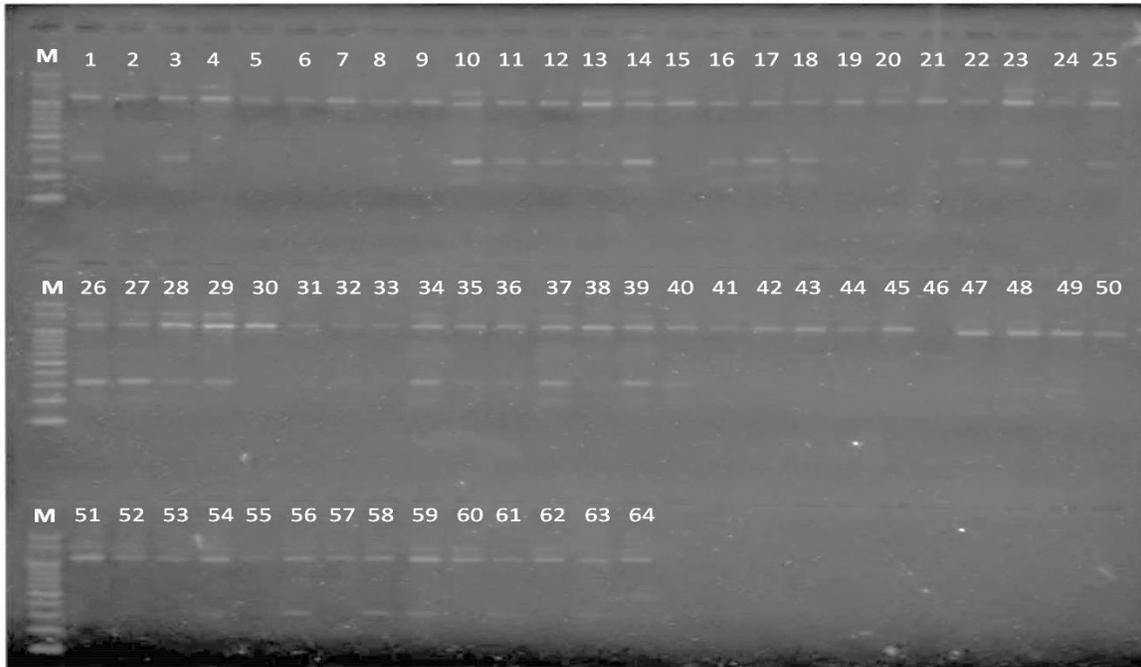

Fig.2 RAPD profile of 64 rice bean genotypes using primer OPB-10, M = 100 bp DNA ladder
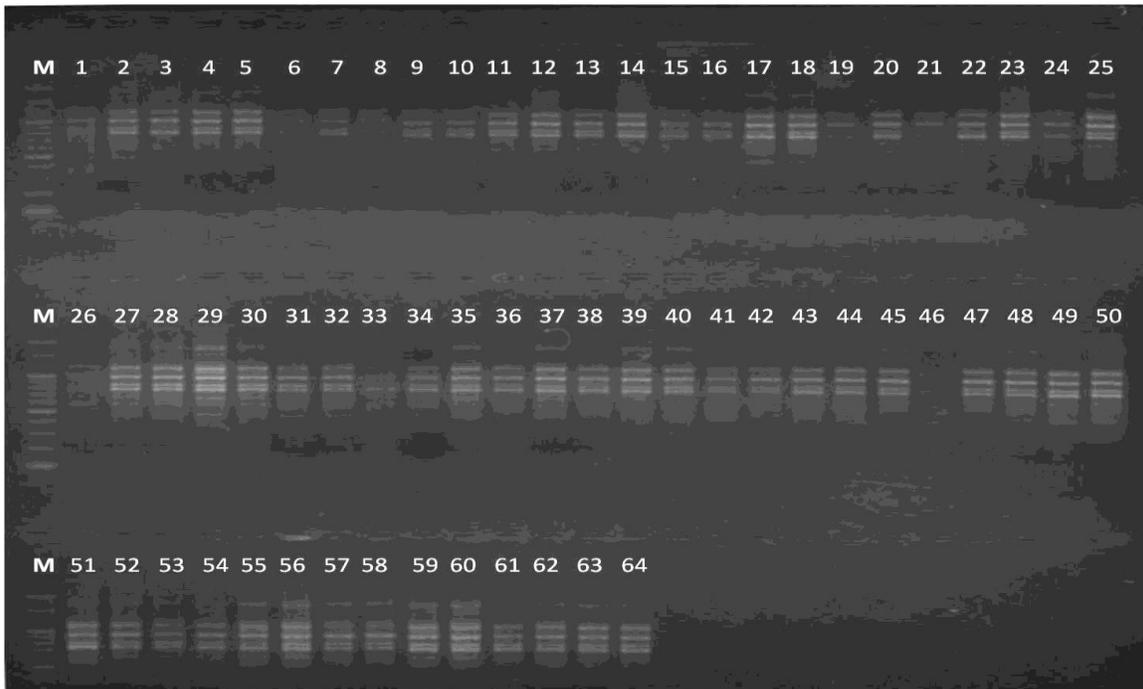
