Original Research Article

Morphological Variability and Molecular Diversity in Blackgram (*Vigna mungo* (L.) Hepper) Genotypes Using SSR Markers

ABSTRACT

A study on the Morphological variability of 25 Blackgram genotypes was carried out in which molecular diversity of 15 genotypes of Blackgram was studied by using 8 SSR markers. The GCV was lower than PCV for all traits, however, there was narrow difference in PCV and GCV, indicating the effect of environment in lesser frequency. The high seed yield was recorded in the genotypes PLU-429(8.06gm) followed by PL-416(7.37gm) and VBN-8(7.29gm). Among all the factors PC 1 to PC 8, the PC 1 (40.610) accounted maximum proportion of variability in the set of all variables. Association studies revealed that there was high and significant positive amount of genotypic as well as phenotypic correlation coefficient for traits viz., primary branches per plant, number of clusters per plant, number of pods per plant, harvest index. Path analysis revealed high positive direct effect was contributed by primary branches per plant, number of clusters per plant, number of pods per plant, harvest index both at genotypic and phenotypic levels. The Polymorphism information content PIC values of 8 SSR markers valued from 31 percent to 71 percent with mean of 51 percent. BG 18 exhibited highest PIC 71 percent and highest heterozygosity of 62 percent. As per Dissimilarity coefficient, 15 genotypes are grouped into five clusters, Cluster II & V has highest 4 genotypes while in compare with Cluster I, III & IV. SSR marker BG18 has highest number of alleles 5. Shannon's information index(I) ranged from 1.465 for BG18 to 0.611 for BG07. Fixation index is ranged from 1.000 to -0.111 with mean 0.696. SSRs with high polymorphism information content successfully assisted in the differentiation of genotypes in this study.

Keywords: Blackgram, GCV, Molecular diversity, PCV, PIC, SSR.

INTRODUCTION

"Blackgram or urd bean (*Vigna mungo* (L.) Hepper) is one of the significant *Vigna* species pulse crop majorly cultivated in India. It is a short-lived, diploid grain legume (2n = 2x=22) that is self-pollinated. Blackgram is thought to have originated from a wild form of *Vigna mungo var*. *silvestris* in India" Kaewwongwal *et al*., (2015). "Blackgram seeds are rich in nutrients, including protein (25–26%), carbs (60%) and fat (1.5%), as well as vitamins, minerals, and amino acids" Singh and Singh, (2013). "Additionally, it is a significant kharif and spring/summer pulse crop in several South Asian nations, including India, Pakistan, Nepal, Bangladesh, Thailand, and Korea" Ghafoor and Arshad, (2008). "Despite its significance, this crop produces surprisingly little. Blackgram is mostly produced and consumed in India. In Andhra Pradesh, Blackgram is grown on an area of 393 thousand hectares with an annual production of 365 thousand tonnes and a productivity of about 929 kg/ha. The annual production of Blackgram in India is 2340 thousand tonnes from 4670 thousand hectares with an average productivity of 501 kg/ha (Ministry of Agriculture, 2020-21). The main obstacles to increasing productivity are a lack of genetic diversity that can be exploited, a lack of suitable ideotypes, a low harvest index, vulnerability to biotic and abiotic stresses, a lack of high-quality seeds of improved varieties, and a limited genetic base as a result of the repeated use of a small number of parents with a high degree of relatedness in crossing programmes" Hadimani *et al*., (2016). The development of new Blackgram cultivars is necessary to increase production. Any research initiative to increase crop output should start with an evaluation of the genetic variability of pulses.

"The exploitation of current genetic variability by calculating various genetic parameters such as genotypic, phenotypic, and environmental variances, their coefficient of variability, genetic advance, and heritability is quite important in order to frame an effective breeding programme in the following generation. Studying the inheritance of different developmental and productive qualities is primarily helpful. A crucial requirement for crop breeding is understanding of genetic diversity, as successful heterosis breeding depends on genetic variety" Kumar *et al*., (2017). A general understanding of heritability gives insight into the roles that additive and non-additive gene actions play in the development of phenotypes. To understand the impact of the greatest individuals, assessments of heritability together with genetic advancement are more crucial than genetic advancement alone Johnson *et al*., (1955). One of the main methods for improving a characteristic is selection, which can effectively address heritable differences and genetic variance among the characters under investigation.

