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Quantifying Genetic Diversity Based on Morphological and Molecular Analysis in Pearl millet [*Pennisetum glaucum* (L.) R. Br.] Inbred Lines

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Abstract: The experiment was carried out with thirty pearl millet inbred lines at Research Farm of ICAR-AICRP on Pearl Millet, Project Coordinating Unit, Mandor-Jodhpur during kharif 2020 in randomized block design (RBD) with three replications. Morphological and molecular diversity was assessed in 30 pearl millet inbred lines by Mahalanobis D^2 analysis and Simple Sequence Repeats (SSR) molecular marker, respectively. Total five clusters were formed from D^2 analysis and seven clusters were generated from SSR markers data analysis. The character *viz.* panicle girth, number of effective tillers per plant and 1000 seed weight contributed maximum to morphological diversity. The clustering pattern based on SSR markers data revealed that genotypes at molecular level had high diversity as compared to that seen at phenotypic level. There was significant differences among the clustering pattern of D^2 and SSR molecular marker analysis. The reason may be environmental influence on phenotypic level. This information can be used as selection criteria to have hybridization between diverse parents to exploit heterosis in pearl millet breeding program.

Key words: Pearl millet, Mahalonobis D^2 analysis, SSR markers

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is the sixth most important cereal in the world, and the fourth most important cereal crop of tropical region, after rice, corn, and sorghum. The global acreage under pearl millet is approximately 30 to 32 million hectares (Anonymous, 2022; Deevi *et al*., 2024). Global production is around 32 million metric tons of which 42% share is from India, 11% from Niger, 8% from China, 6% Nigeria and Sudan 5%, West Ethiopia, Senegal, Bukaria Faso and Chad contribute less than 4%. In India, maximum share is of Rajasthan 39% followed by 17% from Uttar Pradesh, 10% from Gujarat & Madhya Pradesh and 8% from Haryana (Anonymous, 2024). Climate resilience in pearl millet is crucial due to the challenges posed by climate variability, such as

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increasing temperatures, frequent droughts, and unpredictable rainfall. To combat these challenges, breeding programs in India have focused on developing cultivars with traits that enhance resilience to such climatic stresses. Techniques like marker-assisted selection, genomic selection, and participatory breeding are utilized to identify and incorporate climate-resilient traits. These efforts are aimed at producing pearl millet cultivars that can withstand adverse conditions, ensuring food security, nutritional quality, and improved livelihoods for smallholder farmers in the face of climate change (Tara *et al*., 2024).

In hybrid breeding of pearl millet, assessing the diversity of inbred lines is essential for developing superior and stable hybrids, particularly for regions with low-fertility soils and erratic rainfall, where crops like sorghum and maize often fail (Harinarayana *et al*., 1999). The diversity of inbred lines provides the foundation for selecting parental lines that can contribute desirable traits such as high yield, drought tolerance, and disease resistance to the hybrid offspring. Phenotypic diversity offers valuable insights into these observable traits, while molecular diversity, particularly through DNA markers, gives a more precise understanding of the underlying genetic structure and relationships among the lines. Integrating both phenotypic and molecular data in assessing inbred lines enables breeders to make informed decisions, maximizing heterosis and improving the efficiency of hybrid breeding programs. This approach ensures that the resulting hybrids are not only high-performing but also resilient across diverse environments, contributing to sustainable agriculture and food security. Moreover, genetic diversity is fundamental to breeding success; quantifying genetic divergence among germplasm collections and understanding their contributions to different traits provide critical information for yield improvement. Molecular genetic testing is more reliable than phenotypic testing, which can be influenced by unknown genetic mechanisms and environmental factors. Therefore, studying polymorphism at the DNA level, the primary source of all biological information, is essential for accurate diversity estimation. In this study, genetic diversity among inbred lines of pearl millet was analyzed using D^2 statistics and SSR markers.

