



Morphological and molecular genetic diversity analysis of pearl millet (*Pennisetum glaucum*) maintainers and restorers

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ABSTRACT

The success of any pearl millet hybrid programme depends upon the assessment of the structure of parental diversity. In this investigation, polymorphism data generated using 42 simple sequence repeat (SSR) markers and 12 agro-morphological traits were used for genetic diversity assessment among 32 genotypes (29 restorers and 3 maintainers) of pearl millet [*Pennisetum glaucum* (L.) R. Br.]. In contrast to morphological data, SSR based clustering pattern and Principal Coordinate Analysis revealed that the genotypes were found to be consistent with their pedigree and origin. Polymorphism information content (PIC) values based on microsatellites ranged from 0.117 to 0.841 per locus with an average PIC of 0.348. Allelic richness varied from two to five per locus. The highest similarity index (0.74) was observed between restorers WGI 58 and WGI 148, whereas the lowest similarity index (0.27) was observed between seed parent 841 B and PPMI 269. AMOVA analysis observed more genetic variance among the individuals than within individuals. The study led to the identification of maintainer 841 B as a genetically diverse parent from other maintainers as well as among most of the restorers and hence may be used in future hybrid development programmes. Further, the correlation between phenotypic and genetic distance matrices was observed to be very low. Appropriate heterotic combinations were identified based on morphological and molecular diversity.

Key words: Diversity, Dendrogram, Maintainers, Restorers, Simple Sequence Repeat

Millets are the small-seeded grasses which include sorghum, pearl millet, ragi, small millet, proso millet, barnyard millet, kodo millet, foxtail millet, etc. The world's millet production was estimated at 31.01 million tonnes in year 2018 (FAOSTAT). Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is highly nutritious cereal and largely grown under rainfed conditions in India (~8 m ha) and Africa (~18 m ha) (Yadav and Rai 2013). Since the 1960s, pearl millet improvement programmes in India are mainly based on the development of hybrids that currently occupy more than 50% area of total pearl millet acreage. The importance of hybrids in pearl millet in India can be cited from the fact that hybrid adoption led to the improvement of about 300% in crop productivity since 1951 (Yadav *et al.* 2015). Superior hybrids can be developed based on heterosis prediction provided with parental lines of diverse heterotic groups on basis of morphological as well as molecular diversity. Genetic diversity assessment can serve as important criteria

for systematic classification of contrasting parental materials into diverse groups. The diverse parental lines can be further used to enhance heterozygosity or to optimize the genetic heterogeneity in a hybrid population and hence helping to achieve yield stability in changing climatic scenario. Thus, this necessitates the genetic diversity assessment among the available genotypes. Several workers estimated the diversity based on morphological data in pearl millet. However, mostly the morphological data were found to be inadequate in providing reliable information for the calculation of genetic distance and may not correlate well with pedigrees in most cases especially in open-pollinated crops like pearl millet. On contrary to morphological markers, PCR-based co-dominant simple sequence repeats (SSRs) are often considered as one of the most suitable and reliable markers in applied breeding programs and these markers have been extensively employed to assess the extent of genetic diversity in various crop species (Choudhary *et al.* 2016, Bashir *et al.* 2015). With advent of abundant SSR markers, they have been routinely used for genetic diversity assessment in pearl millet (Stich *et al.* 2010, Nepolean *et al.* 2012, Gupta *et al.* 2018). Thus, most of the workers assessed diversity based on either morphological data or molecular data but only a few efforts have been made to

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estimate genetic diversity utilizing morphological as well as molecular data and correlation between them. The present investigation is an attempt to study the genetic diversity at the morphological and molecular level, find their relative effectiveness as well as the establishment of a relationship between them.

MATERIALS AND METHODS

The experimental material consisted of 32 pearl millet genotypes (29 fertility restorers and 3 maintainers). Of these, 21 restorers and two maintainers (411 B and 576 B) were developed at ICAR- Indian Agricultural Research Institute, New Delhi. Six restorers and one maintainer (841 B) from ICRISAT, Patancheru, Telangana and two restorers from CCSHAU, Hisar were also included in the study (Table 1).