Principal component analysis (PCA), a kind of multivariate analysis and cluster

analysis are examples of potential approaches for assessing phenotypic variety, locating genetically remote genotype groups, and choosing significant features that contribute to the overall variation in genotypes. These studies offer data that could aid in developing breeding plans for trait enhancement as well as better parental genotype selection for particular traits. An accurate indication of genetic differences, principal component analysis (PCA) enables natural grouping of the genotypes. Identification of plant features that classify the distinctiveness among potential genotypes using PCA is highly beneficial Chakraborty *et al*., (2013). In contrast to morphological variability, molecular markers can identify genotype differences at the DNA level, offering a more direct, accurate, and effective tool for identification, management, and conservation of germplasm Tanksley *et al*., (1989). "There has only been a small amount of research done on blackgram's nuclear DNA diversity. Simple sequence repeat (SSR) markers, often known as microsatellites, are brief tandem repetitive DNA sequences having repetition lengths of one to five base pairs. Genetic mapping and genome analysis, genotype identification and variety protection, seed purity assessment and germplasm preservation, diversity studies, paternity determination and pedigree analysis, gene and quantitative trait locus analysis, and marker-assisted breeding are just a few of the many applications for which microsatellites have emerged as the preferred molecular markers" Archana and Jawali, (2007). being stable and codominant, numerous and dispersed throughout the genome, repeatable, and possessing a significant amount of polymorphism Powell *et al*., (1996). Many different taxa's genomes contain SSRs, which are tandem repeat motifs of 1-6 nucleotides Beckmann et al., (1992). Using RAPD Lakhanpaul *et al.*, (2000) and amplified fragment length polymorphism (AFLP) Bhat *et al*., (2005) markers, it has been found that the released Indian blackgram cultivars have a small genetic base. Application of molecular methods for Blackgram's genetic advancement has been constrained by the paucity of polymorphic molecular markers. As a substitute for increasing the number of accessible markers, markers from related species can be transferred. The objective of the current study was to use SSR in Blackgram to evaluate the morphological variety and molecular variability of elite genotypes of Blackgram.

MATERIALS AND METHODS

The goal of the current experiment was to determine the morphological variability and molecular variety of many variables related to the crop's potential production. 25 Blackgram genotypes were tested in the study using a random block design at the Department of Genetics and Plant Breeding, SHUATS, Prayagraj, in *kharif*, 2021 followed by 15 blackgram genotypes molecular work is done from Plant Biotechnology Division, Indian Institute of Pulses Research

(IIPR) Kanpur. The morphological data were recorded and statistical analysis of genetic variability and molecular diversity was performed on them. Five randomly selected plants were chosen to gather information on the following: plant height, primary branch count, cluster count, pod count, pod length in cm, seed count, 100 seed weight per pod, biological yield, harvest index, and plant seed yield in blackgram. Burton (1952) gave formulas for calculating the major genetic characteristics, including GCV and PCV, whereas Johnson et al. provided formulas for calculating heritability and GA as % mean (1955).

Table 1: List of Genotypes of Blackgram used for molecular diversity with their pedigree and salient features

Genotypes	Pedigree
VBN-8	VAMBAN3 X VBN-04-008, VAMBAN
PU-19	UPU-1 X UPU-2, GBPUAT Pant Nagar
IPU-10-26	UH-85-5 X PDU-103
ADT-3	Pure line selection from Tirunelveli local (Tamil Nadu)
IPU-94-1	NP19 X T-9, IIPR KANPUR
LBG-752	LBG402 X LBG20, Guntur (LAM)
SHEKHAR -2	7378/2 X T9, (CSAUAT KANPUR)
PGRU-95016	Germplasm collection, UP
KC-153	Germplasm collection, UP
PLU-429	Germplasm collection, UP
IPU-99-16	Germplasm collection, UP
PLU-25	Germplasm collection, UP
PLU-110	Germplasm collection, UP
UH-85-2	Germplasm collection, HAU-Hisar
PDU-3	Advanced breeding line, IIPR

Genomic DNA extraction and quantification

Using the Cetyltrimethyl ammonium bromide (CTAB) extraction method Doyle and Doyle, (1987) which is modified by Nadia Aboul-Ftooh Aboul- maaty *et al*., (2019) adding higher concentration of CTAB and 2-ß-mercaptoethanol. The total genomic DNA was isolated from 15 genotypes and quantified by 0.8 % Ethidium bromide agarose plate method. The concentration of genomic DNA of each genotype was determined by checking with known concentration of λ DNA of~ 200ng as shown in (Fig 1) and concentration of each genotype adjusted to 20 ng μl^{-1} by diluting with Milli-Q water for SSR marker analysis.

Building up of the SSR-PCR 24 SSR or microsatellite repeat primers were used to screen the Blackgram germplasm lines. A 10-µL reaction volume comprising 0.3µl dNTP mix, 1µl Taq buffer(1x), 0.2µl of Taq polymerase, 1µl of primer (Forward and reverse primer), 1µl DNA (20ng) of each sample and make up to the volume 10µl by adding 6.5µl of Milli-Q water used for the PCR amplification. Pre-denaturation at 94 °C for 5 minutes was followed by 35 cycles of denaturation at 94 °C for 40 seconds, annealing at 50.0-60.0 °C for 40 seconds, extension at 72 °C for1 minute, and final extension for 10 minutes at 72 °C with a storage temperature of 10.0°C were the reaction conditions used for the amplification. By 4% agarose (Sigma-Aldrich, India), amplification products were separated using electrophoresis, and gel images were obtained from the Gel documentation unit as shown in (Fig 2).

Data analysis

For each marker allele-genotype combination, amplicons made with SSR primers and resolved on agarose gels were scored quantitatively for presence using binary coding, where presence was denoted by (1) and absence by (0). The binary data was analysed for the number of alleles, Shannon's information index, PIC (Polymorphism information content), and heterozygozity using the Gene Alex 6.5 tool. Utilizing the XLstat ver 2021 programme, PCA (Principal Component Analysis) data was gathered. The NTsys application was used to create a molecular dendrogram cluster analysis for 15 blackgram genotypes using the ward technique and squared Euclidean distance.

