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Molecular characterization and selection of elite maintainer and restorer lines using sales appearance score in pearl millet [*Pennisetum glaucum* (L.) R. Br.]

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Abstract
This study explored the genetic diversity within a collection of 94 economically important pearl millet inbreds, encompassing 45 maintainers and 49 restorer lines, using DNA-based markers. A set of 30 polymorphic SSR primer pairs were utilized, yielding 84 alleles, with alleles per locus ranging from 2 to 4 with an average of 2.8 alleles. Employing DARwin's tree and Neighbor Joining method for cluster analysis, all cultivars were effectively categorized into two major groups (restorer and maintainer), further subdivided into subgroups. Noteworthy variations in Polymorphism Information Content (PIC) values were observed among the markers, indicating varying degrees of genetic diversity. Notably, some markers exhibited substantial diversity, whereas others were highly conserved. Genetic similarity estimates ranged from 0.05 to 0.67, averaging 0.28, underscoring ample diversity among maintainer and restorer inbreds for productive breeding efforts. Intriguingly, the study also detected contamination in three analyzed lines, adding an intriguing facet to the investigation.

Keywords: Genetic diversity, SSR, heterotic pool, inbred *per se* score

INTRODUCTION

Pearl millet (*Pennisetum glaucum* (L.) R. Br.) is an important forage and food crop in arid and semiarid regions of India and Africa. Globally, India is the top producer of millets accounting 13.21 MT production covering 9.76 MHa, followed by China mainland (2.7MT), Niger (2.1MT), and Nigeria (1.9MT) (FAOSTAT, 2021). Pearl millet constitutes approximately two-thirds of millet production in India, accounting for 9.62 MMT in the financial year 2021-2022. (Statista, 2022). It is widely cultivated in many Indian states, including Rajasthan, Maharashtra, Gujarat, Uttar Pradesh, Haryana, Karnataka, Madhya Pradesh, Tamil Nadu, and Andhra Pradesh. Adaptability to diverse environmental conditions has made it a preferred crop in areas where other crops, such as maize and wheat, would not survive (Kalagare *et al.,* 2021). Pearl millet is an affordable protein, iron, and zinc energy source. Given its exceptionally high nutritional value, particularly in micronutrients, pearl millet can significantly improve millions' nutritional status (Govindaraj *et al.,* 2022). Fungal infections, including downy mildew, rust, smut, blast, and ergot, are considered to have a more severe impact on pearl millet production and yield than any other plant disease-causing pathogen. Blast (*Pyricularia grisea*), and rust (*Pucciniasubstriata* var. indica) are also common fungal diseases that affect pearl millet fodder and grain production (Shivhare and Lata, 2017). Over the last two decades, quantum jumps in pearl millet production in India have been obtained due to the development of highly diverse genetically diverse hybrids (Sharma *et al.,* 2020).

The genetic improvement of crop species mainly depends on the extent of variability in their gene pool for economically important traits. Therefore, estimating genetic diversity, and identifying superior genotypes constitute the first important step in crop improvement (Barathi and Reddy, 2022). The first application of molecular markers in pearl millet was the creation of a genetic map using restriction fragment length polymorphism (RFLP) markers (Liu *et al.,* 1994). After that, sequence-independent PCR-based markers, such as RAPD, ISSR, AFLP, and microsatellite probes, were used for genetic diversity studies in pearl millet (Govindaraj *et al.,* 2009). SSR markers have also been developed (Qi *et al.,* 2004) and used for pearl millet genetic diversity and gene mapping studies (Satyavathi *et al.,* 2013; Stich *et al.,* 2010). Decoding the pearl millet genome and advancements in sequencing technologies leading to SNP marker development and exploitation played a significant role in pearl millet breeding (Varshney *et al.*, 2017; Semalaiyappan *et al.,* 2023).

Nevertheless, owing to their advantages, including cost-effectiveness, co-dominance nature, ease of genotyping, and analysis, SSR markers are of immense use in pearl millet for various applications, such as for estimation of genetic diversity, testing genetic purity, cultivar protection, genetic mapping, and tagging studies. Assessing the genetic diversity of restorer and maintainer lines is an important aspect in developing heterotic pools for commercial varieties. Therefore, the objective of the present study was to assess the utility of SSR markers in estimating genetic diversity among Indian pearl millet cultivars, which could be helpful to breeders in selecting diverse parental lines for breeding high-yielding cultivars.

MATERIALS AND METHODS

Plant Material: A set of 94 pearl millet inbreds consisting of 45 maintainers and 49 restorer lines were received from ICRISAT, Hyderabad (**Table 1**). These elite materials were selected during the Scientist's field-day selection. The selections were based on diversity to identify combiners with good general and specific combining abilities. All the genotypes were planted in Randomised Complete Block Design (RCBD) where experimental units of two-row plots with a length of 4.8 m and with 0.70 m between rows, were used. A comprehensive evaluation of commercially relevant traits was conducted at Rallis India Limited's Research and Development facility in Hyderabad, India, during the summer of 2022. The assessed parameters included plant height (cm), panicle length (cm), girth (cm) , productive tillers per plant, leaf length (cm), leaf width (cm), internode count, seed color and scores for Blast and Rust resistance (ranging from poor to excellent), sale appearance score (from poor to excellent) and 1000-seed weight (g). The lines utilized in the study were essential and specific to certain traits, making them highly suitable for use in commercial hybrids and for improving overall crop yield.

