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RESEARCH ARTICLE

SSR Markers based Diversity Analysis in Elite Genotypes of Pearl Millet

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Abstract

Analysis of genetic diversity was carried out using 32 genotypes of pearl millet using SSR markers and phenotypic data of 10 morphological traits. Significant differences were observed for all the traits recorded in the study, indicating the availability of sufficient genetic variability in the materials. Among the 69 simple sequence repeat (SSR) primers screened, 44 primers were found polymorphic. These polymorphic primers amplified 151 alleles. The number of alleles per SSR marker varied from 2 to 5 per locus. Polymorphism information content values (PIC) ranged from 0.058 to 0.608 per locus with an average of 0.375. Genotypes were grouped into three clusters based on molecular and phenotypic data, and the genotypes' distribution was similar in both. Principal coordinate analysis further corroborated the grouping of genotypes obtained on phenotypic and molecular data. Structure analysis was also observed to be in line with cluster grouping as well as principal coordinate analyses.

Keywords: A_4 maintainers and restorers, Dendrogram, Diversity, *Pennisetum glaucum*, Polymorphism information content, Simple sequence repeats.

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Introduction

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is the staple food of the majority of the poor and small landholders. It is also used as feed and fodder for livestock in the rain-fed regions of the country. It requires less input and matures in a short duration. Pearl millet is a highly nutritious cereal with high levels of energy and protein and a more balanced amino acid profile (Andrews and Kumar, 1992). It can play an important role not only in contributing to the food and nutritional security of the poor in the pearl millet growing areas of India and sub-Saharan Africa but could also have potential health value for the affluent (Rai *et al.,* 2012; Jorben *et al*., 2020). Recognizing its value, the Government of India has declared the year 2018 as the 'National Year of Millets' and UN also declared 2023 as the 'International Year of Millets'. It is a drought-tolerant warm-season cereal grown in dry land agriculture on more than 27 million ha in some of the harshest environments in Africa's arid and semi-arid tropical regions (17 million ha) and Asia (10 million ha). In these regions, pearl millet is a staple food of more than 90 million people. In India, pearl millet is the third most widely cultivated food crop after rice and wheat. The area of pearl millet in India was estimated to be 6.93 million hectares, with an average production of 8.61 million tons and productivity of 1391 kg/ha (Directorate of Millets Development, 2020).

Many researchers (Lakshmana *et al.,* 2009; Sharma, 2016) estimated the diversity based on phenotypic traits in pearl millet. But, these data are more influenced by the environment

and other factors and are considered less reliable. Hence, molecular markers have emerged as a more reliable tool for studying the genetic diversity of genotypes. The study of morphological markers and polymerase chain reaction (PCR) based co-dominant markers like simple sequence repeats (SSRs) are often considered to be one of the most suitable and reliable markers in applied breeding programs. These markers have been extensively utilized to assess the extent of genetic diversity among various crops such as sorghum, maize, wheat, barley and cotton (Chandra *et al.,* 2014; *Gupta et al*., 2015). Molecular diversity analysis in pearl millet has been studied often in the recent genomics era (Nepolean *et al.,* 2012; Satyavathi *et al.,* 2013; Chandra *et al*., 2014; Bashir *et al*., 2015; Gupta *et al.,* 2018). Most of these studies have relied on morphological or molecular interpretation. Some studies in other crops (Wang *et al*., 2006; Hegay *et al.,* 2014) are also available, wherein morphological and molecular data have been jointly studied. However, information on the estimation of genetic diversity using both morphological and molecular markers is limited in pearl millet. The present study attempts to classify the elite maintainers and restorers belonging to A_4 cytoplasm using morphological and molecular markers.