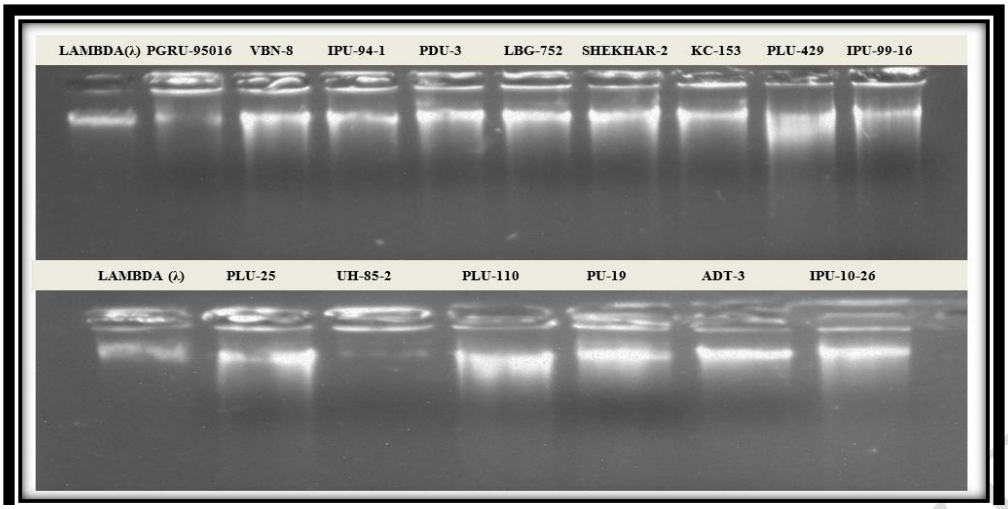

Fig 1: Gel picture of Genomic DNA quantification by Agarose plate method

Table 2: List of Polymorphic SSR markers

S.no	Name of the Primer	Sequence of Forward and Reverse Primers	T_m	AT	No. Of bases
1	BG 06F BG 06R	GCATTTCATCAACCACACAGAT GTCGGTTTCCAGACACGAG	51.1°C 53.3°C	56°C	22 19
2	BG 08F BG 08R	CAAGCATTGAGAGGAAAACGA ACCATACCCTCCACTTTTGGT	50.0°C 52.4°C	51°C	21 21
3	BG 21F BG 21R	GAACTGTATGTAGCAGGGGCTC AGAGGAGACAAAACGCAGAGAT	56.7°C 53.0°C	55°C	22 22
4	BG 16F BG 16R	ACGCCTATGGCTTCAGTAAAAG GCAAGCAAGAAAAGCTCACAA	53.0°C 50.5°C	57°C	22 21
5	BG 18F BG 18R	GTGGTTGTTGTTGGTGATTCTG AGGGTGAGCGTTATGAGGTAGA	53.0°C 54.8°C	54°C	22 22
6	BG 07F BG 07R	CTCTCTGCACCTTCTGCCTTAT GTTTCCTCAAACAGCAACCAAC	54.8°C 53.0°C	54°C	22 22
7	BG 36F BG 36R	TAGAACCACCATAACCCCAATC GAACGAAGCTGGAGAAGTTGTT	53.0°C 53.0°C	59°C	22 22
8	VR 91F VR 91R	ACATATGTATCTGTCTGTGTGCCTA TGGAGATGCAGGACTAAGAAGAG	54.4°C 55.3°C	54°C	25 23

RESULTS AND DISCUSSION

The results of the analysis of variance showed a substantial difference between all of the genotypes for each characteristic. This suggests that there was sufficient room in the current gene pool to select promising lines for yield and its component traits. The presence of a significant amount of variability may result from the use of different sources of data as well as environmental factors that affect the phenotypes. Similar results were found by Panda et al. (2017), Anu et al. (2017), and Tanveer et al. (2018), which revealed significant mean sum of squares values for all the Blackgram characters studied.

"The genotypes PLU-429 (8.06gm), PL-416 (7.37gm), VBN-8 (7.29gm), and IPU-99-16 (7.227gm), which had the highest seed output per plant based on mean performance, are thus recognized as promising lines with high yield potential and good quality for replacing SHEKAR-2 (check) (6.72gm). TLU-326 had the earliest day to 50% flowering and the earliest day to 50% pod setting. PL-416 had the most primary branches, clusters per plant, and harvest index, and CO-6 had the highest biological production". [30]