A total of 32 genotypes were planted in the sandy loam soils in *kharif* season, 2013 in randomized block design with three replications, at ICAR-Indian Agricultural Research Institute, New Delhi, India. Each genotype was planted in a three-row plot of three meter length with a spacing of 75 × 10 cm (Row × Plant) and a standard package of practices were followed to raise a good crop. For phenotypic evaluation, observations were recorded using standard methods on 12 agro-morphological traits that ultimately contribute to plant yield. Agro-morphological observations on each genotype were recorded on five randomly selected competitive plants from each replication for all traits, except for days to 50% flowering, days to maturity, biological yield and grain yield which were recorded on a plot basis.

DNA from 32 genotypes was isolated from young leaves using the CTAB method with suitable modification. A total number of 70 SSR markers covering the whole genome were used to study DNA polymorphism among the genotypes. However, scoring was done for only 42 markers that exhibited polymorphism (Table 3). SSRs were mainly derived from expressed sequence tags and genomic DNA. The amplification reaction was carried out in 15 µl reaction volume containing 20 ng of total genomic DNA (2 µl), 10 µM of primers (2.0µl), 1.0 µl of reaction buffer, 0.2 µl of Taq DNA polymerase (2u/µl) and 10 mM of each dNTP (DNA polymerization mix). PCR amplification was programmed for 38 cycles after an initial denaturation cycle for 3 min at 94°C. Each cycle consisted of a denaturation step at 94°C for 1 min, an annealing step at 54.4-60.6 °C for 1 min, and an extension step at 72°C for 1 min, following by extension cycle for 10 min at 72°C in the final cycle. The amplified fragments were resolved on 3% metaphor agarose. Polymorphism information content (PIC), number

of alleles per locus, observed heterozygosity and probability of identity were calculated for SSR giving polymorphism among the genotypes. Using phenotypic traits that exhibited significant variation among the genotypes, clustering of genotypes was done with Euclidean distance coefficients based on linkage algorithm UPGMA using NTSYS-pc 2.02 programme. Similarly utilizing binary data generated by SSR primers, a Dendrogram was generated with Jaccard's similarity coefficients using NTSYS-pc 2.02. ANOVA and AMOVA analysis were performed for morphological and molecular data respectively using standard procedures. Principal Coordinate Analysis and Mantel's Z statistics were used to establish similarity/dissimilarity among individual genotypes and relationship between them respectively.

RESULTS AND DISCUSSION

Assessment of phenotypic diversity

In the field evaluation trial for assessment of phenotypic diversity, ANOVA (Table 2) revealed the presence of significant variation among the genotypes for all morphological traits except for spike thickness and number of productive tillers per plant. Yield attributing traits like plant height, spike length, weight per panicle, grain yield per panicle and biological yield showed a wide range of variation for trait values. The values of grain yield per panicle ranged from 5.34 to 22.18 g. Such significant variation for grain yield per panicle has also been reported by (Pucher *et al.* 2015, Ramya *et al.* 2017). The clustering based on Euclidean distance scattered the genotypes in four different clusters at 1368.7 Euclidean coefficients. The clustering pattern revealed the clubbing of genotypes across the centers in different four clusters but all three different B lines were categorized in three different clusters (Figure not presented in the manuscript). In phenotypic diversity analysis, genotypes were carefully clustered and compared with the grouping based on phenotypic data, however, the distribution of genotypes was not found as pedigree and origin of genotypes. Similar observations were also reported by Yadav *et al.* (1994) in pearl millet.

Informativeness of SSR Markers

Genotypic data from 42 polymorphic SSR markers covering whole genome were used for statistical analysis which gave amplicons in the range of 98 to 422 bp. The total number of alleles observed was 106 and the number of alleles per locus varied from 2 to 5, with an average number of 2.53 alleles per locus (Table 3). Polymorphism