Materials and Methods

Morphological diversity

The experiment was carried out at Research Farm of ICAR-AICRP on Pearl Millet, Project Coordinating Unit, Mandor-Jodhpur during kharif-2020 in randomized block design (RBD) with three replications. Thirty pearl millet parental lines were grown in plot having row length 4 m and 0.6 m spacing between the rows. Observations were recorded for twelve morphological traits. Days to 50% flowering and days to maturity were recorded on plot basis, while remaining characters were recorded randomly by selecting five plants of each parental line from each replication. Recorded data in each replication was then averaged and mean values were subjected to statistical analysis. The experimental data was analyzed by the method of analysis of variance suggested by Panse and Sukhatme (1985). Multivariate analysis was done by Mahalanobis D2 statistic (Mahalanobis, 1936) and genotypes were grouped into different clusters following Tocher's method suggested by Rao, 1952.

Molecular diversity

SSR marker analysis was conducted to assess the genetic relationships among the pearl millet lines. DNA was extracted from 3-4 healthy, fresh leaves of 12-day-old seedlings using the CTAB (Cetyl Trimethyl Ammonium Bromide) method, as outlined by Murray and Thompson (1980). The DNA was then quantified through agarose gel electrophoresis. Sixty-seven SSR primers were selected from the pearl millet database for this analysis. PCR amplification was performed in 10 µl reaction mixtures containing 1.0 µl of pure DNA, 1.0 µl of 10X buffer, 0.5 µl of dNTPs, 2.0 µl of primers (forward and reverse), 0.2 µl of Taq DNA polymerase, and 5.3 µl of double-distilled water. The PCR process was carried out in a 96-well thermal cycler (Agilent Technologies) under the following conditions: initial denaturation at 94°C for 3 minutes, followed by 35 cycles of 94°C for 1 minute, annealing at 53°C for 50 seconds, extension at 72°C for 2 minutes, and a final extension at 72°C for 1 minute. The resulting PCR products were combined with 6X gel loading dye and electrophoresed on

Table 1. Analysis of variance (ANOVA) for twelve traits studied in pearl millet inbreds

Source	DF	Days to 50% flower-ing	Days to maturity	Leaf area	Flag leaf area	Plant height	Panicle length	Panicle girth	Stem girth	Number of productive tillers	1000 - grain weight	Dry fodder yield / plant	Grain yield/ plant
Replication	2	13.43	20.84	3.34	18.54	287.66	0.17	0.03	0.013	0.08	0.40	15.43	18.30
Treatment	29	74.83*	57.79*	2655.92*	1183.30*	1317.61*	15.33*	0.70*	0.07*	1.63*	3.16*	856.11*	219.41*
Error	58	6.74	10.83	115.91	76.90	116.12	0.94	0.014	0.004	0.08	0.47	51.39	8.71

* represent significant at 5% level

a 3.5% (w/v) agarose gel in 1X TAE buffer. Samples were loaded alongside a 50 bp DNA ladder standard. After electrophoresis, the gel was scanned under UV light using a gel documentation system. The allelic data obtained were analyzed using NTSYS-pc 2.02 software to construct a dendrogram employing the Unweighted Pair-Group Method with Arithmetic Average (UPGMA) algorithm.

Results and Discussion

Analysis of variance (Table 1) depicted significant variation for all the 12 traits studied in the set of pearl millet inbred lines. Contribution of 12 traits to total phenotypic diversity (Table 2) ranged from 0.23 (Days to 50% flowering) to 29.89 (panicle girth). Other than panicle girth, effective tillers (17.93%), dry fodder yield (16.09%) and grain yield (14.48) contributed high to total diversity. All the 30 pearl millet genotypes were grouped into five clusters based on D^2 values by following Tocher's method. Cluster pattern revealed that cluster I and II have most of genotypes, while cluster III, IV and V have solitary genotypes J-2565, PPMI-732 and ICMB 02333. Cluster I have maximum number (22) genotype and cluster II have 5 genotypes (Table 4).