"The phenotypic coefficient of variation was found to be larger than the genotypic coefficient of variation, which suggests that the environment may have an impact on the manifestation of the character under study. The plant height, pod length, and seed yield were the variables with the greatest variations between GCV and PCV". [30] The findings of Chubatemsu *et al*. (2017), Bishnoi *et al*. (2017), Kondagari *et al*. (2017), Bandi *et al*. (2018), Blessey *et al*. (2018), Sushmitharaj *et al*. (2018), and Sarvani *et al*. (2018) were in agreement with the experimental results (2020). "The range of heritability (in the broad sense) was 21.756 to 85.509. The number of major branches per plant (85.509), number of pods per plant (73.841), biological yield (72.282), harvest index (71.379), and number of seeds per pod were shown to have the highest heritability's (70.222). There is no evidence of low or moderate heritability in these traits. The present study's high heritability values for the traits under consideration showed that those traits were less influenced by the environment and helped in the effective selection of traits based on phenotypic expression by using a simple selection method. These high heritability values also suggested the potential for genetic improvement". [30] According to Johnson *et al*. (1955), genetic gain would be more helpful in estimating the effectiveness of selection than just heritability estimates alone.

Harvest index had the highest estimated genetic advance, whereas pod length had the lowest. It

is possible to directly use characters with high heritability and high predicted genetic advance to enhance such characters. The function of additive gene action is also indicated by qualities with high heritability but low genetic progress, and these features may be utilized as an extra selection criterion if necessary. According to Panse and Sukhatme (1957), the function of additive gene action in the successful selection of the concerned characters is supported by a combination of moderate or high values of heritability and genetic progress.

"The range of genetic progress as a percentage of the mean was 2.74% to 36.36%. Number of primary branches, harvest index, and biological yield per plant all showed high genetic advance as a percent of mean, whereas Days to 50% pod initiation showed the lowest genetic advance". [30]

Using the paradigm provided by Sneath and Sokal (1973), principal component analysis was carried out, and factors with eigen values larger than one was taken into consideration. PCA was carried out for 8 SSR markers for 15 Blackgram genotypes based on the polymorphic band results. Of all the factors PC1 TO PC2, PC1 (40.610) accounted for the greatest percentage of variability in the set of all variables, while PC8 (0.769) accounted for progressively less variability. The data above indicate that PC1 has the highest Eigen value (3.249), while PC8 has the lowest Eigen value (0.062). In the past, several crops used a similar application of PCA to create 2D and 3D diagrams and, in turn, to comprehend the genetic diversity. According to the gene action guiding various traits, Jadhav *et al*. (2014) in finger millet; Naik *et al*. (2016) in cotton; Priya *et al*. (2017) in rice; Ayesha and Babu (2018) in foxtail millet; and Priya *et al*. 2019 in Blackgram to indicate the successful hybrid combination to obtain superior hybrids or transgressive segregants. The eight SSR markers are used to cluster the 15 Blackgram genotypes using the ward approach and squared Euclidean distance. The 15 genotypes are divided into five clusters as shown in (Fig 5) Cluster I, II, III, IV, and Cluster V—based on the dissimilarity coefficient. Three genotypes make up Cluster I, which is further divided into two subclusters: IA, which includes KC-153 and LBG-752, and IB, which includes SHEKHAR-2. Four genotypes make up Cluster II, which is broken into two smaller clusters. ADT-3 and PU-19 make up subcluster IIA, whereas PLU-110 and UH-85-2 make up subcluster IIB. Three genotypes make up Cluster III, which is broken into two smaller clusters. PLU-25 and IPU-99-16 make up subcluster IIIA, while PLU-429 is the only genotype in subcluster IIIB. IPU-10-26 is the only genotype that makes up Cluster IV. Four genotypes make up Cluster V, which is separated into two subclusters. Subcluster VA contains PDU-3 and IPU-94-1, while subcluster VB has VBN-8 and PGRU-95016.

The SSR marker BG18 has the maximum number of alleles (5), followed by BG06 and BG08 with 4 alleles each, BG21, BG16, and BG36 with 3 alleles each, and BG07 and VR91 with 2 alleles each. In comparison to the findings of Tantasawat *et al*. (2010) the total and average number of alleles obtained in the current study was small. Due to the relatively small population studied and the lack of polymorphic markers, Deepak *et al*., Kanimozhi *et al*., Souframanien and Gopalakrishna, Ajibade *et al*. The observed heterozygosity has a mean of 0.183 and a range of 0.000 to 1.000. The BG18 marker had the highest observed heterozygosity, which was reported at 100%, and the BG06, BG08, BG21, BG36, and VR91 had the lowest, which was recorded at 0%. The expected heterozygosity has a mean of 0.599 and a range of 0.756 to 0.420. The anticipated heterozygosity measured for BG18 is 78%, while the observed heterozygosity for VR91 is 40%. An essential factor that assesses the effectiveness of polymorphic loci and establishes the capacity of markers to discriminate is the polymorphism information content (PIC).