Fig.3 Dendrogram depicting genetic relationships among the rice bean genotypes
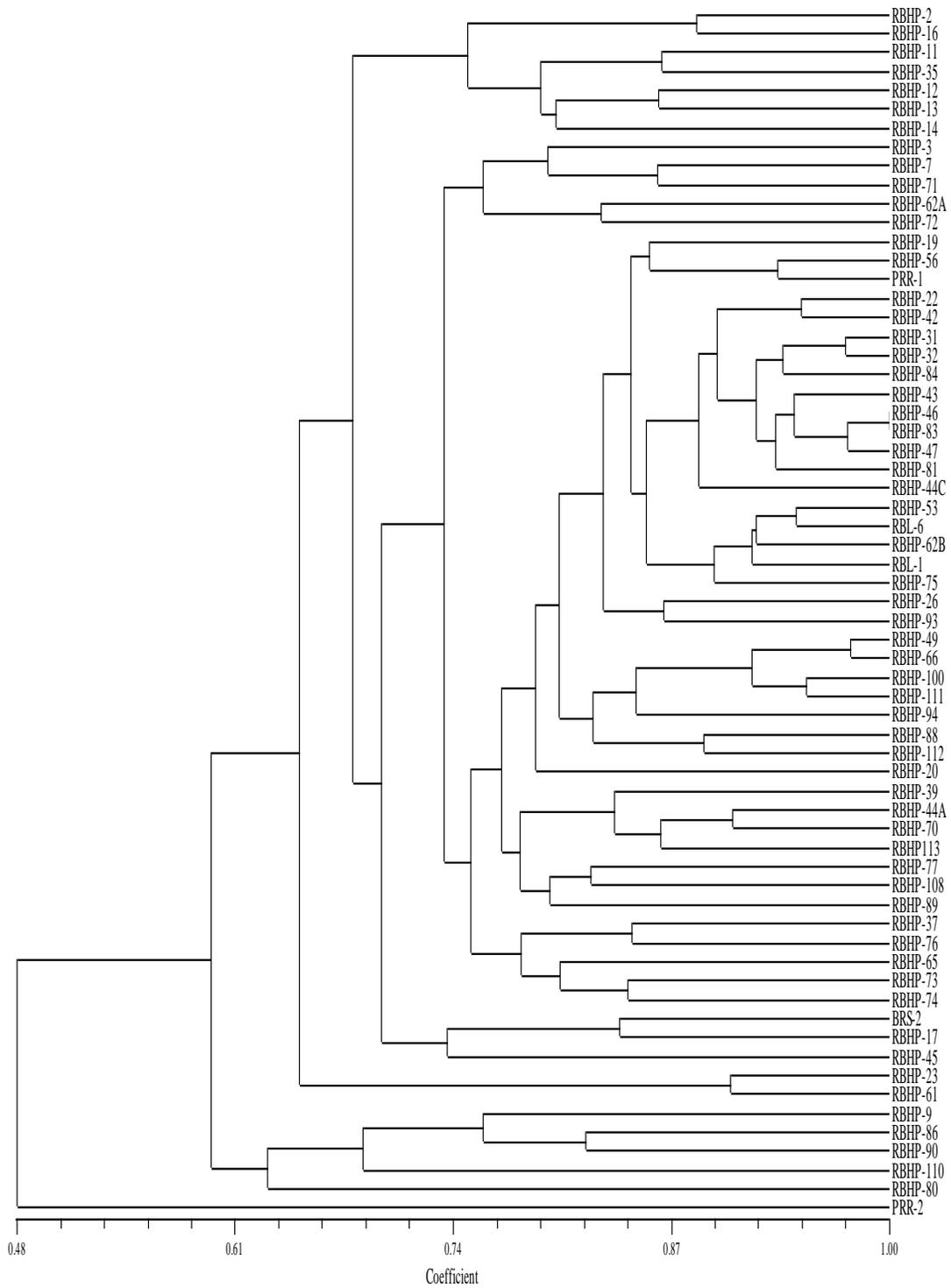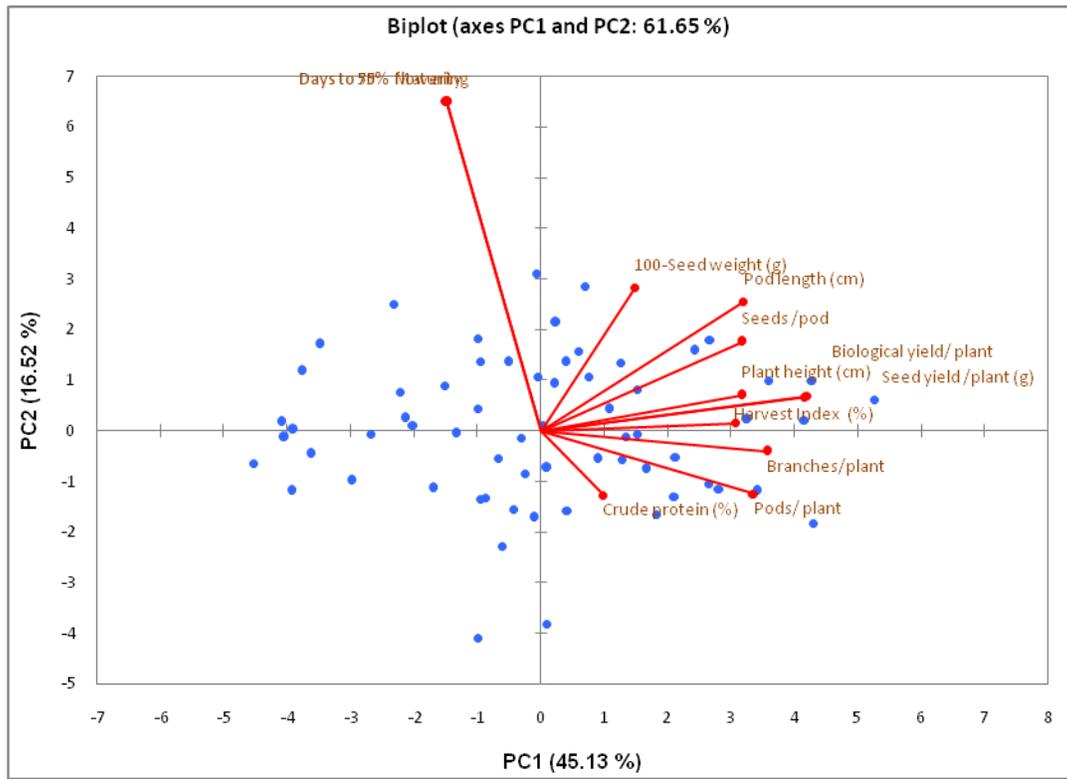Constructed using UPGMA method