Table 1. List of inbred lines used for molecular characterization

S.No.	Name of inbred	S.No.	Name of inbred	S.No	Name of inbred	S.No	Name of inbred	S.No	Name of inbred
1	PMB101	21	PMB121	41	PMB141	61	PMR216	81	PMR236
2	PMB102	22	PMB122	42	PMB142	62	PMR217	82	PMR237
3	PMB103	23	PMB123	43	PMB143	63	PMR218	83	PMR238
4	PMB104	24	PMB124	44	PMB144	64	PMR219	84	PMR239
5	PMB105	25	PMB125	45	PMB145	65	PMR220	85	PMR240
6	PMB106	26	PMB126	46	PMR201	66	PMR221	86	PMR241
7	PMB107	27	PMB127	47	PMR202	67	PMR222	87	PMR242
8	PMB108	28	PMB128	48	PMR203	68	PMR223	88	PMR243
9	PMB109	29	PMB129	49	PMR204	69	PMR224	89	PMR244
10	PMB110	30	PMB130	50	PMR205	70	PMR225	90	PMR245
11	PMB111	31	PMB131	51	PMR206	71	PMR226	91	PMR246
12	PMB112	32	PMB132	52	PMR207	72	PMR227	92	PMR247
13	PMB113	33	PMB133	53	PMR208	73	PMR228	93	PMR248
14	PMB114	34	PMB134	54	PMR209	74	PMR229	94	PMR249
15	PMB115	35	PMB135	55	PMR210	75	PMR230		
16	PMB116	36	PMB136	56	PMR211	76	PMR231		
17	PMB117	37	PMB137	57	PMR212	77	PMR232		
18	PMB118	38	PMB138	58	PMR213	78	PMR233		
19	PMB119	39	PMB139	59	PMR214	79	PMR234		
20	PMB120	40	PMB140	60	PMR215	80	PMR235		

Genomic DNA isolation: The genomic DNA was extracted from healthy young leaves of pearl millet lines using the CTAB procedure developed by Saghai-Maroof *et al.* (1994) with appropriate modifications. In brief, fresh leaves (2-3g) were collected from each genotype and pulverized in liquid nitrogen with a pestle and mortar. The resulting powder was transferred to a 2ml centrifuge tube containing 1ml of pre-warmed (65°C) extraction buffer (100mM Tris-HCl, pH 8.0; 0.5mM EDTA, pH 8.0; 1.4M NaCl; 2% CTAB; 1% β-mercaptoethanol). The suspension was incubated at 65°C for 1 hour with intermittent mixing. Proteins were extracted with one volume of chloroform:isoamyl alcohol (24:1). Nucleic acids were precipitated with 1 volume of cold isopropanol, washed with 70% ethanol, dried, and finally resuspended in TE buffer (10mM Tris HCl, pH 8.0; 0.5mM EDTA) containing RNAase A (50ng/ml) and incubated for 1 hour at 37°C for degradation. Degraded RNA was separated with 1 volume of chloroform:isoamyl alcohol (24:1). This suspension was centrifuged at 10,000 rpm for 5 minutes. The aqueous phase was mixed with 1/10th volume of 3M sodium acetate (pH 5.2) and 2 volumes of chilled ethanol, and then incubated for 2 hours at 20°C. Afterward, it was further centrifuged at 12,000 rpm for 15 minutes. The supernatant was discarded, and the pellet was washed twice with 70% cold ethanol (-20°C). The pellet was then resuspended in TE buffer (10mM Tris HCl, pH 8.0; 0.5mM EDTA). The DNA concentration was determined using a UV spectrophotometer at 280 nm (Sambrook *et al.,* 1989).

Genotyping of inbred lines with SSR markers: A set of 39 SSR markers covering the pearl millet genome was selected from the literature to study the diversity among the genotypes (Patil *et al.,* 2021; Verma *et al.,* 2021). Among these, 30 SSR primers revealed polymorphism, and all the thirty markers were utilized for the further analysis. List of markers studied and polymorphic information are provided in **Table 2**. The polymerase chain reaction (PCR) was performed in 10µl volume. The reaction mix contained 1µl of 10X Taq buffer A, 1µl of 2.5mM dNTPs, 1µl of oligonucleotide primer (forward and reverse), 1µl of 1.2%BSA, 0.5µl of 1.5Mm MgCl2, 2 µl of 50ng genomic DNA, 0.1µl of 5U/µl Taq DNA polymerase (Banglore genei Merck) and 3.4µl of autoclaved doubled distilled water. The PCR was performed using the Eppendorf master X50 PCR Thermal Cycler, USA. The PCR profile included an initial denaturation at 94 °C for 4 minutes, denaturation at 94°C for 30 seconds, annealing at 58°C for 45 seconds (annealing temperatures were standardized for each SSR primer ranging from 54 to 58°C), extension step at 72°C for 1 min was followed by final termination at 72°C for 5 minutes hold 10°C for 10 minutes. The amplified PCR product was resolved on a 3% agarose gel (super fine resolution agarose, LonzaSeaKem). Scoring of the SSR alleles was performed manually in terms of the position of the bands relative to the 100bp ladder sequentially from the smallest to the largest-sized bands. Reconfirmation of the null allele was done. The bands appearing as artifact or bands that were either diffused or highly faint and null alleles even after reconfirmation, were not considered for analysis. SSR bands were scored as present (1) absent (0) for each cultivar.

