Legume Research, 41(2) 2018 : 175-181
Print ISSN:0250-5371 / Online ISSN:0976-0571

AGRICULTURAL RESEARCH COMMUNICATION CENTRE
www.arccjournals.com/www.legumeresearch.in

Assessment of genetic diversity in black gram [V*igna mungo* (L.) Hepper] genotypes based on ISSR

Divya Vyas*, Arunabh Joshi, Ganesh Rajamani and Devendra Jain

Department of Molecular Biology and Biotechnology, Rajasthan College of Agriculture,
Maharana Pratap University of Agriculture and Technology, Udaipur–313 001, Rajasthan, India.
Received: 24-12-2015 Accepted: 21-03-2017 DOI: 10.18805/LR-3401

ABSTRACT

A study was carried out among 22 black gram genotypes to study the genetic diversity using 20 ISSR primers. Screening of the primers revealed that only 15 out of the 20 primers produced amplification. A total of 84 amplified bands were obtained, out of which 72 were polymorphic 85.71 percent polymorphism. The total number of amplified bands varied between 1 (UBC-813 and UBC-878) and 9 (UBC-826) with an average of 5 bands per primer. The overall size of PCR amplified products ranged between 250 bp to 2000 bp. PIC values ranged from 0.00 to 0.51 with an average of 0.285 across all genotypes. Five unique bands were detected in four genotypes, out of which the genotype U-9 gave maximum number of distinct bands. The size of these unique bands ranged from 450 bp to 2000 bp. Based on the UPGMA derived dendrogram and PCA, the 22 genotypes could be divided into four main clusters. While Cluster I included 16 genotypes, the Clusters II, III and IV included two genotypes each. Genotypes IC-16511 and UTTARA, UH-177 and IPU2K-21, STY-2834 and UH-177 were found to be genetically distant from each other with a minimum similarity value of 0.42. The results are encouraging with the suggestion that the ISSR marker could prove to be a versatile tool in further screening of the *Vigna* germplasm pool for study of genetic divergence and the establishment of phylogenetic relationship amongst accessions.

Key words: Black gram, Genetic diversity, ISSR, PCR, Polymorphism, Similarity coefficient.

INTRODUCTION

[Vigna mungo (L.) Hepper] also referred to as the urad, black gram, urdbean, mash, black lentil or white lentil. It is considered to have been domesticated in India from its wild ancestral form (*Vigna mungo* var. *silvestris*) (Lukoki *et al.*, 1980). The seeds of black gram contain a moderately high amount of calories. It is also rich in protein, carbohydrates, essential amino acids, minerals and vitamins for human body (Shafique *et al.,* 2011). Being a short duration crop, it is grown primarily as intercrop and can also be used as green manure crop with residues incorporated into soil after pods have been harvested. It also helps to enrich the soil fertility through symbiotic nitrogen fixation. Major constraints in achieving higher yield of this crop are lack of genetic variability, thermo sensitivity, absence of suitable ideotypes for different cropping systems, poor harvest index and susceptibility to pests and diseases Kanimozhi *et al.*, (2009). Therefore, improvement of this crop is needed through utilization of available genetic diversity.

Molecular assisted breeding, or marker assisted selection are the most recent to be developed and have many advantages over morphological and biochemical markers and have been proved to be a valuable tool for the characterization and evaluation of genetic diversity within and between species and populations (Russel *et al.,* 1997). ISSR markers have been successfully utilized for assessing the genetic diversity in the genus *Vigna* (Ajibade *et al.,* 2000), black gram (Souframanien and Gopalakrishna 2004) and green gram (Sreethi Reddy *et al.,* 2008). ISSR markers are useful in detecting polymorphism among accessions by generating a large number of markers that target multiple microsatellite loci distributed across the genome (Reddy *et al.*, 2002). The ISSR techniques are more informative for estimating the extent of genetic diversity and relationships between black gram varieties. ISSR would be a better tool than RAPD for phylogenetic studies (Pardhe and Satpute 2011). So far, very little attention has been given to varietal improvement of legumes (Sultana *et al.,* 2006; Nisar *et al.,* 2008). The present study was aimed to evaluate and compare the nature and the extent genetic diversity among genotypes of blackgram which were collected from different parts of India using ISSR markers.

