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Genetic diversity analysis using SSR markers in pearl millet (*Pennisetum glaucum*) inbred lines

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Abstract
The extent of genetic variability for grain yield and its attributing traits in forty inbred lines were evaluated at Bajra Section (G&PB), CCSHAU, Hisar. Significant differences were revealed by the ANOVA among the evaluated inbreds for every parameter studied. DNA was extracted for molecular marker analysis. Sixteen polymorphic markers were identified which were dispersed throughout the pearl millet genome. A total of 88 alleles were detected, collectively yielding SSR profiles for forty inbred lines. The average number of SSR alleles per locus was 5.5 with a range of 2 (ICMP 2084 and XCVMP 0018) to 16 (PSMP 2233). PIC values of various SSR loci across the 40 inbred lines ranged from 0.16 (ICMP 3088) to 0.94 (PSM 2233) with an average of 0.59 locus. It is significant to note that 6 out of 16 SSR loci namely PSMP 2233, PSMP 2008, PSMP 2263, ICMP 3050, PSMP 2274 and ICMP 3017 revealed PIC values above 0.70, can be considered highly useful for differentiation of Pearl millet inbred lines. The UPGMA cluster tree analysis led to the grouping of 40 inbred lines in eight major clusters. Clustering pattern revealed that cluster 3 and cluster 7 were the largest consisting of 10 inbred lines each. The inbred line H78/711 and TCP-10-110 grouped in cluster I and PT-1-10-1002 grouped in cluster II were similar on morphological characterization and molecular characterization leading to confirmation of these genotypes in same group.

Keywords: Pearl millet, diversity, molecular markers, millets, crop improvement

Introduction
Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is staple food crop for people living in dry regions of arid and semi-arid tropics. It is well-adapted to nutrient-depleted soil and low rainfall conditions, yet it is capable of grow quickly and vigorously in the right conditions. Pearl millet, an annual diploid, is a highly cross pollinated C_4 monocot species of the family *Poaceae*. It serves as an important forage and food crop in arid and semi-arid Indian and African regions covering more than 26 million ha area. In India, during the year 2020-21, pearl millet was grown in an area of 7.65 million ha with production and productivity of 10.86 million tons and 1420 kg/ha, respectively. In Haryana, area covered by crop was 5.69 lakh ha with production and productivity of 13.5 lakh tons and 2372 kg/ha, respectively (Anonymous, 2021) [2]. It contains starch, fat, protein, calcium, iron, and zinc content and serves as the staple food for poor families. Pearl millet is a requisite source of fodder in many regions of the world. Green fodder from pearl millet is a cherished feed for livestock. Being gluten free, it is famed among the people suffering from gluten allergy (Saini *et al.*, 2022) [10]. It is a rich source of energy (361 Kcal/100 g) which is more than sorghum and just about equivalent to that of brown rice, because of the lipid content which is generally higher *i.e.*, 3 to 6%. It contains antioxidants which can prove to be beneficial for the overall health and wellbeing. (Nambiar *et al.*, 2011) [7]. Pearl millet is a crop normally grown in an area with low and erratic rainfall (200-600 mm), high temperature, high salinity or low pH and impoverished infertile soils. It is tolerant to harsh growing conditions and hence it can be grown in areas where other cereal crops, such as wheat, maize or rice would not survive.
Before starting any breeding or hybridization programme it is important to learn about the yield and its contributing traits. Grain yield and minerals contents are complex attributes that are influenced by the nearby environment and are governed by multiple genes (Owere *et al.*, 2015) [9]. An effective approach for identifying genetic variation in pearl millet genotypes is molecular marker technology. In contrast to morphological features, molecular markers may identify significant genotypic variations at the DNA level, making them a more effective, direct, and reliable tool for managing and conserving germplasm that is unaffected by the

environment. SSR markers can detect a high level of allelic diversity, and they have been extensively used to identify genetic variation among pearl millet genotypes (Gupta *et al.* 2011) [4]. Many SSR markers have been reported to be linked to many biotic, abiotic and yield attributing traits in pearl millet, although several investigations have researched pearl millet germplasm characterization and diversity analysis, variability studies of the common landraces and cultivars grown are limited. Therefore, the study was conducted to assess the genetic diversity of elite inbred lines using molecular markers in pearl millet genotypes.