The average polymorphic information content value (PIC) per locus was 0.519, with values ranging from 0.315 to 0.713 per locus. Eight SSR markers had PIC values ranging from 31% to 71% with a mean of 51%. The highest percent of PIC Value among the eight markers was created by SSR BG18, while the lowest was detected for BG07. These values were discovered to be somewhat lower than those noted by Srivastava *et al*. but comparable to those noted by Tantasawat *et al*. The Shannon's information index (I), which is used to quantify species diversity at random, yielded results in the current study that showed high (I) diversity for BG18 and lowest diversity for BG07, with an average Shannon's index of 1.015 for the eight SSR polymorphic markers. With a mean of 0.696, the fixation index ranges from 1.000 to -0.111. The BG06, BG08, BG21, BG16, BG36, and VR91 all have the maximum fixation index of 1.000, while BG07 has the lowest fixation value of -0.111 and BG-18 has the lowest fixation index of -0.324. Blackgram genotypes with a small number of heterozygous loci accounted for the high fixation index in the majority of the loci by self-pollination. Low variance was found to be correlated with a high fixation index.

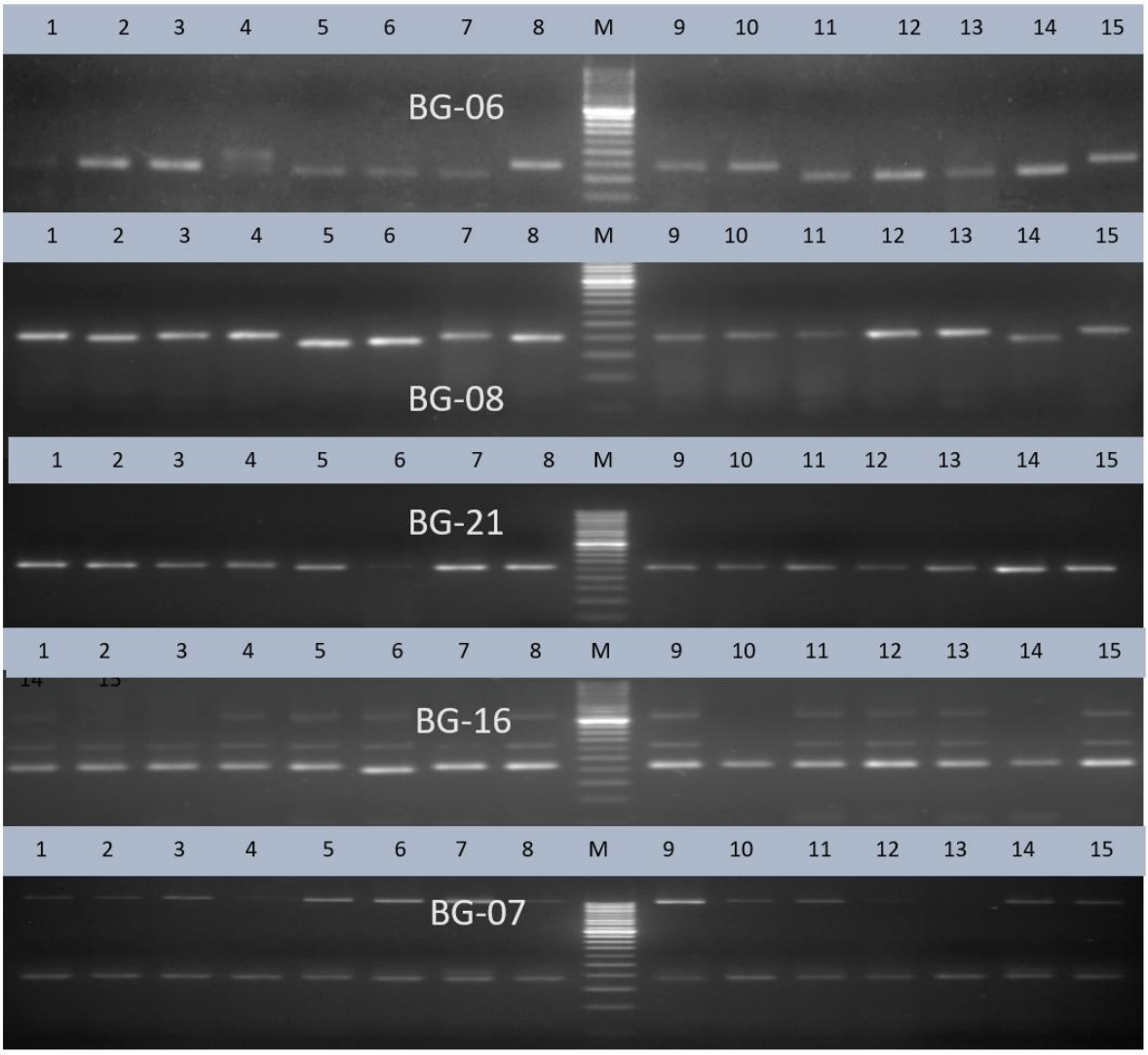

Fig 2: Gel pictures of Agarose stained with Ethidium bromide showing genetic polymorphism among Blackgram (*Vigna mungo* (L.) Hepper) genotypes using SSR primers. M: 50bp Ladder

Table 3: Estimates of genetic parameters for component characters in Blackgram [30]