MATERIALS AND METHODS

Plant Materials: Seeds of 22 genotypes of black gram were procured from ARS, Durgapura, Jobner Agriculture University, Jaipur (Table 1). Laboratory studies were undertaken at the Department of Molecular Biology and Biotechnology, Rajasthan College of Agriculture, MPUAT, Udaipur. DNA was isolated from young seedlings using modified CTAB method (Doyle and Doyle, 1987).

*Corresponding author's e-mail: okdivya@gmail.com

Quantification of DNA using spectrophotometer (UV/ visible UNICAM model). 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 -20^{0}C until further analysis.

ISSR analysis: Total of 20 ISSR primers were used for PCR amplification. PCR amplification was carried out in programmable thermal cycler from Eppendorf AG, Germany. For ISSR analysis, PCR reaction was performed in 20 µl reaction mixture consisting of 100 ng of sample DNA, 200 µM of dNTP mix, 1U of *Taq* polymerase, 1X of reaction buffer, 0.5 µM of primer and double distilled water.

Following protocol used for ISSR primers for PCR amplification

Cycle	Denaturation		Annealing		Extension	
First cycle	94°C	4 min	-	-	-	-
35 cycles	94°C	1 min	*	1 min	72°C	2 min
Last cycle	-	-	-	-	72°C	10 min

* vary between 36 °C to 55 °C

Agarose Gel Electrophoresis (AGE) (Sambrook *et al.*, 1989): Following the PCR amplification, the PCR products of ISSR were loaded on 1.2 per cent agarose gel. Agarose gel was prepared in 1X TAE buffer containing ethidium bromide (10 mg/ml). Electrophoresis 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.

ISSR amplification profile were scored visually, based on the presence (1) or absence (0) of bands for each black gram genotypes. Only clear and unambiguous bands were scored. The size of the amplified bands were observed based on its migration and confirmation relative to standard molecular size markers (100 bp DNA ladder and 1 kb DNA ladder from Bangalore Genei Pvt. Ltd., Bangalore, India).

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, depicting the relationships of the genotypes using computer program 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 information of ISSR 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 study was conducted to assess the extent of genetic diversity and relationships among the 22 blackgram genotypes. The amount of DNA isolated from various genotypes of blackgram ranged from 529 to 5904 ng/µl. The genotype UH-177 yielded the highest amount of DNA (5904 ng/µl), whereas the lowest amount of DNA (529 ng/µl) was obtained from genotype U-9. 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.

Amplification of genomic DNA of the 22 genotypes using 20 ISSR primers, out of which 15 primers showed amplification. DNA banding profile of individual plant DNA samples from 22 genotypes of blackgram after amplification with ISSR primers are depicted in Plate-1.

The 15 ISSR primers yielded a total of 84 amplified bands, of which 72 were found polymorphic. The total number of bands observed for each primer was recorded separately and polymorphic bands percentage was calculated subsequently (Table 2). The total number of amplified bands varied between 1 (UBC-813 and UBC-878) and 9 (UBC-826) with an average of 5 per primer.