Material and Methods

Experimental Location and site

The field experiment was conducted in research area of Bajra Section, Department of Genetics and Plant Breeding, CCS Haryana Agricultural University, Hisar. It is situated in semi-arid sub-tropical region at global geographical position between 29.09°N and 75.43°E with elevation of 215.52 meter above mean sea level with sandy-loam soil. Forty pearl millet germplasm lines were taken as experimental material, collected from CCSHAU, Hisar. The present investigation was carried out by taking 40 genotypes (elite inbred lines) of pearl millet including some checks namely HMS 81B, ICMB 843-22, H77/833-2-202 and HBL 11. The material was evaluated in randomized block design with three replications during *Kharif 2015.* Each plot comprised of two rows of 4 m length with row-to-row distance 45 cm and plant to plant distance of 10-12 cm. All recommended package of practices were adopted to raise a good crop.

Isolation of genomic DNA

Genomic DNA was isolated from 2–3-week-old seedlings of 40 pearl millet genotypes (elite inbred lines) by using CTAB (Cetyl Trimethyl Ammonium Bromide) extraction method given by Doyle and Doyle (1987) [3]. The quality of genomic DNA was observed on 0.8% agarose gel and the concentration and purity of DNA was measured at 260 nm and 280 nm by using nanodrop spectrophotometer. The DNA samples were diluted to a working concentration of 100 ng/µl with nuclease free water and used for the amplifications of Simple Sequence Repeat (SSR) markers in Polymerase Chain Reaction (PCR).

Selection of SSR markers

A total of 40 SSR markers widely distributed on different pearl millet linkage groups were used for studying molecular polymorphism in 40 pearl millet genotypes. All these primers were custom synthesized from Sigma Chemicals Co. USA.

PCR amplification with SSR markers

PCR amplification reaction was carried out in Applied Biosystem's thermalcycler. The optimized PCR reaction with a total volume of 20 µl contained DNA template 100 ng, 10X PCR buffer 2.0 µl, $MgCl_2$ (50 mM) 0.6 µl, dNTPs mix (10 µM) 0.5 µl, Forward primer (10 µM) 0.4 µl, Reverse primer (10 µM) 0.4 µl, *Taq* DNA Polymerase (5 U/µl) 0.3 µl. The PCR reaction was set up in thin walled 0.2 ml PCR tubes in Applied Biosystem's thermalcycler.

PCR amplification was performed with initial denaturation at 94 °C for 5 minutes followed by 10 cycles of denaturation at 94 °C for 25sec, annealing at 64 °C – 54 °C (touch-down cycles with -1 ºC per min/cycle) for 20sec, and extension at 72 °C for 30 sec, followed by 40 cycles of denaturation at 94 °C for 25 sec, annealing at 56 °C for 20 sec and extention at 72 °C for 30 sec, followed by final extension at 72 °C for 20 min. The amplified products were stored at -20 ºC and were resolved on 2.5% agarose gels at 100 V for 2 h and observed under a UV Gel documentation system.

Scoring of alleles and genetic diversity analysis

From SSR amplification profiles, the size of most intensified amplified band of each marker was determined based on electrophoretic mobility relative to molecular weight of 100 bp ladder (Sigma Chemicals Co. USA). Amplified products from microsatellite analysis were scored visually for presence (taken as 1) and absence (taken as 0) of each marker allele and a binary matrix was generated. The binary data was used to calculate similarity genetic distance using JMP 8.0 software, SAS Institute Inc. Dendrogram was constructed by using distance matrix by the unweighted pair group method using arithmetic averages (UPGMA) of JMP 8.0 Software. The polymorphic information content (PIC) value of marker was calculated using the formula given by Anderson *et al.*, (1993) [1]

$$\mathrm{PIC}i = 1 - \sum_{j=1}^{k} P^2 ij$$

Where, Pij is the frequency of the j^{th} allele for I^{th} marker and summation extends over the alleles.

