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Genetic diversity analysis in different genotypes of black gram [*Vigna mungo* (L.) Hepper] using RAPD marker

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ABSTRACT

Amongst the various DNA fingerprinting methodologies, randomly amplified polymorphic DNA (RAPD) was used to estimate genetic diversity and relationship amongst 22 black gram genotypes. A total of 25 randomly selected decamers were screened, out of which only 16 got amplified. A total of 133 amplified bands were obtained, out of which 120 were polymorphic. The average percentage of polymorphism was 90.23. The total number of amplified bands varied between 3 (primer OPK-03) and 15 (primer OPC-08) with an average of 9 bands per primer. The overall size of PCR amplified products ranged between 200 bp to 2600 bp. The average PIC was 0.30 ranging from 0.17 to 0.43. Five unique bands (ranging from 200-1200 bp) were detected in four genotypes using 5 RAPD primers. Jaccard's similarity coefficient values for RAPD primers ranged from 0.58-0.85 with an average of 0.71. Based on dendrogram generated through UPGMA method and PCA, most of the genotypes got divided into three main clusters. Genotypes U-17 and STY-2289 were lying close and thus showed minimum genetic distance while genotypes UH-177 and IPU99-233 had minimum similarity value of 0.42, thus showing maximum divergence. Thus, these results could be used to assess other black gram accessions in the *Vigna* germplasm pool that can provide useful information towards molecular classification and the genetic marker assisted breeding for crop improvement.

Key words: DNA amplification , Genetic diversity, Polymorphism, RAPD, Similarity coefficient.

INTRODUCTION

Pulses are the major source of dietary protein in the vegetarian diet of our country. Besides being the source of quality protein which is nearly three times as much as cereals, they maintain soil fertility through biological nitrogen fixation and thus play a vital role in furthering sustainable agriculture (Kannaiyan 1999). As a pulse crop in India, black gram stands fourth in of production after chickpea, pigeonpea and mungbean. Black gram, also referred to as urad, urdbean, mash, black lentil or white lentil, belongs to the family *Leguminosae* and sub-family *Fabaceae* and has a haploid chromosome number of 11. The seeds of black gram stores a moderately high amount of calories (calorific value of 350 cal/100g), carbohydrates (56.6%), proteins (26.2%) and fat (1.2%). It is also rich in essential minerals and vitamins required by the human body (Shafique *et al.*, 2011).

Black gram varieties can be differentiated into many types based on their morphological and cytological markers, but these markers may not be useful for analysis by breeders. Although iso-zyme and protein markers are useful for characterizing genetic diversity and identifying hybrids of cultivars (Das and Mukarjee 1995; De Vries 1996), the paucity of isozyme and protein methodologies restrict their usefulness in breeding analysis. Molecular markers have been applied widely in genetic analyses and breeding studies, as well as investigations of genetic diversity and relationships between cultivated species (Karuppanapandian *et al*., 2010). The Randomly Amplified Polymorphic DNA (RAPD) technique (Williams *et al*., 1990; Hadrys *et al*., 1992) amongst the PCR based methods has been most popular because of speed, low cost and the use of minute amount of plant material for analysis. The RAPD technique has been applied to assess molecular polymorphism in *Vigna* (Kaga *et al.*, 1996), chickpea (Sonnante and Beckman 1997), *V. ungularis* (Yee *et al.*, 1999), mung bean (Santalla *et al.*, 1998; Lakshanpaul *et al.*, 2000) and black gram (Souframanien *et al.*, 2002). RAPD has been used extensively for classification of varieties, identification of cultivars and diversity estimation in various crops such as green gram (Karuppanapandian *et al.*, 2006a), cow pea (Karuppanapandian *et al.*, 2006b), and black gram (Karuppanapandian *et al.*, 2007). The present study was aimed to evaluate and compare the genetic diversity in the elite genotypes of black gram using RAPD markers.

MATERIALS AND METHODS

Plant Materials: Seeds of twenty two genotypes of black gram were procured from Rajasthan Agriculture Research Institute (RARI), Durgapura, Jobner Agriculture University, Jaipur. Source details of the materials used are given in

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Table 1. Crop was grown during *Kharif*, 2013 at Plant Breeding Research Farm, Rajasthan College Agriculture, MPUAT, Udaipur. Soil is of sandy-loam in nature and average temperature condition was between 24-33^{0}C.