Table 1: Source of the Blackgram (*V. mungo* L.) genotypes

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

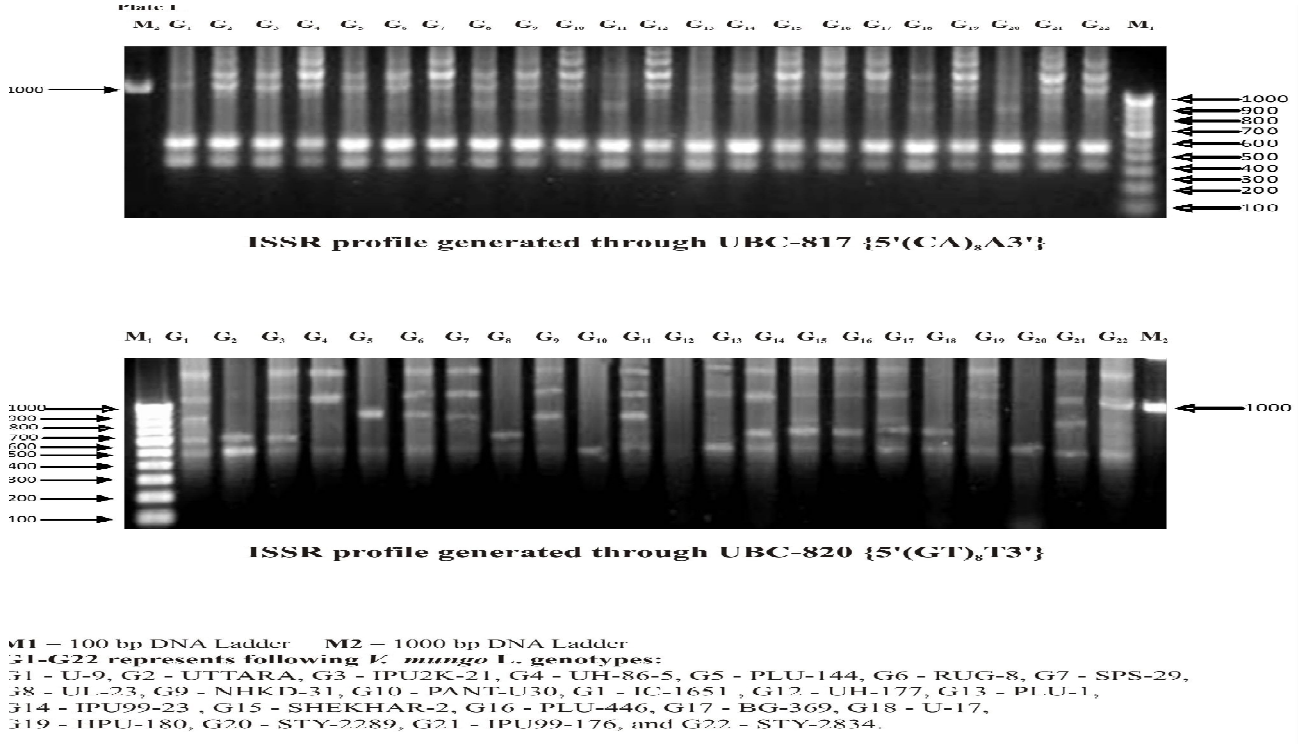

Table 2: DNA amplification profile and polymorphism generated in Blackgram (*V. mungo* L.) using 15 ISSR primers

Primer Code	Sequence 5' to 3'	Ta*(°C)	Molecular weight range(bp)	Total no. of bands amplified	Polymorphic bands		PIC
					Number	Frequency (%)	
UBC-810	$(GA)_8T$	42.9	2000-500	7	7	100	0.318
UBC-811	$(GA)_8C$	43.3	1500-800	3	3	100	0.443
UBC-813	$(CT)_8T$	43.3	700	**1**	1	100	**0.51**
UBC-815	$(CT)_8G$	44.9	1000-350	5	3	60	0.114
UBC-817	$(CA)_8A$	52	1600-325	7	5	71.42	0.282
UBC-818	$(CA)_8G$	52	1500-500	7	6	85.71	0.334
UBC-820	$(GT)_8T$	50	1600-500	6	6	100	0.345
UBC-822	$(TC)_8A$	45	2000-300	5	3	60	0.252
UBC-826	$(AC)_8C$	52	2000-400	**9**	9	100	0.36
UBC-836	$(AG)_8YA$	43.3	1800-550	5	5	100	0.256
UBC-840	$(GA)_8YT$	45	1000-350	6	5	83.33	0.257
UBC-845	$(CT)_8R*G$	47.7	2000-400	7	7	100	0.296
UBC-848	$(CA)_8RG$	55.5	2000-250	7	7	100	0.349
UBC-873	$(GACA)_4$	45	2000-550	8	5	62.5	0.160
UBC-878	$(GGAT)_4$	60	800	1	0	0	**0**
Total				**84**	**72**	**85.71**	**0.285**