Results and Discussion

Genetic diversity among pearl millet inbred lines using SSR markers

In the present study, out of 40 SSR markers, only16 markers were found polymorphic and were dispersed throughout the pearl millet genome (Table 1.). A total of 88 alleles were detected, collectively yielding SSR profiles for all the forty inbred lines. Summarized data for the number of alleles detected per SSR locus analyzed as well as polymorphism information content (PIC) values for each of the SSR loci are presented in Table 1. The average number of alleles per locus was 5.5, with a range from 2 (ICMP2084 and XCUMP0018) to 16 (PSMP2233). PIC values of 16 polymorphic SSR loci across all the 40 inbred lines ranged from 0.16 (ICMP3088) to 0.94 (PSMP2233) with an average of 0.59 per locus. 6 out of 16 SSR loci, namely PSMP2233, PSMP2008, PSMP2263, ICMP3050, PSMP2274 and ICMP3017 possessed PIC values above 0.70, can be considered highly significant and useful for differentiation of pearl millet inbred lines. Out of 40 primers, 16 polymorphic primers (Table 4.29) were found, which produced a total of 88 alleles. The number of alleles detected per primer pair ranged from 2 to 16 with an average of 5.5 per primer. PIC value was highest for the SSR primer *PSMP2233* (0.94) followed by *PSMP2008* (0.86) and lowest for the primer *ICMP3088* (0.16). The higher the PIC value, the more informative is the SSR marker and hence, primer *PSMP2233* was found to be highly informative. Based on the dendrogram (Figure 1), the forty inbred lines of pearl millet were grouped into eight main clusters (Table 4.30). Among these clusters, clusters 3 and 7 were the largest comprising of ten genotypes each. The first cluster comprised of nine inbred lines followed by four genotypes, ten genotypse, three genotypes, one genotype, one genotype, ten genotypes and

two genotypes in the subsequent clusters. Clustering using SSR markers was also done by Sumanthi *et al.* (2010) [11]; Kapila *et al*. (2007) [6]; Kannan *et al*. (2014) [5] and Nepolean *et al*. (2012) [8].

Table 1: Details of 16 polymorphic SSR markers used in the study of 40 pearl millet genotypes for genetic diversity analysis.

S. No.	Polymorphic primers	Number of alleles	Band size (bp)	Linkage group	PIC values
1	PSMP 2008	10	180-1300	5	0.86
2	PSMP 2229	5	220-320	NA	0.52
3	PSMP 2233	16	200-1200	5	0.94
4	PSMP 2263	8	120-1100	NA	0.83
5	PSMP 2274	8	220-900	NA	0.79
6	ICMP 3017	6	180-1000	1	0.74
7	ICMP 3018	4	140-250	NA	0.58
8	ICMP 2084	2	400-500	NA	0.29
9	ICMP 3050	7	120-1200	NA	0.80
10	ICMP 3088	3	170-300	1	0.16
11	ICMP 10	3	300-600	NA	0.54
12	XCUMP 001	4	150-600	NA	0.38
13	XCUMP 006	3	120-500	NA	0.52
14	XCUMP 0016	3	200-300	NA	0.57
15	XCUMP 0018	2	250-500	NA	0.29
16	CTM 27	4	300-330	1	0.59

NA= not available

Cluster analysis of pearl millet inbred lines

The cluster analysis led to the grouping of 40 pearl millet inbred lines in eight major clusters (Figure 1). These eight clusters grouped all the 40 lines in such a way that inbred lines within each cluster had high similarity than those in other clusters (Table 2). Cluster 3 and cluster 7 were the largest each consisting of 10 inbred lines. This was followed by cluster 1 (9 inbred lines), cluster 2 (4 inbred lines), cluster 4 (3 inbred lines), cluster 8 (2 inbred lines), cluster 5 (1 inbred line) and cluster 6 (1 inbred line).

The inbred lines H78/711 and TCP-10-110 grouped in cluster 1 and inbred line PT- 1 -10-1002 grouped in cluster 2 were similar based on morphological characterization (DUS criteria and yield attributes) and molecular characterization (based on UPGMA software) leading to confirmation of these genotypes in same group. A more conclusive pattern may be established by using more number of markers across all linkage groups and generation of phenotypic data by taking more number of plants in each replication. The study reveals that enough variation is present in the inbred lines at both genotypic and phenotypic levels. Similar consonances were earlier reported by Kannan *et al*. (2014) [5] and Nepolean *et al*. (2012) [8] in pearl millet.

Table 2: Distribution of forty pearl millet inbred lines in different clusters based on SSR marker analysis.