Plant DNA Extraction: DNA was isolated from fresh leaves (45 days old seedlings) using modified CTAB method (Doyle and Doyle, 1987). Three grams of leaf tissues were transferred into prechilled mortar, liquid nitrogen was used to ground. The fine powder was allowed to thaw in the presence of 10 ml of pre-heated extraction buffer [CTAB 2% (w/v), Tris HCl (pH 8.0) 100 mM, Sodium chloride 1.4 M, EDTA (disodium, pH 8.0) 20 mM, β-mercaptoethanol 2%] and incubated for 45-60 min. at 65^{0}C with occasional mixing. 3 ml of chloroform: isoamyl alcohol mixture (24:1 v/v) was added and mixed by inversion for 1 hr. This was followed by centrifugation at 10,000 rpm for 15 min. at 4^{0}C. The clear aqueous phase was transferred to a new sterile tube. An equal volume of ice cold isopropanol was added and mixed gently by inversion and then kept in the freezer until DNA got precipitated out. Using blunt end tips, the precipitated DNA was spooled out into an eppendorf tube. The spooled DNA was air dried after removing the supernatant by a brief spin. 200 µl of TE was added to dissolve the DNA which was followed standard RNase treatment procedure. Finally DNA pellet was dissolved in 150-250 µl of TE (depending on the pellet size). Quantification of DNA was done by using spectrophotometer (Nanospectrophotometer, Implen Germany). The quality of genomic DNA was checked by using 0.8 per cent agarose gel in presence of EtBr. The DNA samples were stored at 4^{0}C until further analysis.

RAPD Analysis (Srivastava *et al.*, 2013) : PCR amplification was carried out in programmable thermal cycler from Eppendorf AG, Germany. Total of 25 RAPD primers were used for PCR amplification. Based on the published RAPD primer sequences (Operon Inc.), the primers were synthesized at Sigma and used in the present study. For RAPD analysis, PCR reaction was performed in 20 µl reaction mixture which is detailed in Supplementary Table 1. Amplification program consisted of a pre-denaturation step at 94^{0}C for 4 min followed by 44 amplification cycles, consisting of denaturation at 94^{0}C for 1 min, annealing at 37^{0}C for 1 min and extension at 72^{0}C for 2 min and a final post extension step for 10 min at 72^{0}C.

Agarose Gel Electrophoresis (AGE) (Sambrook *et al.*, 1989): Following the PCR amplification, the PCR products of RAPD were loaded on 1.2 per cent agarose gel. Agarose gel was prepared in 1X TAE buffer containing ethidium bromide (10 mg/ml). Electrophoresis (Bangalore Genei Pvt. Ltd., Bangalore, India) was carried out at a constant voltage (3V/cm of gel) till bromophenol blue/loading dye migrated to other end of the gel. The gel was visualized on a UV-transilluminator and photographed using gel documentation system (Alpha innotech corporation).

Table 1: Source of the twenty two black gram genotypes

Genotype	Source
U-9	IIPR, Kanpur(U.P)
UTTARA	IIPR, Kanpur
IPU2K-21	IIPR, Kanpur
UH-86-5	HAU, Hisar
PLU-144	IARI, Delhi
RUG-8	RAU, Durgapura
SPS-29	IIPR, Kanpur
UL-23	Uttar Pradesh
NHKD-31	IIPR, Breeding line
PANT-U30	GBPAU&T, Pant nagar
IC-16511	NBPGR, New Delhi
UH-177	HAU, Hisar
PLU-1	IARI, Delhi
IPU99-233	IIPR, Kanpur
SHEKHAR-2	CSAUAT, Kanpur
PLU-446	IIPR, Kanpur
BG-369	Andhra Pradesh
U-17	IIPR, Kanpur
HPU-180	Himachal Pradesh
STY-2289	IIPR, Breeding line
IPU99-176	IIPR, Kanpur
STY-2834	IIPR, Breeding line

Supplementary Data

Table 1: PCR reaction mixture content

Components	Final Concentration	Single tube (20 µl)
DNA template	100 ng	1.00 µl
Master mixture		
1. dNTP mix	200 µM	1.60 µl
2. *Taq* DNA polymerase	1U	0.5 µl
3. Reaction buffer (10X)	1X	2.00 µl
4. Primer	0.5 µM	2.00 µl
5. dd H_2O		12.9 µl

RAPD amplification profile were scored manually, based on the presence (1) or absence (0) of bands for each black gram genotype. Only clear and unambiguous bands were scored. The band size was compared with 100 bp and 1 kb ladder (MERCK, Bioscience).