Table 1 Details of the genotypes used for diversity study in pearl millet

Genotypes	Breeding centre
411B, 576B, DPR 1, DPR 2, DPR 9, PPMI 162, PPMI 214, PPMI 269, PPMI 295, PPMI 479, PPMI 638, PPMI 719, PPMI 720, PPMI 724, PPMI 759, PPMI 760, PPMI 823, PPMI 882, PPMI 893, PPMI 85, TPMP 1213, WGI 58, WGI 148	ICAR-IARI, New Delhi
841B, EGPB 423, IPC 1266, IPC 1518, ICMR 06111, ICMR 06222, ICMR 07999	ICRISAT, Hyderabad
HTTP 94/54, H77/833-2-202	CCSHAU, Hisar

Table 2 Analysis of variance for 12 phenotypic traits

Trait	Replication	Genotypes	ESS	CV
Degrees of freedom (df)	2	31	62	
Plant height (cm)	176.781	1206.88**	70.889	4.652
Spike length(cm)	3.219	55.472**	1.896	6.003
Spike girth (cm)	0.0085	0.609	0.02	5.794
No. of productive tillers/plant	5.323	1.224	0.57	19.593
Days to 50% flowering	1.698	33.418**	1.074	2.067
Days to maturity	3.1355	32.295**	2.533	1.991
Weight per panicle (g)	28.2315	126.582**	3.49	7.383
Grain yield/panicle (g)	15.459	36.807**	2.479	11.793
Biological yield (q/ha)	30.4755	1949.31**	27.976	4.674
Grain yield (q/ha)	7.744	133.273**	2.434	7.233
Harvest Index (%)	90.807	104.047**	13.028	17.304
1000 seed weight (g)	0.089	15.51**	0.246	5.138

** Significant at $P \leq 0.01$; df: Degree of Freedom; C V = Coefficient of variation.

information content (PIC) in the present study ranged from 0.117 to 0.841 per locus with an average PIC of 0.348. PIC calculated was highest for three SSR primers, viz. PSMP 2070 (0.841), PSMP 2273 (0.642) and PSMP 2088 (0.635) and lowest for the primer IPES 52 (0.117). Probability of identity, or the probability that two unrelated individuals would have an identical genotype ranged from 0.155 (PSMP 2070) to 0.888 (IPES 153). Out of the 42 SSR markers used, 30 revealed heterozygosity in different inbreds. The five SSR primers, viz. IPES 0179, IPES 0206, PSMP 2088, PSMP 2070 and PSMP 2090 could identify more than 10 heterozygotes. The observed heterozygosity per primer ranged from 0 to 0.56. The amount of heterozygosity found among the genotypes collected from different centres indicates that greater efforts are needed in maintenance breeding in highly cross-pollinated crops like pearl millet. The number of alleles has a positive correlation with PIC which implies that alleles amplified can be indirectly used to assess PIC in pearl millet (Kapila *et al.* 2008). Although alleles per locus were less than Napoleon *et al.* (2012) and Gupta *et al.* (2018) but comparable with those reported by Sumanth *et al.* (2013). Use of agarose metaphor for genotyping and region-specific inbreds might have resulted in less number of alleles. Both the number of alleles detected per marker and gene diversity of markers depends on the number of genotypes analyzed (Burstin *et al.* 2001) that was comparably less in the present study.

AMOVA was generated using genotyping data from 42 microsatellite loci for 3 B-lines and 29 R-lines. Genetic variation among individuals for B- and R- lines (67%) was significantly higher than the variance between B- and R-lines (2%)(Table 4). AMOVA analysis revealed more genetic variance among individuals than within individuals which conform with the results presented earlier (Ramya *et al.* 2018). In AMOVA to assess the variability among, within suggested-groups and individuals, variability among groups was low due to high fixation index and small group size.