Cluster	Inbred lines	Number of inbred lines
Cluster1	TCP-10-110, HTP03/13/901-78, HTP92/80, PT1-10-1021, IH8, H78/711, SGP10-120, HPT-1-12-84, H12/009	9
Cluster 2	S97/120, SGP10-111, H12/011, ICMB843-22	4
Cluster 3	ISK51, 99HS-139, HFePPT2/12-141, HFePPT2/12-152, HTP93/109-1, G73/107, PT-1-10-1002, HPT-10-129, RAJ3, TPC-1	10
Cluster 4	H77/833-2-202, HBL11, HPT-2/12-06	3
Cluster 5	HPT-2-12-62	1
Cluster 6	HPT-1-12-44	1
Cluster 7	PT-2-10-173, AC-04/13, SGRLT14/106, LPRLT-14/104, A5RLT14/109, PT-1-10-1047, HFeT-3-11-125, EMRLT14-116, HMS81B	10
Cluster8	HPT-10-144, TCH-26-1	2

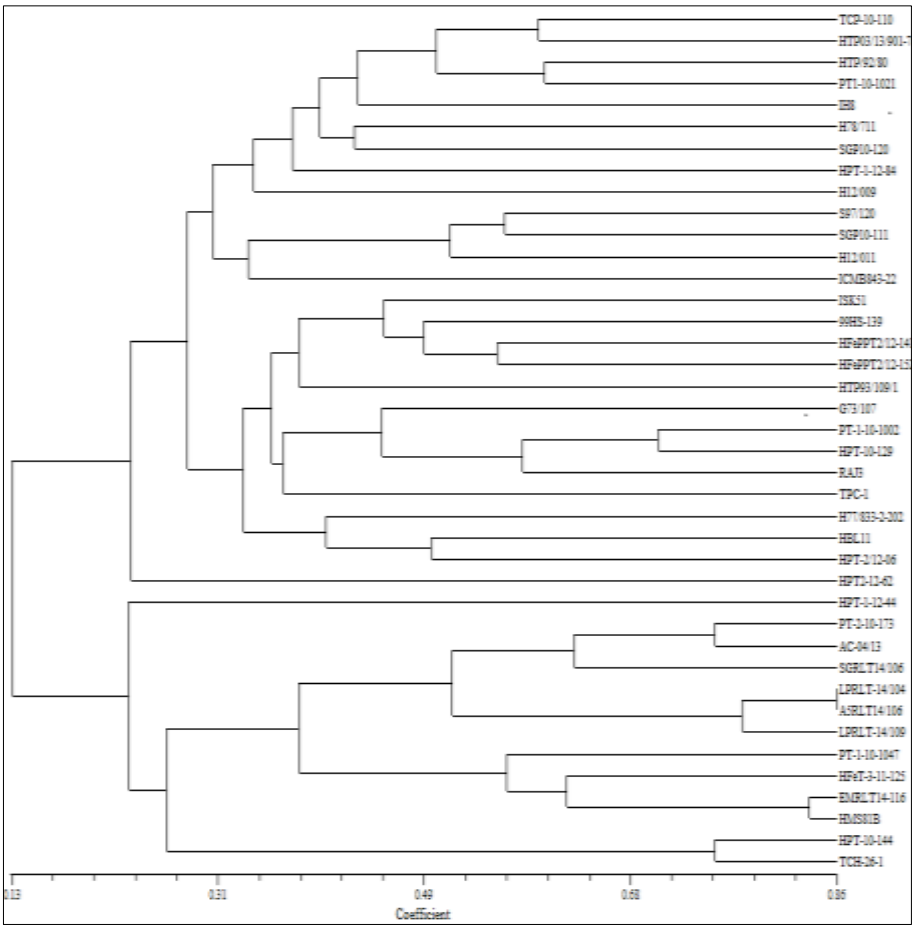

Fig 1: Dendrogram showing the clustering pattern of forty inbred lines of pearl millet based on genotyping by SSR markers.

Conclusion

The present study illustrated the existence of wide range of variations for most of the characters among the pearl millet inbred lines and opportunities of the genetic gain through selection or hybridization. However, grain yield being a complex trait, it is pertinent to have more detailed analysis on the genetic basis at the molecular level involving the present inbred lines. The present study also demonstrates that SSRs are effective markers for the assessment of genetic diversity in inbred lines of pearl millet. The study reveals that the number of alleles detected for a SSR marker can be a good indicator to access PIC/diversity and selection of the markers based on higher repeat number will be more efficient for genetic diversity studies. Further, the diversity assessed can be manipulated to broaden the genetic base of inbred lines for the development of commercial hybrid varieties. This study shows that the inbred lines developed at CCS HAU over years have sufficient diversity at phenotypic and genotypic levels.

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