Ta*=Annealing temperature, * Code of primers, Y* Pyrimidine and R* Purines

The polymorphism percentage ranged from as low as 0 per cent (UBC-878) to as high as 100 per cent in eight primers and the average polymorphism was 85.71 per cent. Pardhe and Satpute (2011) also reported the similar findings, which per cent polymorphism using ISSR primers ranged from 20 per cent (UBC-819) to 88.23 per cent (UBC-821). The overall size of PCR amplified products ranged between 250 bp to 2000 bp. The PCR amplification using ISSR primers gave rise to reproducible amplification products. The number of potential ISSR markers depends on the variety and frequency of microsatellites, which tends to change with species and the SSR motifs that are targeted (Despeiger *et al.,* 1995). Polymorphism information content (PIC) values ranged from 0.0 to 0.51 with an average of 0.285 across all the genotypes. Five unique bands were detected in four genotypes *viz.,* U-9, UTTARA, IPU 2K-21 and SHEKHAR-2 with 3 ISSR primers (UBC-826, UBC-845 and UBC-873). The genotype U-9 gave maximum number of distinct bands *i.e.,* 2. The size of these unique bands ranged from 450-2000 bp. (Table 3).

Table 3: Genotype specific bands as detected by 3 ISSR primers in 4 different genotypes of Blackgram (*V. mungo* L.)

Primer code	Total no. of unique bands	Genotypes	No. of unique bands	Size of bands (bp)
UBC-826	2	U-9	1	2000
		SHEKHAR-2	1	1800
UBC-845	2	IPU2K-21	1	500
		U-9	1	450
UBC-873	1	UTTARA	1	900
Total			**5**	

In black gram, Souframanien and Gopalakrishna (2004) identified a set of ISSR primers with high PIC scores which would be useful in surveying genetic diversity among accessions of black gram. Similarly, Kanimozhi *et al.,* (2009) studied the genetic diversity among 23 black gram genotypes using twelve ISSR primers. The number of alleles produced by different ISSR primers ranged from 8 to 17 with an average of 11.5 per primer and the level of polymorphism was found to be 82.05 per cent. Similarly, Abd El-Hady *et al.,* (2010) selected 11 ISSR primers which produced 128 bands across seven genotypes of which 89 were polymorphic with an average of 11.64 per primer. The size of amplified bands ranged from 264 to 2838 bp.

Genetic Relationship and Cluster Tree Analysis: Based on ISSR similarity matrix data, the values of similarity coefficient ranged from 0.42 to 0.85 *i.e.,* 42-85 per cent or genetic diversity ranged from 15 to 58 per cent (Table 4). Similar results were obtained by Singh *et al.,* (2011) and Das *et al.,* (2014). The average similarity across all the genotypes was found out to be 0.63, showing that genotypes were moderately similar. Maximum similarity value of 0.85 was observed between genotypes IPU 2K-21 and U-9 followed by IPU99-176 and HPU-180 with a similarity coefficient of 0.84. Genotypes IC-16511 and UTTARA, UH-177 and IPU 2K-21, STY-2834 and UH-177 were found to be genetically diverse with a minimum similarity value of 0.42 followed by UH-177 and U-9, PLU-710 and U9 having similarity values of 0.44.

The ISSR data were used to obtain a similarity matrix (Table 4). The similarity coefficient was found to lie between 0.52-0.86. The result showed that they could be divided into 4 major clusters at a similarity coefficient of 0.62 (Fig. 1). Cluster I was the main one that included sixteen genotypes *viz.,* U-9, IPU 2K-21, UH-86-5, SPS-29, PLU-144, UL-23, PLU-1, IPU99-233, SHEKHAR-2, HPU-180, IPU99-176, STY-2834, RUG-8, NHKD-31, PLU-446 and BG-369. It could be divided into 6 sub-clusters. In sub-cluster I, U-9 and IPU 2K-21 were related to each other at 0.86 similarity coefficients. In sub-cluster II, genotypes UH-86-5 and NHKD-31 were related to each other at 0.83 similarity coefficients. In sub-cluster III, genotypes RUG-8 and SPS-29 were related to each other at 0.80 similarity coefficients. In sub-cluster IV, genotypes PLU-144 and UL-23 were related to each other at 0.82 similarity coefficients. In sub-cluster V, genotypes IPU99-233 and SHEKHAR-2 were related to each other at 0.79 similarity coefficients. In sub-cluster VI, genotypes HPU-180 and IPU99-176 were related to each other at 0.84 similarity coefficients.