Materials and Methods

For the assessment of genetic diversity, six seed parents (B-Lines)/maintainers and 26 A_4 restorers (R-Lines) were obtained from ICRISAT, Patancheru, India. Details of these parental lines are given in Table 1. All 32 genotypes were sown at ICAR-Indian Agricultural Research Institute, New Delhi, and evaluated in randomized complete block design with three replications. In the case of phenotypic evaluation, observations were recorded using standard methods on ten agro-morphological traits, which ultimately contributed to the plant yield. Observations on each genotype were recorded for yield and related characters on five randomly selected competitive plants from each replication, except for days to 50% flowering, days to maturity and grain yield, which were recorded on plot basis. The average values were used for statistical analysis.

DNA was isolated from young expanding leaves of 32 genotypes using the modified CTAB method (Saghai Maroof *et al*., 1984) with suitable modification. About 69 SSR primers developed for pearl millet were selected in such a way as to have 8 to 10 primers from each of the seven chromosomes (Rajaram *et al.,* 2013). The utility of a marker for detecting genetic variation was estimated using power marker software as reported by Liu and Muse, 2005). It includes a) polymorphism information content (PIC), which is equal to 1- ΣPij^2 where Pij is the frequency of j^{th} allele at i^{th} locus summed across all alleles in the locus, b) observed heterozygosity c) genetic diversity and d) allelic richness which means the number of alleles per primer.

A simple matching allele frequency-based distance matrix was used in the DARwin-6.0 program (Perrier *et al*., 2003) to construct a tree diagram to examine the genetic structure and diversity among maintainer and restorer lines using molecular marker scores. The grouping into clusters and sub-clusters was done at 5% dissimilarity level with a bootstrap analysis of 1000 times for statistically supporting the branches of the cluster. Similarly, using morphological data, pair-wise genetic distance comparisons of genotypes were calculated by Euclidean distance coefficients (Bakonyi and Johnson, 1995) using SAS 9.3 program (SAS Institute, Cary NC). Based on an average linkage algorithm (UPGMA, unweighted pair group method with arithmetic average), clustering of genotypes was done to depict the similarity or dissimilarity among groups or individual genotypes.

Bayesian clustering model was also performed using STRUCTURE software version 2.3.4 (Pritchard *et al*., 2000). A continuous series of K were tested from 1 to 10 in 10 independent runs. The initial burn-in period for each run was set to 1,50,000, followed by 1,50,000 Markov Chain Monte Carlo (MCMC) iterations. The online available program, STRUCTURE HARVESTR (Earl and von Holdt, 2012) was used to calculate the optimum k value based on an ad hoc quantity ΔK proposed by Evanno *et al.,* 2005 based on second order rate of change of the likelihood function with respect to K estimated to get the final population structure.

Results and Discussion

Assessment of Phenotypic Diversity

The mean, range, and coefficient of variation of 10 phenotypic traits recorded on 32 genotypes were estimated. Significant differences were observed for all the 10 traits recorded, indicating the existence of sufficient genetic variability in the material (Figure 1). Information on the genotypic coefficient of variation, phenotypic coefficient of variation, heritability in a broad sense and genetic advance were also obtained. The genotypic coefficient of variation varied from 4.29 to 47.12. The range of phenotypic coefficient of variation was 4.33 to 47.32. High heritability estimates were observed for all the traits except the number of productive tillers per plant. The highest heritability (99.17%) was observed for blast score and the lowest for the number of productive tillers per plant (67.05%). In the same way, the highest genetic advance was observed for blast score and it was lowest for days to maturity. Similar results for phenotypic diversity were also reported by Shanmuganathan *et al*., 2006, Lakshmana *et al*., 2009 and Sharma, 2016. The clustering based on Euclidean distance distributed the genotypes in three different clusters at 1.0 Euclidean coefficient. On the basis of morphological data, most diverse genotype was ICMB 03999, placed in cluster III alone. The distribution of genotypes was not found in

Table 1: Details of genotypes used for morphological and molecular characterization