In SSR marker analysis, out of 67 markers, 47 markers were polymorphic which amplified 156 alleles ranging from 2 to 8 with a mean value of 3.32 alleles per locus (Table 3). A comparable value reported by Singh *et al*., 2012. Polymorphic Information Content (PIC) ranged from 0.153 to 0.828 with a mean value 0.396. A comparatively high value was reported by Ambawat *et al.* (2020) which were ranged from 0.33 to 0.76 with mean of 0.55. This suggested that a significant variability was reported by SSR markers. A dendrogram was constructed by using the UPGMA cluster tree analysis based on similarity coefficient which led to the grouping of 30 parental lines in to seven major clusters. Earlier also, different clustering pattern was reported by Ponnaiah *et al.* (2019) observed four and three distinct clusters of pollinator and seed parent. The maximum number of genotypes clustered under cluster A (18) followed by cluster D (4), cluster G (3), cluster B (2) and cluster C, E and F have solitary genotypes (Table 4).

Table 2. Per cent contribution of different characters toward total diversity

Characters	Contribution (%)	Time ranked
Days to 50% flowering	0.23	1
Days to maturity	-	-
Leaf area	6.21	27
Flag leaf area	1.15	5
Plant height	4.14	18
Panicle length	5.29	23
Panicle girth	29.89	130
Stem girth	1.84	8
Effective number of tillers per plant	17.93	78
Dry fodder yield per plant	16.09	70
1000 grain weight	2.76	12
Grain yield per plant	14.48	63

Comparison of morphological and molecular diversity

Dendrograms developed on the basis of 12 morphological traits and 47 polymorphic SSR markers data as shown in (Fig. 1) and (Fig. 2). The clustering pattern at SSR analysis revealed that genotypes at molecular level had high diversity as compared to that at phenotypic level. Twelve genotypes (MIR 606, MIR 710, MIR 916, MIR 920-1, MIR 1261, MIR 1255, MIR 1259, H 77/833-2, H 77/29-2, G 73-107, RIB 102-108-S/19, RIB 109-120-S/19) were clustered together in both D^2 and SSR marker analysis in cluster I/A. The genotypes MIR 714, MIR 901, MIR 612 and MIR 705-1 also grouped together but in SSR analysis it

Table 3. Polymorphism pattern observed for SSR primers studied in pearl millet inbreds

S.No.	Primers	No. of Alleles	Range	PIC Value	S.No.	Primers	No. of Alleles	Range	PIC Value
1	PGIRD43	4	75-90	0.640	25	ICMP3056	2	140-150	0.185
2	PGIRD46	2	50-90	0.456	26	ICMP3078	3	50-250	0.461
3	PGIRD49	5	200-700	0.589	27	ICMP3088	3	150-450	0.580
4	PGIRD56	3	140-325	0.571	28	ICMP3091	7	75-600	0.642
5	IPES004	2	110-115	0.426	29	ICMP3093	3	75-250	0.620
6	IPES008	2	350-700	0.493	30	ICMP4006	3	250-300	0.554
7	IPES009	5	60-375	0.655	31	ICMP4010	3	200-350	0.609
8	IPES0010	2	50-70	0.358	32	XCTM08	4	50-250	0.564
9	IPES0011	3	75-240	0.550	33	XCTM09	2	70-90	0.346
10	IPES0014	2	200-600	0.375	34	XCTM27	4	60-400	0.476
11	IPES0019	2	50-110	0.499	35	XCTM21	3	175-400	0.625
12	IPES0020	3	75-450	0.364	36	XCTM25	2	75-80	0.500
13	IPES0022	6	75-300	0.771	37	XCTM26	2	350-400	0.500
14	IPES0029	7	75-600	0.828	38	ICMP3033	2	70-200	0.480
15	IPES0036	2	145-150	0.172	39	ICMP3022	2	70-200	0.444
16	IPES0040	3	75-500	0.623	40	ICMP3029	2	60-350	0.165
17	IPES0043	8	120-700	0.783	41	IPES0197	2	50-300	0.302
18	IPES0044	5	125-750	0.565	42	IPES0103	2	300-690	0.480
19	IPES0047	2	95-100	0.153	43	IPES0226	5	210-440	0.710
20	XICMP3080	7	70-800	0.790	44	IPES0101	6	60-700	0.411
21	XICMP3086	5	100-600	0.689	45	Xcump 002	3	60-100	0.432
22	ICMP3017	2	200-500	0.278	46	Xcump 005	2	50-70	0.480
23	ICMP3018	2	140-240	0.494	47	Xcump 017	3	50-125	0.169
24	ICMP3019	2	210-250	0.500		Total	156	Average	0.396

clustered separately under cluster D but in D^2 analysis they clustered in cluster I. A similar pattern was observed with MIR 915 and MIR 1106, however PPMI 732 showed concordance in both the phenotypic and molecular analysis. It is clear from the both analyses (D^2 and SSR analysis) that, there is adequate amount of diversity among the 30 pearl millet inbred lines studied. But significant difference among the grouping pattern of genotypes based on D^2