The cluster II included 2 genotypes U-17and STY-2289. Both of the genotypes were related to each other at 0.79 similarity coefficients. The cluster III includes 2 genotypes IC-16511 and UH-177 that were related to each other at 0.61 similarity coefficients. The cluster IV included 2 genotypes UTTARA and PANT-U30. Both the genotypes were related to each other at 0.63 similarity coefficients.

Similar results were reported by Kanimozhi (2009) used ISSR marker for genetic diversity analysis and presented a dendrogram that distributed the 23 blackgram genotypes into five main clusters with a dice similarity coefficient ranging from 0.62 to 0.92. The highest genetic similarity coefficient (0.92) was measured between genotypes CBG 671 and CBG 632.

Principal Component Analysis Based on ISSR Data: Two and three dimension principal component analysis based on ISSR data (Fig. 2 and 3, respectively) showed similar clustering of 22 genotypes as evident from cluster tree analysis. Dice similarity coefficients ranged from 0.65 to 0.88, indicative of an average level of variation among the genotypes. As visible in the dendrogram, the genotypes that

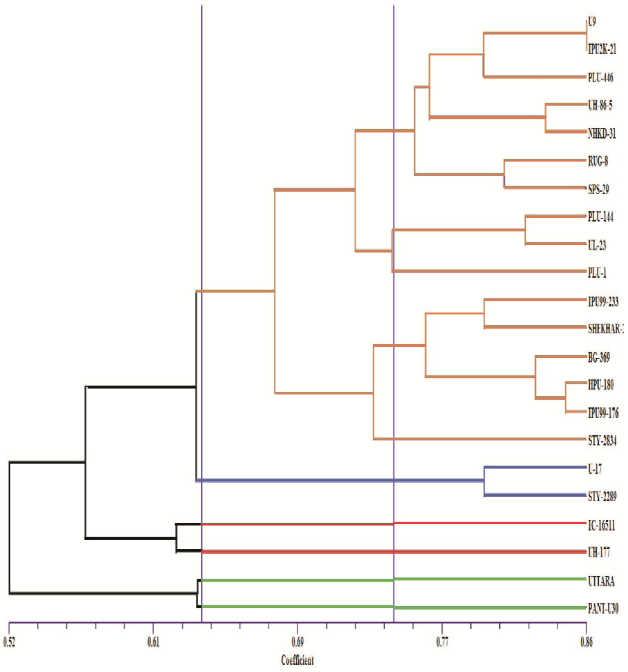

Fig 1: Dendrogram constructed with UPGMA clustering method among 22 genotypes of V.mungo *L.*using ISSR markers