S. No.	*Genotype*	*Parentage/pedigree*
1	ICMB 99111	(843B x ICTP 8202-161-5)-17-1-2-B-1
2	ICMB 99444	(SPF3/S91-327 x SPF3/S91-5)-6-2-2
3	ICMB 03999	ICMB 89111 x IP 9402-2-1-1-2)-31-1-B-B
4	ICMB 04111	(81B x 4017-5-4-B)-12-3-1-3
5	ICMB 05888	(SRC II C3 S1-1-1-2 x HHVBC)-5-1-1-2
6	ICMB13222	[(ICMB 95111 x 9035/S92-B-3)-17-5-1-B-B-B x ICMB 99111]-3-2-1-3
7	ICMR 06111	MC 94 C2-S1-3-1-3-3-2-2-B
8	ICMR 06222	SDMV 90031-S1-3-3-2-1-3-2-2-1-B
9	ICMR 06333	SDMV 90031-S1-93-3-1-1-3-2-2-1-1-B
10	ICMR 06444	[((MC 94 S1-34-1-B × HHVBC)-16-2-1) × (IP 19626-4-2-3)]-B-37-1-1-1-2-B
11	ICMR 07222	MRC HS-130-6-1-1-B-B-B-B-B-B
12	ICMR 07555	ICMS 8511 S1-17-2-1-1-4-1-B-3-2-2-B
13	ICMR 08888	ICMS 7704 S1-52-3-1-2-1-2-1-6-B-B
14	ICMR 08999	JBV 3 S1-18-2-2-1-3-2
15	ICMR 09666	[(((IP 12322-1-2) x B-Lines)-B-14) x (MRC S1-156-2-1-B)]-B-1-3-3-B-B
16	ICMR 09777	JBV 3 S1-35-2-1-2-B
17	ICMR 09888	[((MC 94 S1-34-1-B x HHVBC)-16-2-1) x (IP 19626-4-2-3)]-B-34-1-3-3-1-1-B-2
18	ICMR 10888	ICMV 93074-S1-9-1-1-1-3-B-B-B-B
19	ICMR 12222	JBV-3-S1-33-2-1-3-3-B-3-B-1-B
20	ICMR 12333	(E 298 x LCSN 282-4-1-2)-12-2-1-2-B-B-B-1
21	ICMR 12444	(ICMS 7704-S1-127-5-1 × RCB-2 Tall)-B-19-3-2-1-1-1-B
22	ICMR 12555	(MC 94 C2-S1-3-2-2-2-1-3-B-B x AIMP 92901 S1-488-2-1-1-4-B-B)-B-28-1-1
23	ICMR 13444	(IAC-ISC TCP6 S1-9-1-2-B-4-2-B x AIMP 92901-S1-488-2-1-1-4-B-B)-B-11-3-1-B
24	ICMR 13555	GB 8735-S1-15-3-1-1-3-4-2-2-1-1-B-B
25	ICMR 13666	ICMS 8511-S1-17-2-1-1-4-1-B-3-2-3-2-B-1-1-B
26	ICMR 13999	MDMRRC S1-329-1
27	A_4RT-13-2/DPR 7	PPMI 2354 x PPMI 112/PMMI 217-89-52-2-2
28	A_4RT-15-13	(MC 94 C2-S1-3-2-2-2-1-3-B-B x AIMP 92901 S1-488-2-1-1-4-B-B)-B-28-1-1-B
29	A_4RT13-3/DPR 8	P1977x P85/PPMI 69-2310-256-5-3-1
30	A_4RT-13-5	(ICMS 7704-S1-127-5-1 ∀ RCB-2 Tall)-B-19-3-3-1-2-1
31	A_4RT-15-10	(IPC 337 x SDMV 90031-S1-84-1-1-1-1)-12-5-2-4-B-B-B
32	A_4RT-15-12	(MC 94 C2-S1-3-2-2-2-1-3-B-B x AIMP 92901 S1-488-2-1-1-4-B-B)-B-23-4-4-2

accordance with pedigree and source/origin of genotypes. It may be because environmental conditions highly influence morphological traits.