S.No.	TRAITS	GCV	PCV	h^2 (Broad Sense) %	Genetic Advance 5%	Gen. Adv as % of Mean 5%
1	**Days to fifty percent flowering**	3.054	3.964	59.351	2.222	4.846
2	**Days to fifty percent pod setting**	2.085	3.267	40.719	1.486	2.74
3	**Days to maturity**	2.522	3.644	47.896	2.318	3.595
4	**Plant height (cm)**	4.593	8.238	31.089	3.118	5.276
5	**Number of primary branches per plant**	19.092	20.646	85.509	1.83	36.368
6	**Number of clusters per plant**	7.027	9.782	51.601	1.174	10.398
7	**Number of pods per plant**	12.332	14.352	73.841	5.015	21.831
8	**Number of seeds per pod**	9.125	10.889	70.222	0.85	15.752
9	**Pod length (cm)**	3.839	7.038	29.747	0.18	4.313
10	**Seed yield per plant (g)**	3.272	7.015	21.756	0.202	3.144
11	**Biological yield per plant (g)**	12.252	14.411	72.282	4.388	21.458
12	**Harvest Index (%)**	13.353	15.805	71.379	7.429	23.239
13	**Seed Index (g)**	8.514	11.212	57.661	0.575	13.318

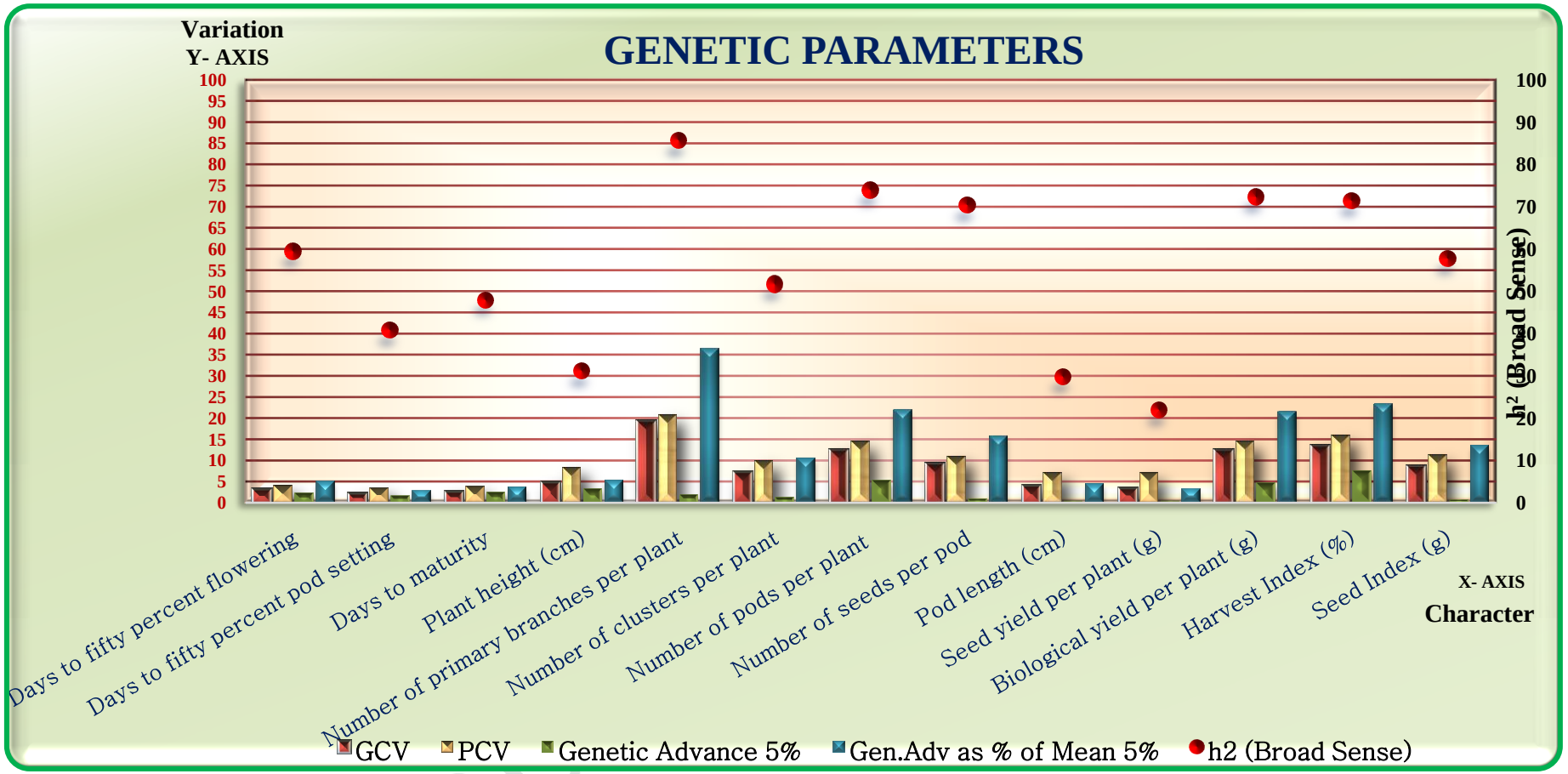

Fig.3: Graph depicting GCV, PCV, Genetic advance and Heritability for 13 Quantitative characters of Blackgram Genotypes