Table 4: Jaccard's similarity coefficient for ISSR 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	446	PLU-BG-369	U-17	HPU-180	STY-2289	IPU99-176	STY-2834
U-9	1.00																					
UTTARA	0.46	1.00																				
IPU2K-21	0.85	0.47	1.00																			
UH-86-5	0.76	0.54	0.73	1.00																		
PLU-144	0.66	0.53	0.66	0.73	1.00																	
RUG-8	0.77	0.48	0.77	0.76	0.76	1.00																
SPS-29	0.75	0.48	0.72	0.79	0.77	0.80	1.00															
UL-23	0.70	0.57	0.77	0.72	0.82	0.70	0.76	1.00														
NHKD-31	0.76	0.46	0.76	0.83	0.71	0.78	0.70	0.72	1.00													
PLU-710	0.44	0.63	0.47	0.51	0.53	0.46	0.51	0.54	0.45	1.00												
IC-16511	0.61	0.42	0.61	0.60	0.66	0.67	0.63	0.67	0.70	0.55	1.00											
UH-177	0.44	0.46	0.42	0.57	0.59	0.55	0.60	0.58	0.48	0.58	0.61	1.00										
PLU-1	0.71	0.47	0.69	0.78	0.76	0.71	0.75	0.72	0.71	0.47	0.66	0.55	1.00									
IPU99-233	0.70	0.59	0.73	0.72	0.71	0.75	0.76	0.73	0.69	0.63	0.54	0.54	0.69	1.00								
SHEKHAR-2	0.66	0.55	0.70	0.66	0.60	0.71	0.62	0.60	0.63	0.57	0.51	0.45	0.63	0.79	1.00							
PLU-446	0.78	0.46	0.80	0.79	0.65	0.72	0.77	0.71	0.77	0.47	0.60	0.48	0.72	0.67	0.66	1.00						
BG-369	0.64	0.53	0.63	0.66	0.60	0.66	0.72	0.63	0.58	0.60	0.52	0.51	0.65	0.75	0.80	0.66	1.00					
U-17	0.54	0.48	0.61	0.55	0.71	0.63	0.67	0.75	0.59	0.46	0.57	0.52	0.59	0.65	0.58	0.60	0.58	1.00				
HPU-180	0.66	0.57	0.65	0.78	0.67	0.67	0.72	0.65	0.75	0.60	0.58	0.54	0.72	0.77	0.73	0.70	0.83	0.55	1.00			
STY-2289	0.59	0.52	0.64	0.61	0.76	0.70	0.70	0.75	0.64	0.59	0.66	0.58	0.73	0.70	0.64	0.59	0.61	0.79	0.61	1.00		
IPU99-176	0.72	0.54	0.71	0.70	0.59	0.70	0.71	0.64	0.71	0.60	0.59	0.47	0.66	0.73	0.77	0.70	0.82	0.53	0.84	0.58	1.00	
STY-2834	0.61	0.50	0.65	0.69	0.57	0.66	0.67	0.54	0.69	0.54	0.50	0.42	0.60	0.72	0.67	0.67	0.70	0.50	0.75	0.53	0.80	1.00

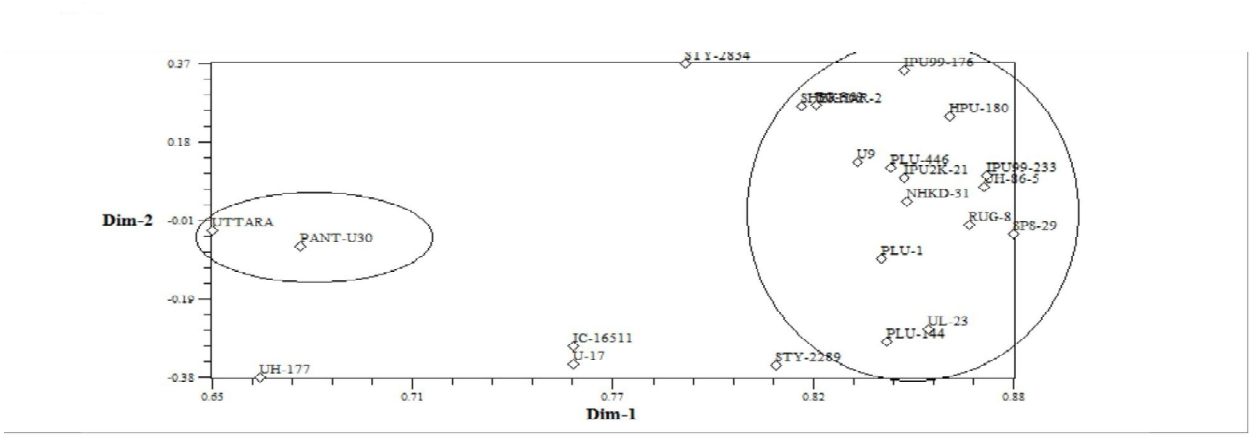

Fig. 2: Two Dimensional PCA (Principal Component Analysis) scaling of 22 genotype of *V.* mungo L.using ISSR markers.