Table 4. Cluster formed from Mahalonobis D^2 analysis and SSR markers analysis

D^2 analysis		SSR marker analysis	
Clusters	Genotypes	Clusters	Genotypes
I	MIR 606, H 77/833-2, MIR 714, MIR 710, MIR 901, MIR 920-1, MIR 1261, MIR 1259, MIR 1255, MIR 612, MIR 915, H 77/29-2, MIR 916, MIR 1106, ICMB 97444, ICMB 95222, RIB-102-108-S/19, G 73-107, RIB-109-120-S/19, RIB-121-127-S/19, MIR 705-1, ICMB 88004	A	MIR 606, PPMI 1267, RIB-102-108-S/19, H 77/29-2, ICMB 92777, MIR 1255, MIR 1259, H 77/833-2, MIR 710, MIR 1261, MIR 916, PPMI 1239, J 2565, RIB-109-120-S/19, G 73-107, J2290, J2691and ICMB 88004
II	PPMI 1267, J 2290, ICMB 92777, J 2591, PPMI 1239	B	MIR 915 and MIR 1106
III	J 2565	C	PPMI 732
IV	PPMI 732	D	MIR 612, MIR 901, MIR 714 and MIR 705-1
V	ICMB 02333	E	MIR 920-1
		F	RIB-121-127-S/19
		G	ICMB 95222, ICMB 97444 and ICMB 02333

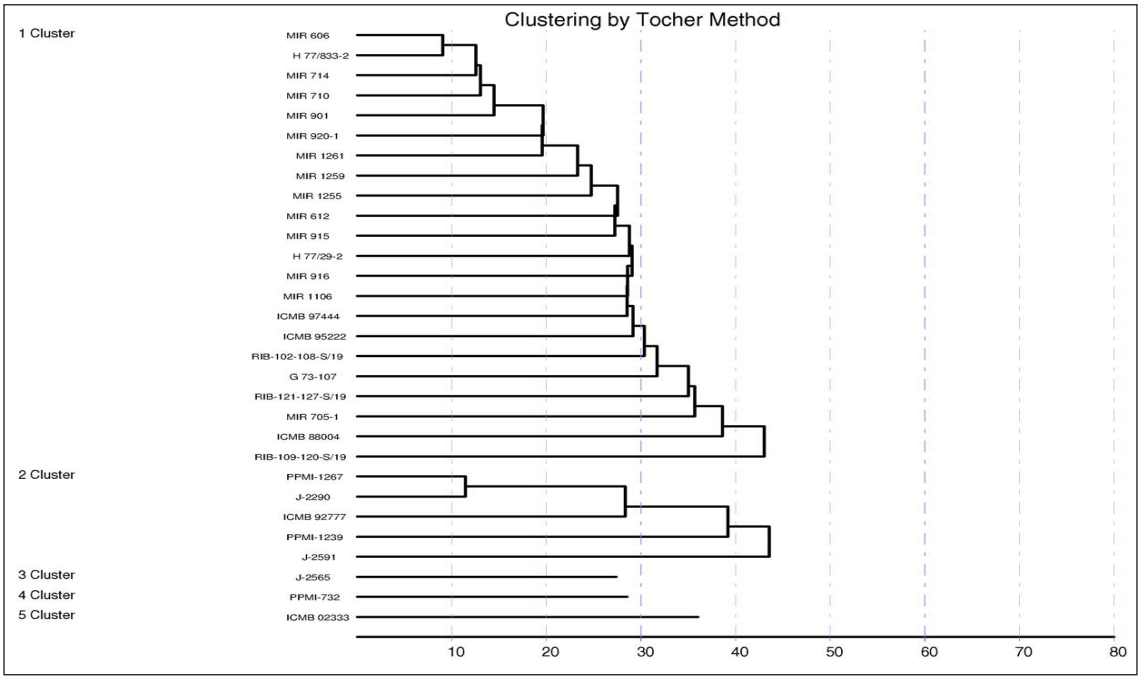

Fig. 1. Dendrogram generated from D^2 analysis of 30 inbred lines of pearl millet.