Data Analysis (Jaccard, 1908) : The scores (0 or 1) for each band obtained from photograph were entered in the form of a rectangular data matrix (qualitative data matrix). The pair-wise association coefficients were calculated from qualitative data matrix using Jaccard's similarity coefficient. Cluster analysis for the genetic distance was then carried out using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering method (Sneath and Sokal, 1973). The genetic distances obtained from cluster analysis through UPGMA were used to construct the dendrogram,

Table 2: DNA amplification profile and polymorphism generated in black gram using 16 RAPD primers

Primer Code*	Sequence 5' to 3'	Molecular weight range (bp)	Total no. of bands amplified (x)	Polymorphic bands		PIC
				Number	Frequency (%)	
OPA-1	CAGGCCCTTC	2000-300	10	8	80	0.315
OPA-02	TGCCGAGCTG	2200-400	7	7	100	0.341
OPC-08	TGGACCGGTG	2100-200	15	15	100	0.326
OPD-12	CACCGTATCC	2100-200	14	14	100	0.358
OPE-03	CCAGATGCAC	1600-350	8	6	75	0.207
OPF-17	AACCCGGGAA	1200-350	4	4	100	0.34
OPK-05	TCTGTCGAGG	2400-600	9	7	77.77	0.281
OPK-06	CACCTTTCCC	2200-650	6	6	100	0.315
OPK-07	AGCGAGCAAG	2000-800	3	3	100	0.43
OPP-03	CTGATACGCC	2600-400	6	6	100	0.275
OPP-04	GTGTCTCAGG	2000-300	7	4	57.14	0.177
OPP-05	CCCCGGTAAC	2100-250	11	11	100	0.32
OPP-06	GTGGGCTGAC	1600-200	8	6	75	0.311
OPP-07	GTCCATGCCA	2000-350	5	5	100	0.284
OPP-08	ACATCGCCCA	2000-500	8	8	100	0.332

depicting relationships of the genotypes using software NTSYSpc version 2.02 (Rohlf, 2004). A two dimensional and three dimensional principal component analysis (PCA) was constructed to provide another means of testing the relationship among the cultivars using the EIGEN programme (NYSTS-pc).

Polymorphism Information Content (PIC) (Smith *et al.*, 1997) : To measure the polymorphism information of RAPD marker system. The PIC was calculated according to following formula:

$$PIC = \sum_{i=1}^{n} 1 - pi^2$$

Where, N= total number of allele detected for a locus of a marker
Pi= frequency of the 1st allele

RESULTS AND DISCUSSION

The objective of the present study was to assess the extent of genetic diversity and relationships among the 22 black gram genotypes which were collected from ARS, Durgapura, Jaipur. The amount of DNA isolated from various genotypes of black gram ranged from 529 ng/µl (U-9) to 5904 ng/µl (UH-177). The ratio of absorbance (A260/A280) ranged from 1.70 to 2.0 revealed that the DNA obtained was free from contaminants like polysaccharides, protein and RNA.

Polymorphism in black gram using RAPD Primers (Williams *et al.*, 1990): Twenty five RAPD primers having 60 per cent or more GC content were used for the present investigation. Out of 25 primers, only 16 were amplified except OPD-05, OPF-19, OPJ-04, OPK-04, OPP-01, OPP-02, OPP-10, OPP-12 and OPP-16. A total of 133 amplified bands were obtained out of which 120 were polymorphic and showed 90.23% polymorphism. Similar results have been reported by Samarajeewa *et al.,* 2002, who used 5 random primers that produced 134 bands out of which 104 were polymorphic.

The DNA amplicon size and polymorphism generated among various genotypes of black gram using RAPD primers are presented in Table 2. The total number of bands observed for every primer was recorded separately and polymorphic bands checked subsequently. The total number of amplified bands varied between 3 (primer OPK-03) and 15 (primer OPC-08) with an average of 9 bands per primer. The overall size of PCR amplified products ranged between 200 bp to 2600 bp. The per cent polymorphism ranged from as low as 57.14 (OPP-04) to as high as 100 (OPA-02, OPC-08, OPD-12, OPF-17, OPK-06, OPK-07, OPP-03, OPP-05, OPP-07 and OPP-08). The average PIC was 0.304 ranging from 0.17 to 0.43. The lowest and the highest PIC value were recorded for primer OPP-04 and OPK-07 respectively. Similar results have been reported by Talebi *et al.*, (2008).