Yadav *et al.* (1994) also did not find accordance between phenotypic diversity and pedigree/origin of genotype.

Informativeness of SSR Markers

Total 69 SSR loci were analyzed to differentiate among 26 restorers and 6 maintainers of A_4 cytoplasm. About 44 SSR markers were found to be polymorphic and data derived from these polymorphic SSR markers was used for further statistical analysis, which gave amplicons in the range of 130 to 340 bp. Total number of alleles observed were 151 and the number of alleles per locus varied from 2 to 5 with an average number of 3.43 alleles per locus. The gels for visualizing the amplification products of two primers are shown in Figure 2. PIC ranged from 0.058 to 0.608 per locus with an average PIC of 0.375. PIC calculated was highest for four SSR primers *viz*. PSMP 2081 (0.61), PSMP 2237 (0.59), IPES 191 (0.58) and IPES 233 (0.58) and lowest for the primer IPES160 and IPES175 (0.06) (Table 2). Seven out of the 44 SSR markers revealed heterozygosity in different inbreds. The seven SSR primers *viz.,* IPES 200, IPES 225, IPES 236, IPES 198, IPES 208, PSMP 2089 and PSMP 2235 have detected heterozygosity. The observed heterozygosity per primer ranged from 0 to 0.81. PIC values observed in the study were comparable with those reported by Satyavathi *et al.,* 2009, Sumanth *et al.,* 2013, Chandra *et al*., 2014 and Kifouli *et al*., 2017. The number of alleles detected per marker and the gene diversity of markers depend on the number of genotypes analyzed, which were comparably less in the present study.

Assessment of SSR Marker-based Genetic Diversity

Simple matching dissimilarity coefficients were calculated using SSR primer amplification scoring data generated in 32 genotypes using Darwin 6. Accordingly, these genotypes were placed in three clusters at 5% dissimilarity coefficient

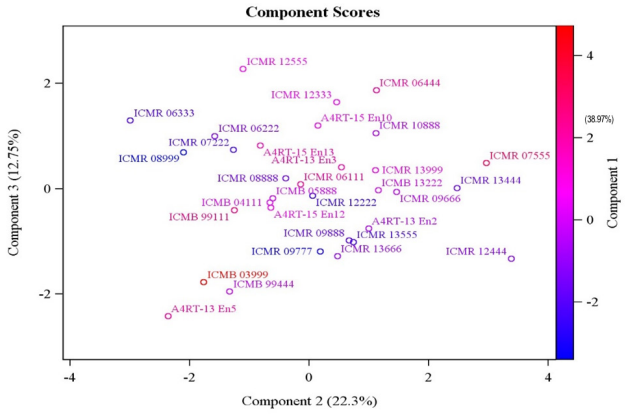

Figure 1: PCA plot based on phenotypic diversity analysis of 32 genotypes using 10 morphological traits

Table 2: List of SSR primers, their allelic size range, allelic richness, major allele frequency, gene diversity, PIC and observed heterozygosity