analysis and molecular marker analysis. The experimental results were in agreement with the finding of several authors in different crops (Sunder *et al.,* 2014 on cotton, Yadav *et al.,* 2015 on barley, Murthy *et al*., 2017 on cotton and Poker *et al*., 2019 on cowpea).

Conclusion

In this study, a comparison between the clustering patterns obtained from Mahalanobis D^2 statistics and SSR markers revealed differences in the number of clusters and the distribution of inbred lines within each cluster. These variations may be attributed to environmental influences on the phenotypic traits used for Mahalanobis D^2 clustering. The findings suggest that while morphological data, based on visual observations of plant groups, provide valuable insights, SSR marker analysis offers a more reliable method for

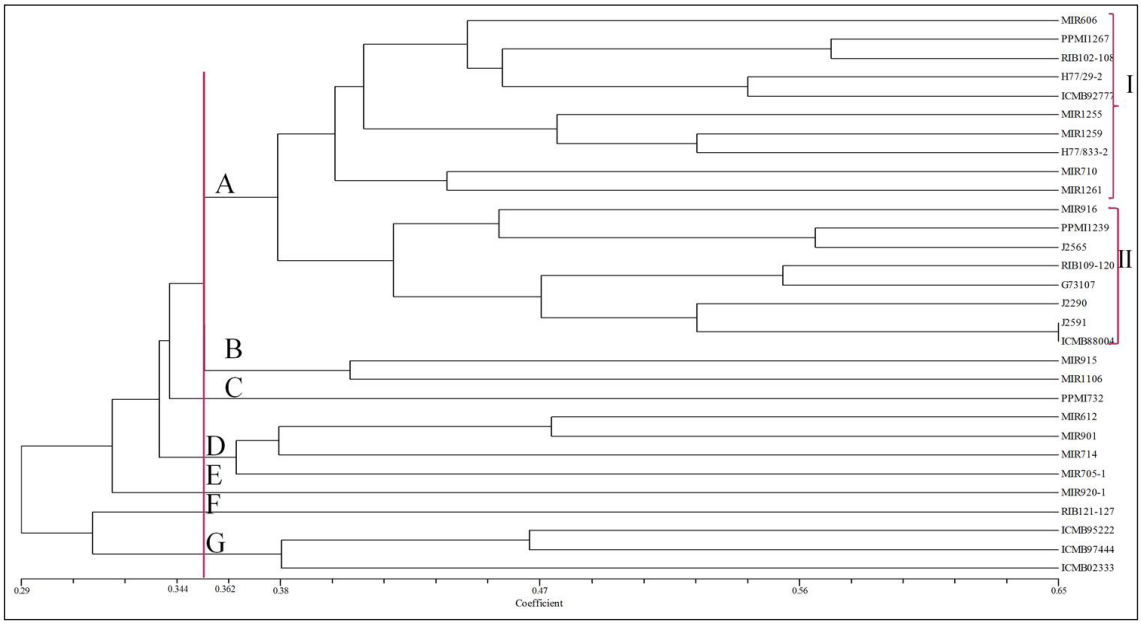

Fig. 2. UPGMA dendrogram generated based on SSR data of 30 inbred lines of pearl millet.

estimating genetic similarities among pearl millet inbred lines. This combined information is particularly valuable for breeders involved in hybrid breeding, as it aids in the selection of genetically diverse and complementary parental lines, ultimately enhancing the effectiveness of breeding programs. The approach can be especially useful when working with large numbers of pearl millet inbred lines, ensuring more informed decisions in the development of high-performing and resilient hybrids.

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