Assessment of SSR marker-based diversity: Based on SSR data, 32 genotypes were placed in four different clusters at 50% Jaccard's similarity coefficient (Fig 1). Cluster I was further divided into four sub-clusters. Among them, sub-cluster Ia consisted of one maintainer 411 B and nine restorers, viz. PPMI 760, HTP 94/54, TPMP 1213, EGP 423, WGI 58, WGI 148, IPC 1518, PPMI 162 and PPMI 893. Out of nine restorers, two were from ICRISAT and one from CCSHAU, Hisar. Sub-cluster Ib comprised of one maintainer 576 B and 4 restorers, viz. DPR 1, PPMI 295, IPC 1266, PPMI 720. Sub-cluster Ic and Id were having five and four IARI derived elite restorers, viz. PPMI 479, PPMI 269, PPMI 882, PPMI 759, PPMI 823 and PPMI 85, PPMI 683, PPMI 724, PPMI 719, respectively. Cluster II was divided into two sub-clusters, of which sub-cluster IIa comprised two restorers, viz. DPR 2 and H77/833-2-202 from IARI and CCSHAU, Hisar respectively. Sub-cluster IIb comprised two restorers, viz. DPR 9 and PPMI 214 from IARI. The maintainer 841 B developed at ICRISAT alone remained as a solitary individual (outlier) forming cluster III. This implied that 841 B is genetically diverse from other maintainers used this study and hence may be used for the new male sterile line development programme. Cluster IV comprised three elite restorers (ICMR 06111, ICMR 06222 and ICMR 07999) developed at ICRISAT. The molecular diversity-based grouping indicated that lines developed from a common centre were genetically similar to a larger extent, with only a few exceptions. These findings agree with those reported by Yadav *et al.* (2013). Thus, genetic information based on molecular data enables a precise grouping of genotypes that accords well with that of common lineage or origin.

Principal Coordinate Analysis

Principal Coordinate Analysis (PCoA) was done to visualize the similarity or dissimilarity among groups or individual genotypes. The PCoA based on phenotyping data