S. No.	*SSR loci*	*Allelic richness*	*Allele size range*	*Major allele freq.*	*Gene diversity*	*Observed hetero zygosity*	*PIC*
1	IPES230	3	10 (170–180)	0.84	0.28	0	0.26
2	IPES076	4	30 (200–230)	0.78	0.37	0	0.35
3	IPES210	4	20 (170–190)	0.81	0.33	0	0.31
4	IPES017	3	10 (140–150)	0.72	0.43	0	0.38
5	IPES144	3	20 (140–160)	0.84	0.27	0	0.25
6	IPES219	3	20 (130–150)	0.91	0.17	0	0.17
7	IPES174	3	20 (230–250)	0.88	0.23	0	0.21
8	IPES200	3	20 (170–190)	0.52	0.53	0.03	0.42
9	IPES225	3	10 (250–260)	0.81	0.32	0.06	0.30
10	IPES185	4	20 (150–170)	0.69	0.47	0	0.42
11	IPES166	4	20 (210–230)	0.75	0.42	0	0.39
12	IPES206	3	10 (230 –240)	0.84	0.28	0	0.26
13	IPES087	3	10 (220–230)	0.63	0.53	0	0.47
14	IPES089	4	20 (310–330)	0.69	0.48	0	0.44
15	IPES236	4	20 (210–230)	0.45	0.6	0.22	0.51
16	IPES198	4	20 (300–320)	0.52	0.63	0.09	0.57
17	IPES195	3	10 (180–190)	0.56	0.52	0	0.41
18	IPES151	4	20 (170–190)	0.88	0.23	0	0.22
19	IPES213	4	20 (220–240)	0.75	0.41	0	0.39
20	IPES127	4	20 (270–290)	0.63	0.55	0	0.50
21	IPES208	2	10 (280–290)	0.64	0.46	0.03	0.35
22	IPES175	2	10 (270–280)	0.97	0.06	0	0.06
23	IPES095	4	20 (200–220)	0.88	0.23	0	0.22
24	IPES118	3	10 (180–190)	0.63	0.52	0	0.45
25	IPES229	2	10 (180–190)	0.81	0.3	0	0.26
26	IPES154	2	10 (300–310)	0.84	0.26	0	0.23
27	IPES233	4	20 (230–250)	0.53	0.63	0	0.58
28	IPES191	4	20 (260–280)	0.56	0.62	0	0.58
29	IPES186	4	30 (270–300)	0.69	0.48	0	0.44
30	IPES160	2	10 (150–160)	0.97	0.06	0	0.06
31	IPES093	4	110 (40–150)	0.75	0.4	0	0.36
32	IPES052	4	20 (260–280)	0.44	0.64	0	0.57
33	IPES019	5	30 (250–280)	0.56	0.61	0	0.56
34	PSMP2081	4	40 (180–220)	0.41	0.67	0	0.61
35	PSMP2089	3	10 (270–280)	0.8	0.33	0.03	0.30
36	PSMP2077	5	30 (150–180)	0.59	0.59	0	0.54
37	PSMP2229	4	20 (230–250)	0.84	0.28	0	0.26
38	PSMP2235	3	10 (140–150)	0.53	0.55	0.81	0.45
39	PSMP2246	4	30 (150–180)	0.69	0.49	0	0.45
40	PSMP2237	4	20 (270–290)	0.53	0.64	0	0.59
41	PSMP2090	4	20 (150–170)	0.72	0.45	0	0.41
42	PSMP2030	2	10 (170–180)	0.84	0.26	0	0.23
43	PSMP2202	3	20 (170–190)	0.72	0.43	0	0.38
44	PSMP2027	3	20 (250–270)	0.78	0.37	0	0.34
Mean		3.4		0.71	0.42	0.03	0.37

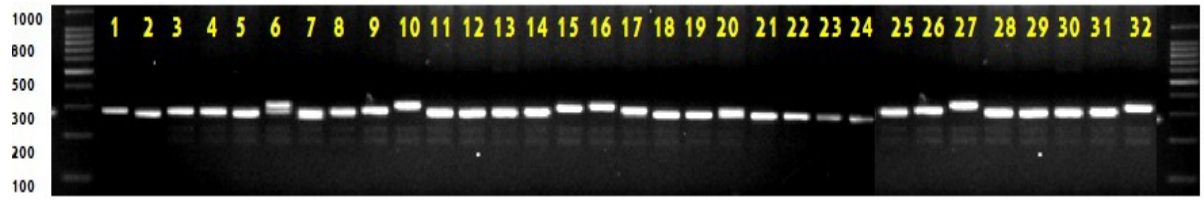

Figure 2: Ethidium bromide-stained DNA amplification profile of 32 genotypes of pearl millet using microsatellite marker PSMP 2237