The RAPD technique has been applied to assess molecular polymorphism in *Vigna* (Kaga *et al.*, 1996), chickpea (Sonnante and Beckman 1997), *V. ungularis* (Yee *et al.*, 1999), mung bean (Santalla *et al.*, 1998; Lakshanpaul *et al.*, 2000) and black gram (Souframanien *et al.*, 2002).

DNA banding profile of individual plant DNA samples from 22 genotypes of black gram after amplification with RAPD primers were depicted in Figure-1.

Five unique bands (band which is present in a particular genotype but absent in rest of the genotypes) were detected in four genotypes *viz.*, STY-2289, IPU99-233, NHKD-31 and PLU-144 with 5 RAPD primers (OPC-08, OPD-12, OPF-17, OPP-03 and OPP-07) were presented in Table 3. The genotype STY-2289 gave maximum number of distinct bands *i.e.*, 2. The size of these unique bands ranged from 200-1200 bp. However, Reddy *et al.*, (2008) reported that accessions with most distinct DNA profiles are likely to

Table 3: Distinct, genotype specific bands as detected by 5 RAPD primers as shown by 4 different genotypes of black gram

Primer code	Total no. of unique bands	Genotype	Size of bands (bp)
OPC-08	1	STY-2289	200
OPD-12	1	STY-2289	200
OPF-17	1	IPU99-233	1200
OPP-03	1	NHKD-31	400
OPP-07	1	PLU-144	350
Total	**5**		

contain the greatest number of novel alleles. It is these accessions that are likely to uncover the largest number of unique and potential agronomically useful alleles.

Genetic Relationship and Cluster Tree Analysis

(A) Similarity Matrix Values Based on RAPD Data: Based on RAPD similarity matrix data, the values of similarity coefficient ranged from 0.42 to 0.84 *i.e.,* 42-84 per cent or genetic diversity ranged from 16 to 58 per cent (Table 4). A similar result was observed by Suneja *et al.,* (2012). The average similarity across all the genotypes was found out to be 0.63 showing that the genotypes were moderately diverse. Maximum similarity value of 0.84 was observed between genotypes STY-2289 and U-17 followed by IPU99-233 and PLU-144 with a similarity coefficient value of 0.82. Similarly, minimum similarity value of 0.42 was observed between genotypes UH-177 and IPU2K-21 followed by PLU-1 and IPU2K-21 with a similarity coefficient of 0.43.

(B) RAPD Marker Based Cluster Tree Analysis: The RAPD data were used to obtain a similarity matrix (Table 4). The similarity coefficient was found to lie between 0.58-0.85. The RAPD cluster tree analysis of 22 black gram genotypes showed that they could be mainly divided into 3 major clusters at a similarity coefficient of 0.67 (Fig. 2). Cluster I included five genotypes *viz.,* U-9, RUG-8, NHKD-31, PLU-446 and BG-369. It could be divided into 2 sub-clusters. In sub-cluster I, RUG-8 and NHKD-31 were related to each other at 0.80 similarity coefficient. In sub-cluster II, genotypes PLU-446 and BG-369 were related to each other at 0.77 similarity coefficient value. Genotype IPU2K-21 was out-grouped from the first cluster having a similarity coefficient of 0.67. The cluster II included 4 genotypes *viz.,* SHEKHAR-2, HPU-180, IPU99-176 and STY-2834. It was divided into 2 sub-clusters. In sub-cluster I, SHEKHAR-2 and HPU-180 were related to each other at 0.82 similarity coefficient. In sub-cluster II, genotypes IPU99-176 and STY-2834 were related to each other at 0.77 similarity coefficient.