Table 4: Total variance explained by different principal components in Blackgram

Observations	Eigenvalue	Variability (%)	Cumulative %
PC1	3.249	40.610	40.610
PC2	1.790	22.377	62.987
PC3	1.524	19.054	82.041
PC4	0.640	8.000	90.041
PC5	0.315	3.943	93.984
PC6	0.281	3.512	97.497
PC7	0.139	1.735	99.231
PC8	0.062	0.769	100.000

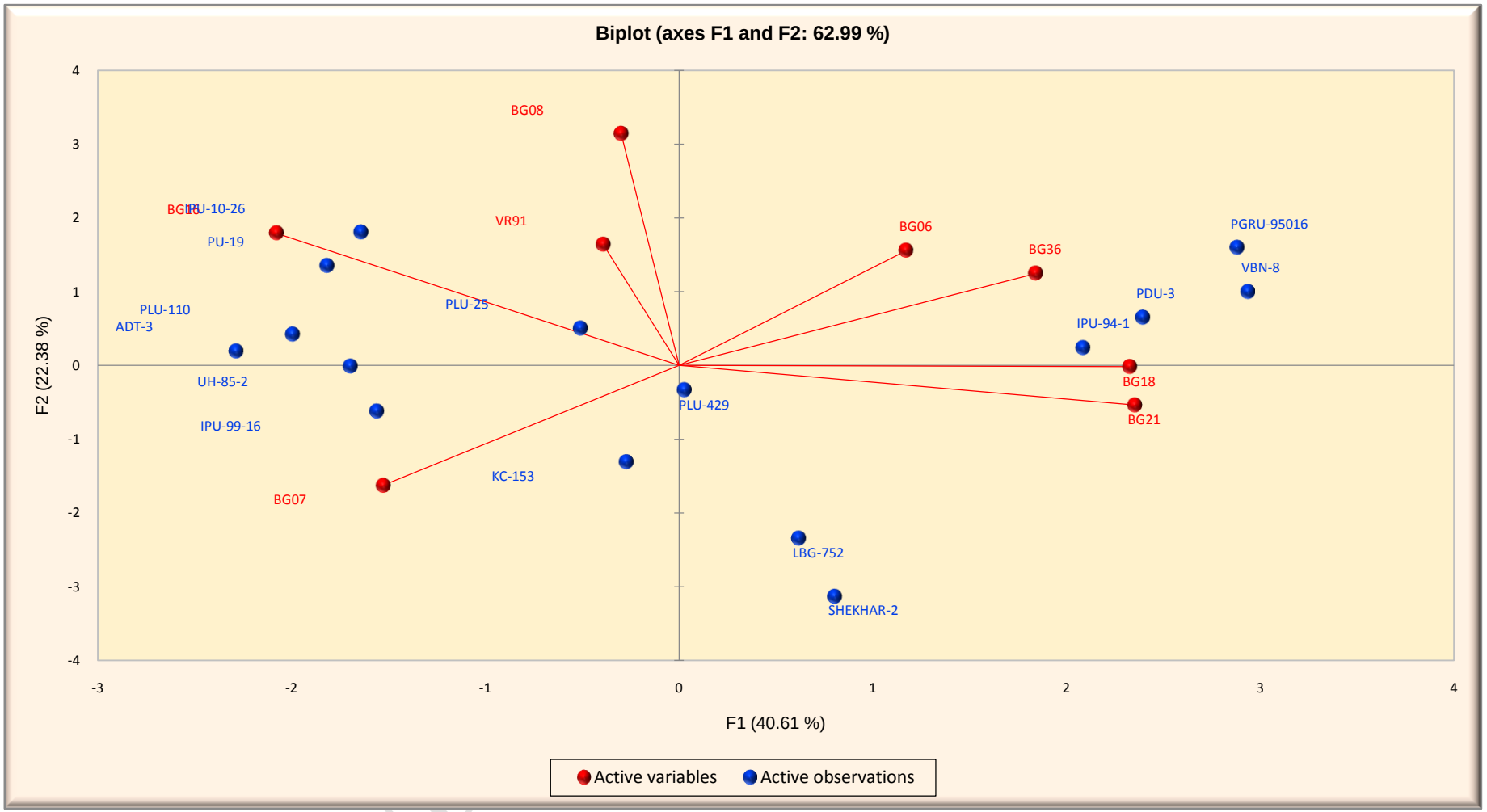

Fig. 4: Distribution of 15 genotypes on the bi-plot axes in PC 1 and PC2 of principal component analysis

Table 5: Information generated by using 8 SSR markers in Blackgram genotypes

Markers	Alleles count	Number of effective alleles	Observed heterozygosity	Expected heterozygosity	PIC	I	Fixation index
BG06	4	3.261	0.000	0.693	0.638	1.270	1.000
BG07	2	1.724	0.467	0.420	0.315	0.611	-0.111
BG08	4	2.848	0.000	0.649	0.586	1.171	1.000
BG21	3	2.711	0.000	0.631	0.556	1.044	1.000
BG16	3	2.273	0.000	0.560	0.461	0.892	1.000
BG18	5	4.091	1.000	0.756	0.713	1.465	-0.324
BG36	3	2.528	0.000	0.604	0.521	0.991	1.000
VR91	2	1.923	0.000	0.480	0.365	0.673	1.000
Total	26	21.359	1.467	4.793	4.155	8.117	5.565
Mean	3.250	2.670	0.183	0.599	0.519	1.015	0.696