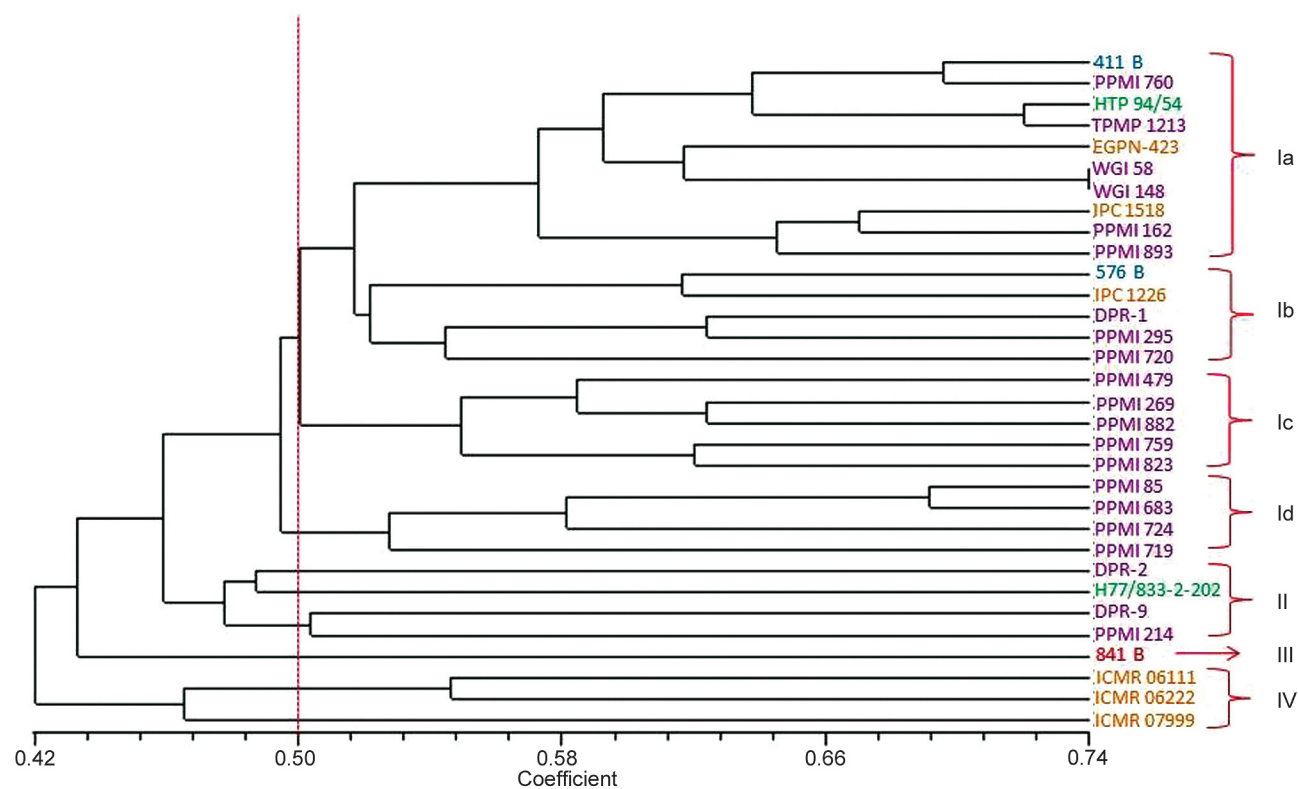


Fig 1 Dendrogram showing Jaccard's dissimilarity produced using UPGMA cluster analysis demonstrating association among 32 genotypes of pearl millet.