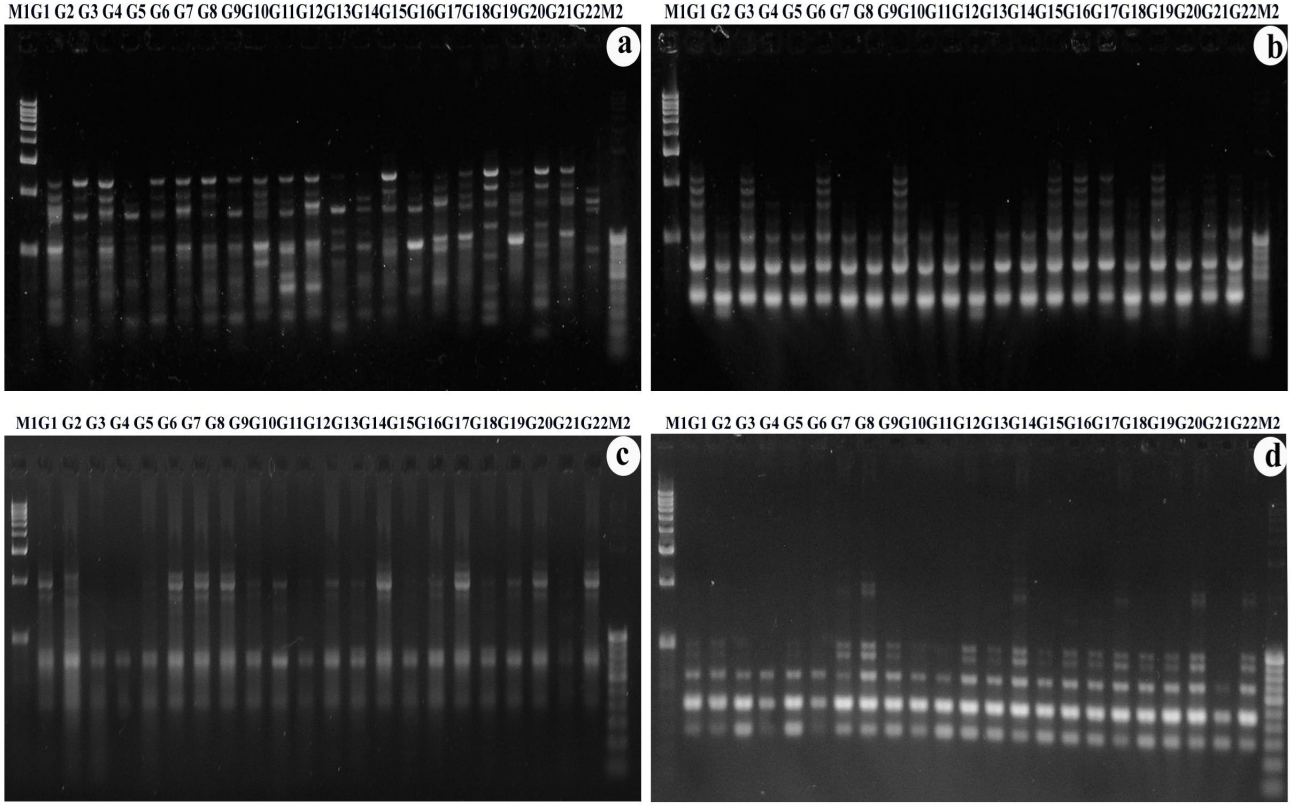

Fig 1: RAPD profiles generated through (a) OPC-08 (b) OPA-01 (c) OPA-02 (d) OPP-04.
G1-G22 represents G1–U-9; G2-UTTARA; G3-IPU2K-21; G4-UH-86-5; G5-PLU-144; G6-RUG-8; G7–SPS-29; G8-UL-23; G9-NHKD-31; G10–PANT-U30; G11–IC-1651; G12–UH-177; G13–PLU-1; G14-IPU99-2; G15–SHEKHAR-2; G16–PLU-446; G17–BG-369; G18–U-17; G19-HPU-180; G20-STY-2289; G21- IPU99-176 and G22-STY-2834; M1-100bp DNA ladder; M2-1000bp DNA ladder (MERCK, Biosciences, Bangalore India)

Table 4: Jaccard's similarity coefficient for RAPD profile generated by agarose gel electrophoresis