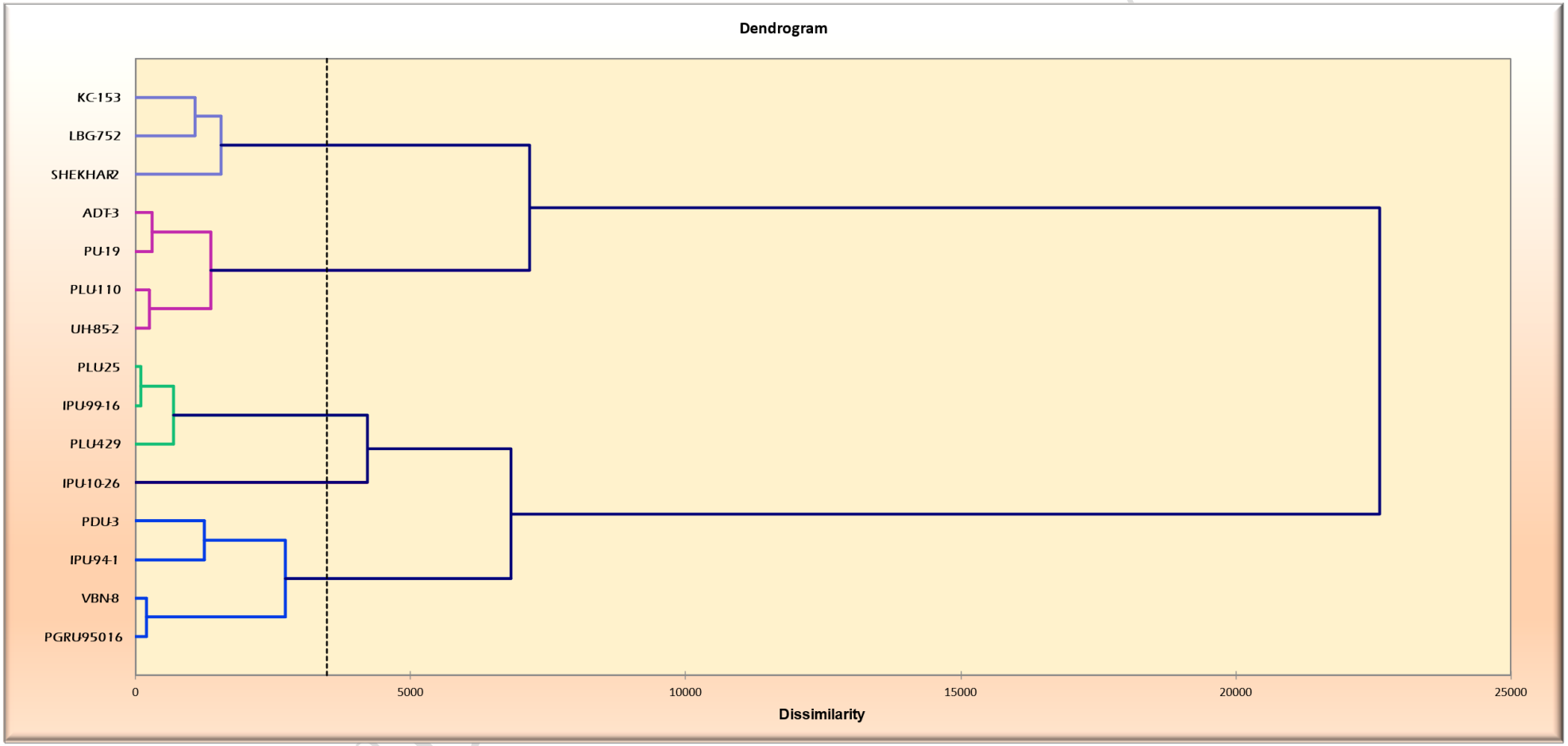

Fig. 5: Cluster analysis using ward method and squared Euclidean distance for 15 Blackgram genotypes.

CONCLUSION

According to the results of the inquiry, there were substantial differences between the 25 genotypes as shown by the Analysis of Variance, which suggests that it may be possible to choose promising lines from the available germplasm. PLU-429, PL-416, and IPU-99-16 genotypes produced the most amount of seeds. For the number of primary branches and harvest index, high PCV, GCV, heritability, and genetic progress were recorded as a percentage of the mean. The largest amount of variability in the set of all variables was accounted for by PC 1, the first of all the factors (PCs) (numbered 1 to 8). With a PIC of 71% and a heterozygosity of 62%, BG-18 had the greatest levels of both. Comparatively to Cluster I, III, and IV, Cluster II and V have the highest number of genotypes. In this work, SSRs with high polymorphism information richness successfully aided in genotype discrimination. The findings of this study indicate that molecular diversity among Blackgram genotypes can be estimated with success using SSR analysis.

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