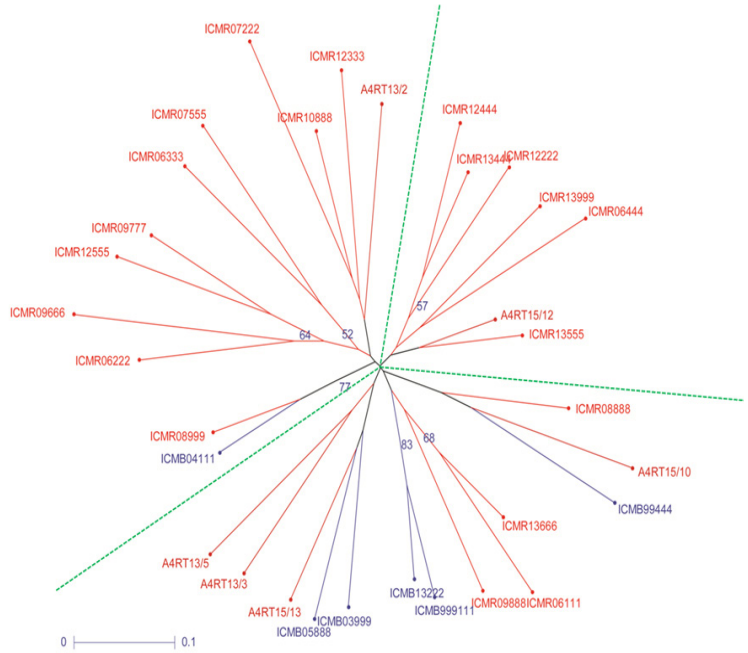

Figure 3: Unweighted neighbor-joining tree based on a simple matching dissimilarity matrix for allele sizes, detected by 44 SSR primers across 32 genotypes (B-lines are shown in blue and R-lines in red color).

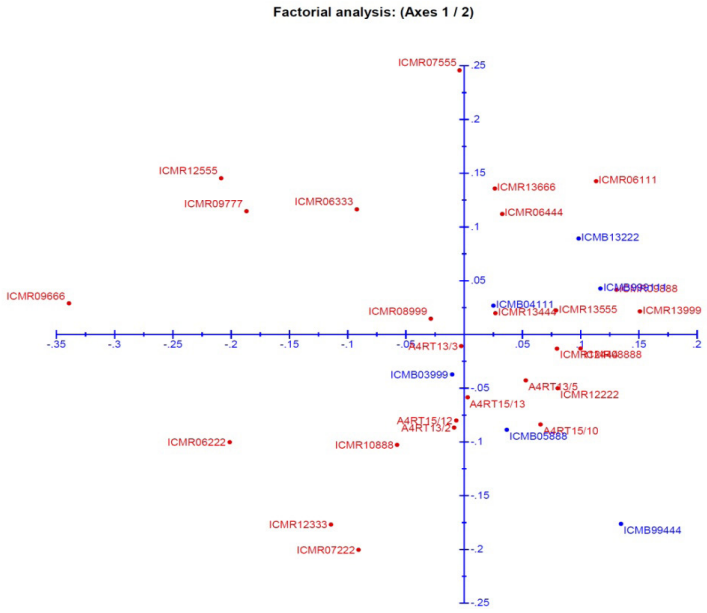

Figure 4: Factorial analysis of 32 genotypes using 44 SSR primers (B-lines are shown in blue and R-lines in red color)