Genotypes	U-9	UTTARA	IPU2K-21	UH-86-5	PLU-144	RUG-8	SPS-29	UL-23	NHKD-31	PLU-710	IC-16511	UH-177	PLU-1	IPU99-233	SHEKHAR-2	PLU-446	BG-369	U-17	HPU-180	STY-2289	IPU99-176	STY-2834
U-9	1.00																					
UTTARA	0.46	1.00																				
IPU2K-21	0.72	0.48	1.00																			
UH-86-5	0.61	0.66	0.59	1.00																		
PLU-144	0.55	0.72	0.56	0.78	1.00																	
RUG-8	0.72	0.58	0.70	0.72	0.77	1.00																
SPS-29	0.61	0.64	0.55	0.76	0.81	0.70	1.00															
UL-23	0.56	0.67	0.54	0.72	0.70	0.57	0.75	1.00														
NHKD-31	0.77	0.51	0.59	0.66	0.63	0.80	0.73	0.57	1.00													
PLU-710	0.48	0.68	0.49	0.71	0.72	0.61	0.57	0.70	0.53	1.00												
IC-16511	0.55	0.71	0.56	0.72	0.77	0.68	0.72	0.72	0.65	0.81	1.00											
UH-177	0.54	0.68	0.42	0.65	0.72	0.61	0.76	0.75	0.61	0.62	0.65	1.00										
PLU-1	0.52	0.64	0.43	0.67	0.64	0.53	0.68	0.69	0.55	0.68	0.68	0.70	1.00									
IPU99-233	0.48	0.79	0.54	0.69	0.82	0.63	0.69	0.71	0.54	0.75	0.77	0.64	0.66	1.00								
SHEKHAR-2	0.72	0.55	0.59	0.69	0.64	0.77	0.65	0.53	0.78	0.59	0.60	0.54	0.56	0.57	1.00							
PLU-446	0.75	0.49	0.66	0.60	0.56	0.72	0.61	0.57	0.78	0.51	0.59	0.54	0.57	0.51	0.67	1.00						
BG-369	0.69	0.58	0.63	0.63	0.62	0.74	0.60	0.54	0.72	0.60	0.61	0.54	0.50	0.60	0.67	0.77	1.00					
U-17	0.48	0.78	0.51	0.68	0.78	0.63	0.70	0.69	0.56	0.60	0.69	0.67	0.58	0.75	0.53	0.45	0.55	1.00				
HPU-180	0.65	0.53	0.56	0.63	0.60	0.71	0.60	0.51	0.71	0.54	0.57	0.49	0.50	0.57	0.81	0.65	0.66	0.45	1.00			
STY-2289	0.47	0.79	0.52	0.67	0.78	0.63	0.71	0.73	0.54	0.63	0.72	0.70	0.66	0.77	0.50	0.49	0.51	0.84	0.46	1.00		
IPU99-176	0.65	0.56	0.53	0.69	0.70	0.69	0.71	0.60	0.72	0.64	0.73	0.54	0.60	0.63	0.75	0.61	0.56	0.61	0.67	0.58	1.00	
STY-2834	0.56	0.60	0.47	0.63	0.60	0.59	0.58	0.55	0.56	0.63	0.62	0.52	0.60	0.62	0.75	0.53	0.61	0.57	0.72	0.51	0.76	1.00