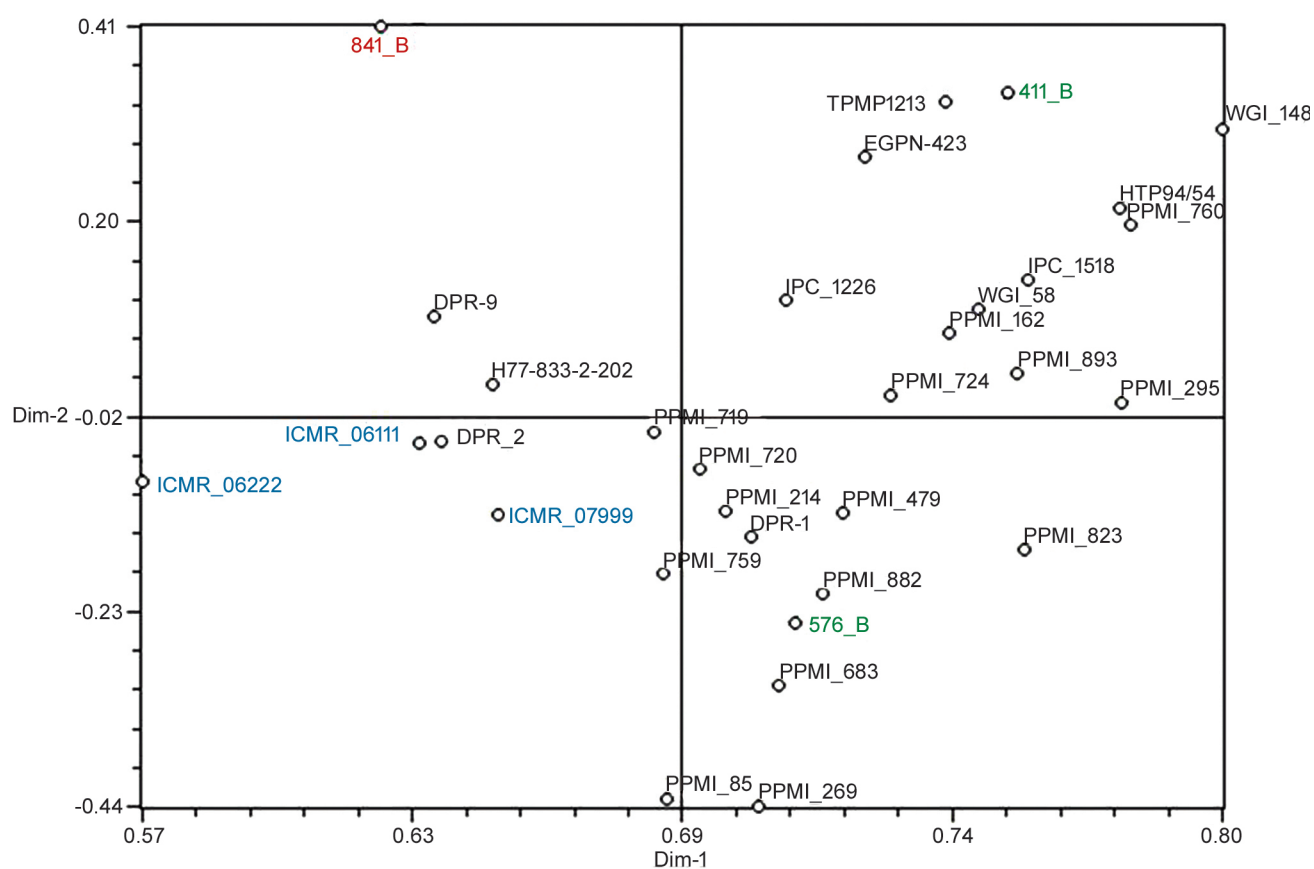


Fig 2 Principal Coordinates Analysis (PCoA) using SSR marker-based similarity coefficient matrix of 32 genotypes.

Table 3 List of SSR primers used with their linkage group (LG), repeat motifs (RM), allelic richness (AR), polymorphic information content (PIC), observed heterozygosity (Obs. He) and the probability of identity (PI)

Primer	Linkage group	Repeat motif	AR	PIC	Obs. He	PI
IPES0017	1	(TA)10	2	0.284	0.031	0.633
IPES0052	6	(TGCA)5	2	0.117	0	0.799
IPES0076	4	(TGGTT)5	2	0.391	0.031	0.6
IPES0087	6	(AGA)7	2	0.492	0.031	0.619
IPES0089	5	(GCT)8	2	0.451	0	0.606
IPES0117	2	(TAGC)5	2	0.341	0	0.608
IPES0126	1	(AGC)8	2	0.218	0	0.682
IPES0144	6	(CTGTG)5	2	0.194	0.031	0.662
IPES0151	6	(TCGA)5	3	0.304	0	0.57
IPES0153	7	(GCGAT)5	2	0.118	0.062	0.888
IPES0179	7	(TGGAC)5	3	0.477	0.437	0.466
IPES0180	3	(TGTAT)4	3	0.629	0.312	0.309
IPES0185	3	(GTTTT)5	3	0.599	0.281	0.41
IPES0193	-	(ATGT)5	2	0.468	0	0.611
IPES0195	7	(ATGG)5	2	0.499	0.062	0.616
IPES0198	7	(AATACC)8	2	0.218	0	0.682
IPES0200	6	(GTAC)11	2	0.451	0.031	0.609
IPES0205	7	(GCGGT)3	2	0.145	0.093	0.841
IPES0206	7	(AGC)6	2	0.323	0.406	0.614
IPES0208	4	(CTC)4TA(CTA)5	2	0.482	0.062	0.616
IPES0210	2	(AT)5	2	0.136	0	0.888
IPES0225	4	(GATC)4	2	0.169	0.093	0.841
IPES0227	6	(GAT)4	3	0.56	0.125	0.49
IPES0230	5	(TGGT)5	2	0.218	0	0.682
IPES0236	2	(TGG)11	2	0.429	0	0.602
PSMP2030	1	(CA)11, (GA)10	3	0.435	0.25	0.484
PSMP2070	3	(CA)25, (TA)6	5	0.841	0.5	0.155
PSMP2089	2	(AC)14 imp.	4	0.503	0.031	0.387
PSMP2090	1	(CT)12	3	0.627	0.562	0.395
PSMP2201	2	(GT)6 imp.	2	0.194	0.031	0.705
PSMP2202	5	(GT)8	2	0.241	0.281	0.662
PSMP2203	7	(GT)18 imp.	3	0.38	0	0.493
PSMP2207	-	(GT)5	2	0.304	0.031	0.594
PSMP2208	5	(GT)10	4	0.327	0.062	0.413
PSMP2209	-	(CT)9	2	0.263	0	0.646
PSMP2229	5	(GT)5	2	0.468	0.031	0.609
PSMP2235	-	(TG)6	3	0.174	0.062	0.706
PSMP2237	2	(GT)8	3	0.539	0.062	0.46
PSMP2249	3	(GT)6	2	0.241	0.281	0.662
PSMP2068	-	(AC)14	3	0.604	0.125	0.384
PSMP2273	1	(GA)12	4	0.642	0.312	0.336
PSMP2088	2	(CA)24	4	0.635	0.5	0.34