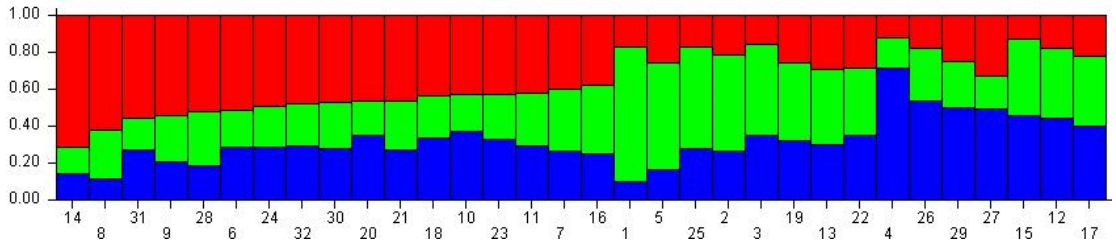

Figure 5: Bar diagram for 32 genotypes arranged based on inferred ancestry at K = 3; Color codes represent the three subpopulations. Values in the left indicate the membership coefficient (Q). Proportions of colors in each bar indicate the allelic affiliation of the sub-populations

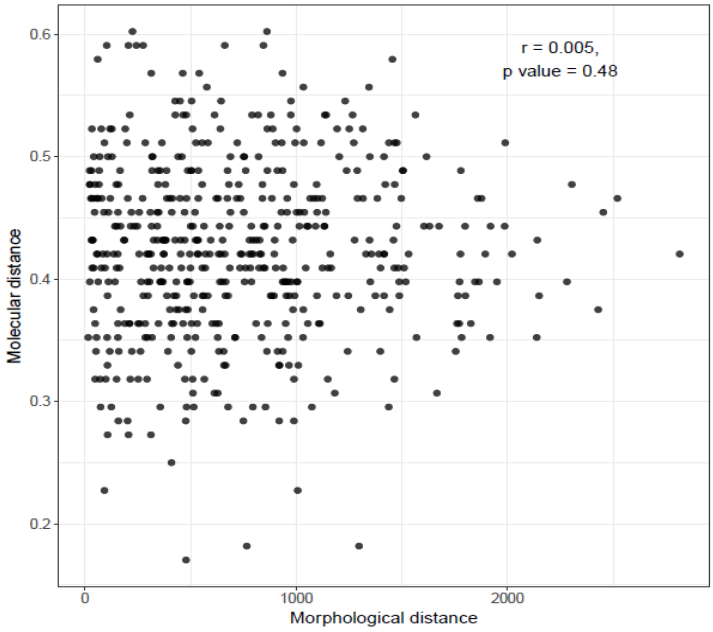

Figure 6: Correlation between morphological and molecular genetic distance matrices values (X Label: Euclidian similarity distance & Y Label: Simple matching dissimilarity distance

(Figure 3). On the basis of molecular grouping, in cluster I, five maintainers viz., ICMB 99444, ICMB13222, ICMB 99111, ICMB 03999 and ICMB 05888 were placed with eight restorers namely A4RT15/13, ICMR 08888, ICMR 06111, ICMR 13666, ICMR 09888, A4RT15/10, A4RT13/5, and A4RT13/3. This indicates these maintainers and restorers are genetically more similar and hybrid developed using female parents of these maintainers and restorers may not result in a heterotic combination. One maintainer (ICMB04111) was placed in cluster II along with 11 restorers (ICMR07555, A4RT13/2, ICMR06333, ICMR08999, ICMR10888, ICMR07222, ICMR12333, ICMR09666, ICMR06222, ICMR09777 and ICMR12555). This again indicates a similarity between this maintainer and these 11 restorers. These eleven restorers can be crossed with 5 maintainers placed in cluster I. Similarly, restorers available in cluster I can be crossed with a female parent of the maintainer placed in cluster II. Seven restorers, namely ICMR13444, ICMR06444, ICMR13999, ICMR13555, ICMR12444, ICMR12222, and A4RT15/12, which were placed in the third and last cluster, can be crossed with a female parent of maintainers available in cluster II and cluster II. This analysis indicates that genetic diversification of parental lines should be given priority for realizing maximum heterosis.