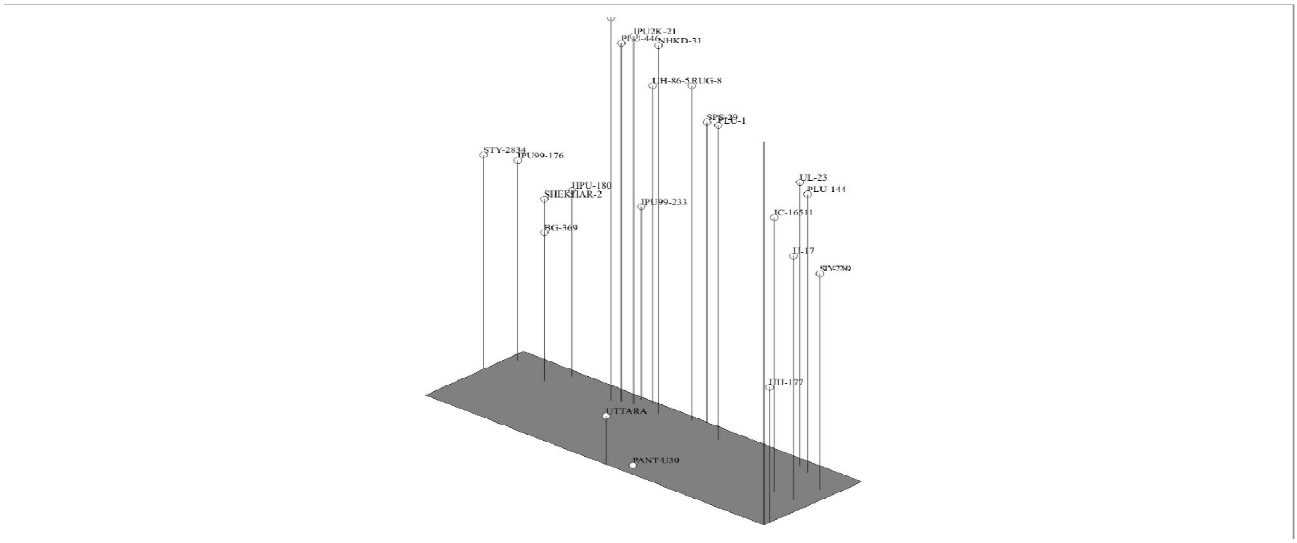

Fig. 3: Three Dimensional PCA (Principal Component Analysis) scaling of 22 genotype of *V.* mungo L.using ISSR markers.

were closer were more similar than those that were lying apart. Similar observations were recorded with PCA as well. Cluster I included sixteen genotypes *viz.,* U-9, IPU2K-21, UH-86-5, SPS-29, PLU-144, UL-23, PLU-1, IPU99-233, SHEKHAR-2, HPU-180, IPU99-176, STY-2834, RUG-8, NHKD-31, PLU-446 and BG-369; and cluster IV included 2 genotypes *viz*., UTTARA and PANT-U30 that lay closer to each other. Genotype UH-177 was lying far apart followed by three other genotypes STY-2834, IC-16511 and U-17. Similar results have been obtained by Kanimozhi *et al.,* (2009).

In conclusion, cross breeding between genetically different individuals is a recommended, rather than involving individual belonging to related genetic group (Rocha *et al.,* 2002). ISSR analysis reported in the present work that genotypes IC-16511 and UTTARA, UH-177 and IPU 2K-21, STY-2834 and UH-177 could be useful to select as a parents to be crossed for generating appropriate populations intended for both genome mapping and breeding purposes.

ACKNOWLEDGMENT

I acknowledged DST Inspire Fellowship for providing financial support during doctoral program. Authors gratefully acknowledged the financial assistance from RKVY project "Validation of important crop varieties through DNA fingerprinting".

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