Fig.4 Biplot of 64 rice bean genotypes based on PC1 and PC2

Two genotypes (RBHP-46, RBHP-83) were found to be genetically similar and probably duplicates, since they were collected from nearby locations. Yoon *et al.,* (2007) studied genetic variation and relationships among members of the adzuki bean complex (*Vigna angularis*) including rice bean (*V. umbellata*) but was unable to find much polymorphism in rice bean accessions.

In a similar study, Bajracharya *et al.,* (2010) studied genetic diversity among 112 rice bean genotypes using selective simple sequence repeats (SSR) markers developed from adzuki bean (*V. angularis*) and observed that 35 SSR primer pairs (out of 109) were polymorphic and were used further to characterize genotypes. Muthusamy *et al.,* (2008) also evaluated genetic variation between 10 landraces of rice bean using RAPD and ISSR markers, thus indicated that both the marker systems were equally effective in determining polymorphisms.

Analysis of variance for quantitative traits showed the significant differences among the genotypes for all the quantitative traits. This indicates that sufficient amount of variability was present among the genotypes for all quantitative traits. The 12 principal components were obtained through principal component analysis, out of which 9 explained 99% of the total variability (Table.3). The First four principal components were accounted for more than 80% of the total variability and first two were accounted for 61.65% of the total variability. In principal component analysis, the variance-covariance matrix was used to transform all the quantitative attributes into a single index of similarity in the form of principal component. Most of the variation was distributed up to the

seventh principal component, being responsible for 94.07% of the relative variation observed. Examining the Eigen vectors of individual components, level of association with the original traits are indicated. The characters with highest weight in first component were biological yield per plant, seed yield per plant followed by branches per plant, pods per plant, pod length, plant height, and seeds per pod and harvest index (Table-4). Similarly, characters with highest weight in second component were days to 50% flowering and days to 50% maturity followed by 100 seed weight. Further, third component, the highest contributor was 100-seed weight and in fourth it was crude protein. Yield and some yield contributing traits appear strongly in the first three components (Katiyar and Dixit 2009). The relationship between different yield contributing traits and genotypes behavior is plotted in the biplot graph (Fig-4). Since, the biplot provides a useful tool for data analysis (Gabriel 1971; Gower and Hand 1996). If the angle and directions between vectors or lines which indicated yield contributing traits are less than 90 ^{o}C, it represents a positive correlation and if the angle between the lines is more than 90 ^{o}C, it indicates negative correlation. According to the biplot, there was a positive correlation between most of the traits that appeared in graph close to each other except for crude protein with days to fifty percent flowering and days to fifty percent maturity.

It may concluded even though rice bean genotypes were obtained from nearby geographical locations (except for few), 56.6% polymorphic bands obtained in RAPD indicated it as a potential marker for identification of molecular diversity among rice bean genotypes. Most of the sub-clusters formed in the dendrogram were of genetically more similar genotypes as indicated from their origin. Rice bean being an underutilized but highly potential crop, very few studies have been conducted to assess their genetic relationship and therefore, there is huge scope to obtain their genetic variation through molecular markers like RAPD or preferably by SSRs for further exploration in the crop improvement programme.

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