Based on molecular data, the dissimilarity was again subjected to factorial analysis to explore and establish similarity or dissimilarity coefficient matrices among groups or individual genotypes. The same trend of genotype distribution was observed as shown in a dendrogram. There were only six restorer lines, which were not grouped with any maintainers. These lines are ICMR 06333, ICMR 07555, ICMR 08999, ICMR 09666, ICMR 09777 and ICMR 12555. A cross between these lines and the female parent of maintainers included in this study may result into a heterotic one

Principal Coordinate Analysis

Using same similarity coefficient, principal coordinate analysis was performed. The total genetic variation existing in the material was divided into principal components. Principal components 1, 2, and 3 explained the 73.84% total variability used to plot the genotypes (Figure 4). Based on

colors and position, the same trend in the distribution of genotypes was observed as depicted in the dendrogram.

The dissimilarity coefficient matrices were again subjected to factorial analysis to explore and establish similarity or dissimilarity among groups or individual genotypes. The same trend of genotype distribution was observed as shown in the dendrogram. Clustering pattern as well as PCoA formed three groups which broadly clustered the majority of B- and R-lines into two separate clear-cut groups with minor exceptions, as shown by AMOVA also, indicating that B- and R-lines exist as two distinct broad-based gene pools.

Structure Analysis

Population structure refers to all genetic patterns of individuals within a population. In fact, the genetic structure in a natural population is characterized by the number of possible subpopulations within it, the frequencies of different alleles in each subpopulation, and the degree of genetic isolation of the subpopulations. The population structure of the 32 genotypes was analyzed based on generated polymorphism data of each marker system and pooled data using STRUCTURE analysis for K = 1 to K = 10. In all the analyses, the results obtained by STRUCURE HARVESTER indicated that maximum ΔK was reached at K = 3 (Figure 5). At this K, all accessions were assigned to three main subpopulations. The inferred population structure for K = 3 indicated that most of the accessions had a membership coefficient (qi) to one of the subpopulations equal or higher than 0.6 (qi = 0.6). These results further confirmed the PCoA and cluster analysis.

Correlation between Phenotypic and SSR-based Genetic Distance Matrix

Mantel's correlation test statistic (Z) was applied to determine the relationship between molecular marker-based and phenotypic marker-based distances. This test indicated a non-significant association between phenotypic and molecular-based analysis. The overlapping trend of dots was not observed, indicating low correlation between two types of matrices. The present study revealed a low correlation between phenotypic traits and SSR-based matrices, indicating that the two methods were different in assessing genetic diversity. Furthermore, the simple correlation between both the genetic distance matrices was also low (r = 0.005) (Figure 6). This indicates that both morphological and molecular data should be utilized in the estimation of genetic diversity in pearl millet. Despite the low correlation, many genotypes were placed together in the same clusters in both the dendrograms.

Low association between phenotypic and molecular diversity may be due to fewer morphological traits (10 traits) and fewer polymorphic markers (44 only), which may also be from non-genic regions. Morphological traits are also highly influenced by environmental factors and may be a cause of low association between morphological and molecular diversity analysis. As in the present study, weak correlation between phenotypic data and molecular data was reported by Vinu *et al*., 2016 and Karuri *et al*., 2009 in mustard and sweet potato. The low association indicated that molecular markers (SSR) did not adequately sample the relevant genomic regions for phenotypic differentiation. In contrast to the present study, Rahman *et al*., 2011, and Hegay *et al*., 2014 reported a significant association between phenotypic and molecular diversity in rice and common bean. The co-phenetic correlation can be further improved by considering the trait-linked molecular markers, particularly from genic regions such as SNPs.

Conclusion

Fundamental of any crop improvement program is existence of genetic diversity among parental lines. Exploitation of genetic diversity will result into the development of hybrids and varieties with high yield potential. In current study, high genetic diversity was observed among 32 restorers and maintainers of A4 cytoplasm using morphological and molecular data. Placement of maintainers and restorers into different clusters indicates that they are highly diverse and may be used for generating hybrids and transgressive segregants with higher yield potential.

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