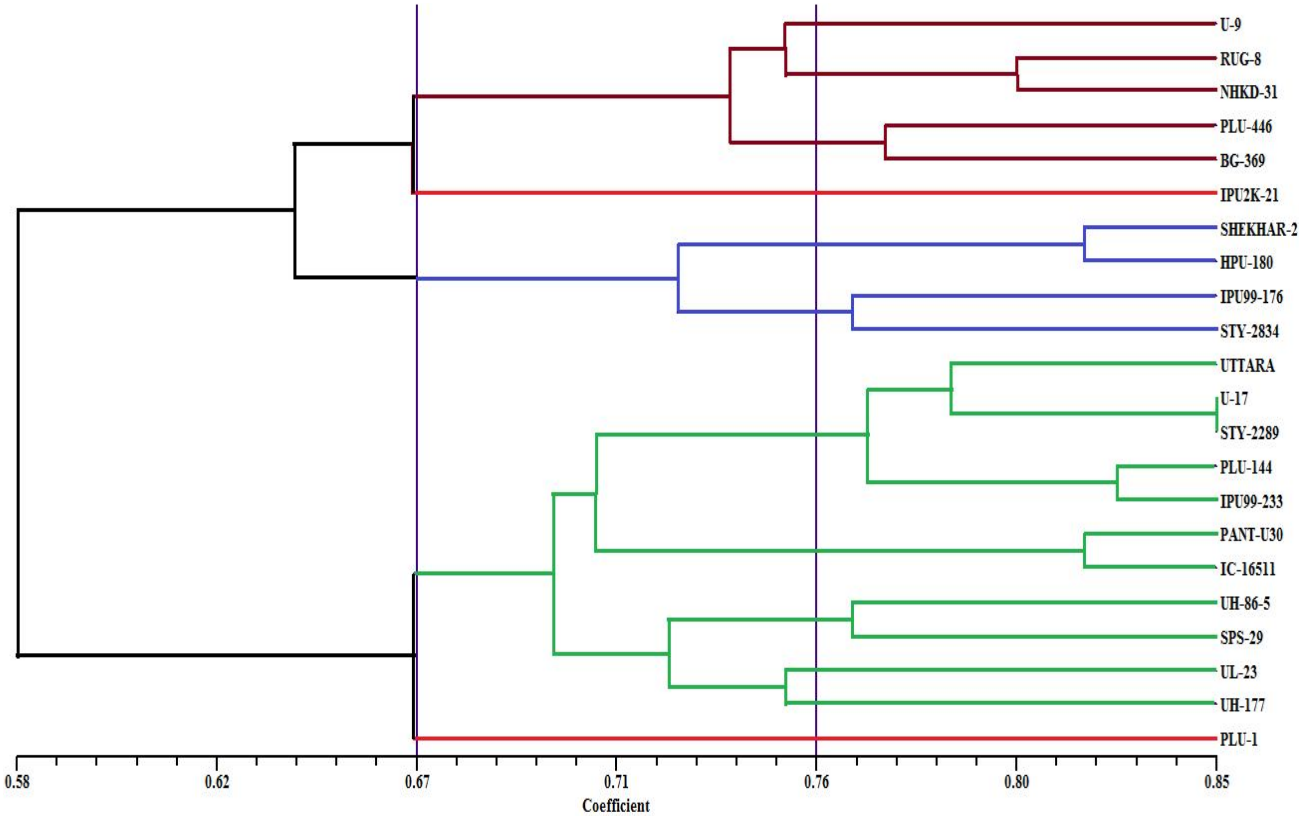

Fig 2: Dendrogram constructed with UPGMA clustering method among 22 genotypes of *V. mungo* L. using RAPD.

The cluster III included 11 genotypes *viz.*, UTTARA, U-17, STY-2289, PLU-144, IPU99-233, PANT-U30, IC-16511, UH-86-5, SPS-29, UL-23 and UH-177. It could be divided into 5 sub-clusters. In sub-cluster I, U-17 and STY-2289 were related to each other at 0.85 similarity coefficient. In sub-cluster II, genotypes PLU-144 and IPU99-233 were related to each other at 0.82 similarity coefficient. In sub-cluster III, genotypes PANT-U30 and IC-16511 were related to each other at 0.81 similarity coefficient. In sub-cluster IV, genotypes UH-86-5 and SPS-29 were related to each other at 0.76 similarity coefficient. In sub-cluster V, genotypes UL-23 and UH-177 were related to each other at 0.75 similarity coefficient. The genotype PLU-1 was out-grouped from the third cluster at a similarity coefficient of 0.67. Genotypes U-17 and STY-2289 were found closest among all genotypes and related at 0.85 similarity coefficient.

Similar results have been reported by Srivastava *et al.*, (2013) who analyzed genetic diversity among 8 black gram genotypes. The resulting dendrogram yielded two main clusters. The highest similarity index (0.76) was observed between PLU-839 and PLU-456 genotypes while the lowest similarity index (0.43) was observed between the genotypes LBG-20 and PLU-839. In a similar study, Abd El-Hady *et al.*, (2010) studied genetic diversity among 7 *Vigna* species. Similarity coefficient matrices were used to generate a dendrogram of *Vigna* species based on UPGMA analysis, which grouped the seven genotypes into three main clusters. The highest similarity value was 0.91 which recorded between *V. unguiculata* L. Ger. and *V. unguiculata* L. Egy while the lowest similarity value was 0.120 between *V. sinesis* L. Egy. and *V. radiate* L., respectively.

(C) Principal Component Analysis Based on RAPD Data: Two and three dimension principal component analysis based on RAPD data (Fig. 3) showed similar clustering of 22 genotypes as evident from cluster tree analysis. Dice similarity coefficients ranged from 0.71 to 0.88. Most of the genotypes tended to cluster mainly into three clusters. Cluster I and II included 5 (U-9, RUG-8, NHKD-31, PLU-446 and BG-369) and 4 genotypes (SHEKHAR-2, HPU-180, IPU99-176 and STY-2834) that lay closer. Cluster III was the major that included 11 genotypes *viz.*, UTTARA, U-17, STY-2289, PLU-144, IPU99-233, PANT-U30, IC-16511, UH-86-5, SPS-29, UL-23 and UH-177. While the genotypes IPU2K-21 and PLU-1 were lying apart showing maximum genetic distance. Similar results have been observed by Saini *et al.*, (2010).