Table 4 Analysis of molecular variance (AMOVA) of 3 maintainer lines (B-lines) and 29 restorer lines (R-lines) using 42 SSRs

Source of variation	Degree of freedom	Sum of squares	Mean sum of squares	Estimated variance	Variance percentage (%)	P (9999 per mutations)
Between B and R lines	1	14.824	14.824	0.124	2%	0.219
Among individuals	30	404.207	13.474	5.479	67%	0.000
Individuals	32	80.500	2.516	2.516	31%	0.000
Total	63	499.531		8.119	100%	

was less persuasive than PCoA based on DNA based data, so not presented in this manuscript. Based on SSR marker similarity coefficient matrix, three elite restorers developed by ICRISAT and one from HAU, Hisar were placed in left-hand quadrant while most of the restorers developed by IARI were grouped in right-hand quadrant (Fig 2). Hence, SSR based PCoA revealed that the genotypes were found to be consistent with their pedigree or origin. From a comparison of both PCoA of morphological and molecular data, it was revealed that the variation in morphological traits is more due to environmental influences and hence superfluous and less effective in a proper grouping of genotypes. On the contrary, SSR based data is less influenced by the environment and hence more reliable for grouping of genotypes effectively (Choudhary *et al.* 2016).

The information available on genetic distances between the parental inbreds can be used to predict hybrid performance, as is done in several other crops such as maize (Mohammadi *et al.* 2008) and pearl millet (Gupta *et al.* 2018). However, an earlier study conducted on parental lines of pearl millet hybrids indicated molecular marker-based genetic distance as a non-reliable tool for predicting heterotic combinations (Chowdari *et al.* 1998). This could be due to the use of a limited number of parental lines (only 12 lines). However, in the present study, several pairs of B- and R-lines with wide genetic distance from 0.27 to 0.74 were identified, and hybrids can be generated to investigate the association of genetic distance with hybrid performance. Four clusters of heterotic pools have been found and it signifies that certain combinations among genotypes between these clusters can help in the development of heterotic hybrids. For example, the crossing of restorers namely ICMR 06222, PMMI 85, PMMI 269 with male sterile line 411A; restorers ICMR 06111, ICMR 06222, ICMR 07999 with male sterile line 576 A and restorers PPMI 214, PPMI 269, PMMI 85 with male sterile line 841A are expected to result into superior heterotic combinations. In our study, maintainer 841 B was found to be more discriminative based on both yield and yield attributing traits and marker-based genetic distance. The existence of enormous diversity in maintainer line 841B can be understood by the fact that it has been used as the seed parent in development and release of three hybrid viz. Pusa 23, Pusa 322 and Pusa 605.

Correlation between phenotypic and molecular genetic distance matrices

Mantel test values indicated a non-significant but

positive correlation between phenotypic and SSR marker variation. Furthermore, a very low correlation ($r=0.01$) indicated that the two methods were quite different in assessing genetic diversity. The low correspondence between the similarity matrices as obtained with morphological and molecular data has been reported in other studies also (Handerson *et al.* 2014) in different crops. The low correlation between morphological and SSR based similarity matrices could be due to the fact that a large portion of variation detected by SSRs is non-adaptive and is, therefore, not subject to either natural or artificial selection as compared with phenotypic characters. Mean genetic distance estimate for molecular markers was higher than that of the morphological markers indicating that the SSR marker data set had higher discriminating power compared to the morphological data set. Hence, SSR based diversity assessment more effective in the precise grouping of genotypes especially when pedigree information is not available.

The marker-based analysis implemented in this study identified substantial genetic diversity existing among pearl millet parental lines (maintainer and restorers) developed by different centers. The germplasm lines from opposite heterotic groups can be crossed to develop heterotic populations and hybrid varieties, whereas the germplasm lines from within heterotic groups can be crossed for further synthesis and derivation of new inbred lines. Phenotypic traits and molecular markers analysis showed that molecular marker-based distance was not strongly correlated with phenotype-based distance, a conclusion that invites further investigation with a higher number of markers evenly distributed across all linkage groups. Detection of heterozygosity among the genotypes also indicates the importance of maintenance breeding in cross-pollinated crops like pearl millet.

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