The markers generated by RAPD assays can provide practical information for the management of genetic resources and these results could also provide useful information for the molecular classification and breeding of new black gram varieties.

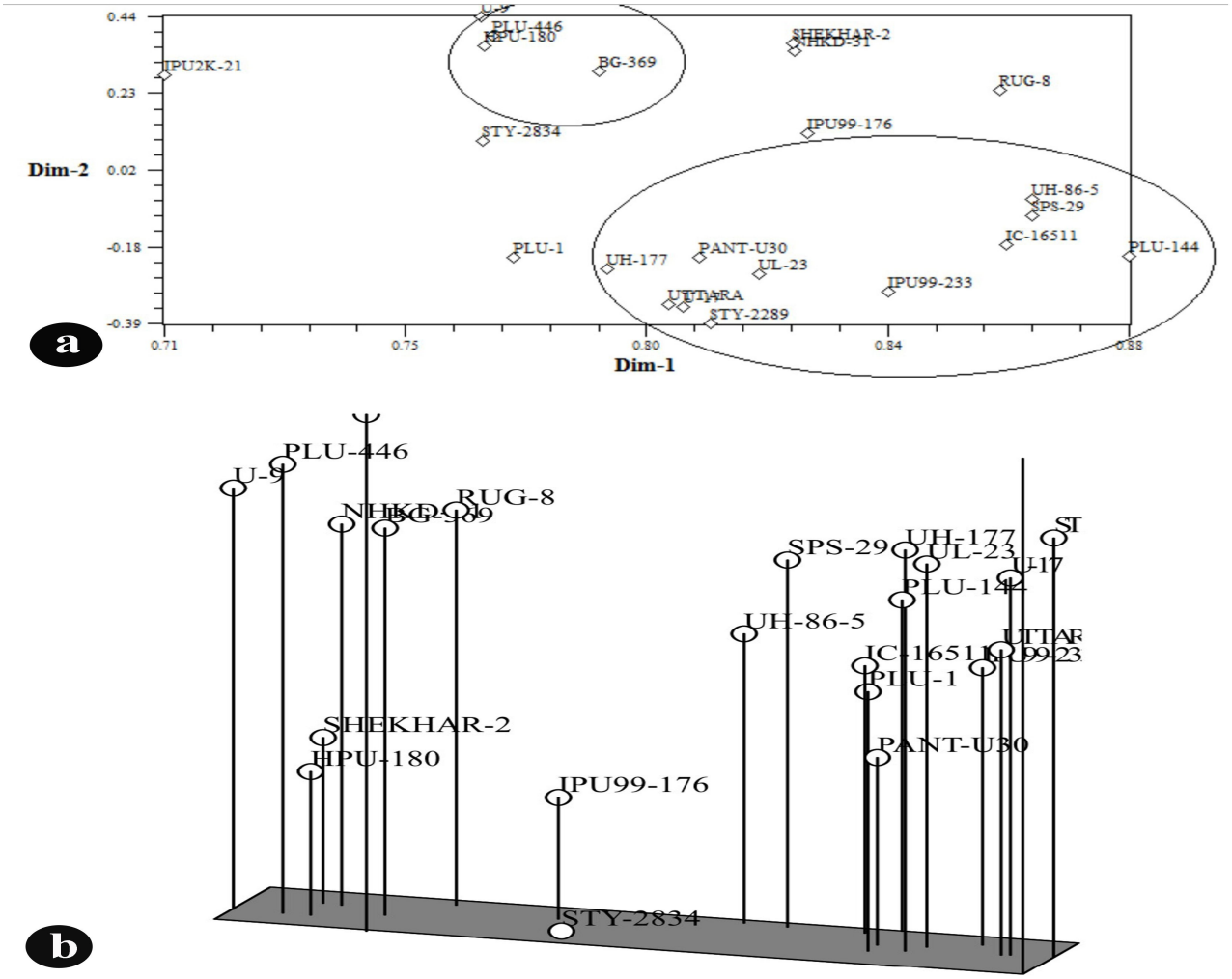

Fig 3: (a) Two Dimensional Principal Component Analysis (b) Three Dimensional Principal Component Analysis based on Euclidean Cluster Analysis Using RAPD Markers.

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