Data analysis: PowerMarker V3.0 software (Liu and Muse, 2005) was used to predict the total number of alleles, alleles per locus, and occurrence of common, most frequent, rare, and unique alleles; gene diversity, and heterozygosity. DARwin (version 6.0.21) (Perrier and Jacquemoud-Collet, 2003) was used to measure the accession's genetic diversity. Neighbour-Joining tree method was used to visualize the phylogenetic tree from the dissimilarity coefficient based on a simple matching approach. DARwin was used to perform Principal Coordinates Analysis (PCoA) and to find the genetic distance between accessions.

RESULTS AND DISCUSSION

Assessment of genetic diversity among the materials will assist in understanding crop heterosis. The development of molecular markers and their utilization in linkage map development and trait mapping has revolutionized the field of crop breeding. Since developing and utilizing SSR markers in pearl millet breeding in 2004 (Qi *et al.,* 2004), they have played a prominent role in diversity studies and molecular characterization (Gupta *et al.,* 2018). Out of 39 SSR markers screened, a total of 30 SSR markers showed polymorphism and were employed for assessing genetic diversity among the pearl millet inbred lines. Genotyping of 45 maintainers and 49 restorer lines using the polymorphic markers detected 84 alleles. The number of alleles per locus varied from 2 to 4, with an average of 2.8 alleles per locus (**Fig. 1**). The allele size varied from 102 bp (*Xpsmp2030*) to 385 bp (*Xpsmp2203*). The PIC value of the SSR primers ranged from -0.253 (Xpsmp2207) to 0.761 (Xpsmp2079.2), with an average of 0.369 (**Table 2**). Seven of the thirty SSRs *Xpsmp2203, Xpsmp2086, Xpsmp2018, Xpsmp2030, Xpsmp2070, Xpsmp2081,* and *Xpsmp2079.2* were highly polymorphic, and their PIC values ranged from 0.609 to 0.761.

In the current study, the overall polymorphism among cultivars was 92%. However, we observed a lower average number of alleles per locus than those found in previous studies in pearl millet. Kapila *et al.* (2008) found an average of four alleles per locus in a diverse set of maintainer and restorer lines. Similarly, Stich *et al.* (2010) also observed a higher number of alleles per locus while characterizing diversity in germplasm lines. The lower number of alleles/loci recorded in this study could be explained by two accounts generally, inbred lines represent low genetic diversity in comparison to the germplasm lines, and 2). Furthermore, the majority of the SSR primers were highly polymorphic. The high levels of polymorphism revealed that the inbred lines included in this study were genetically diverse. This was obvious as different parental lines were used to develop these

Table 2. Features of 30 SSR marker used in the study

S. No.	SSR Locus	Repeat motif	No of alleles	Size range of the PCR products (bp)	LG	PIC
1	Xicmp3043	(AGC)5	3	198-228	7	0.327
2	Xpsmp2079.2		3	117-179	7	0.761
3	Xpsmp2275	(GTT)10	3	267-300	6	0.523
4	Xipes0082	(AGGAG)7	2	154-209	7	0.012
5	Xipes0203	(ATC)16	4	229-307	1	0.330
6	Xpsmp2220	(GT)11	2	136-156	5	0.279
7	Xpsmp2232	(TG)8	2	247-255	1 & 2	-0.077
8	Xpsmp2070	(CA)25 (TA)6	3	204-278	3	0.666
9	Xipes0220.1		3	172-216	3	0.575
10	Xpsmp2207	(GT)5	2	315-333	7	-0.253
11	Xipes0186	(TTG)10	3	169-293	4	0.405
12	Xipes0152.2		2	109-137	5	-0.077
13	Xpsmp2089	(AC)15	3	117-159	2	0.517
14	Xipes0200	(GTAC)11	3	174-210	6	0.525
15	Xpsmp2203	(GT)18 imp.	3	351-389	7	0.609
16	Xpsmp2085	(AC)11	3	181-199	4	0.274
17	Xpsmp2068	(AC)14	4	112-162	2	0.576
18	Xpsmp2201	(GT)6	3	363-385	2	0.499
19	Xctm10	(CT)22	3	183-219	3	0.353
20	Xicmp3032	(GCT)8	2	202-217	1	0.387
21	Xipes0213	(GAT)4	2	163-181	3	0.327
22	Xipes0236	(TGG)11	2	215-263	2	-0.012
23	Xpsmp2249	(GT)7 imp.	3	154-182	3	0.427
24	Xpsmp2086	(AC)14	3	107-149	4	0.628
25	Xpsmp2030	(CA)11 (GA)10	4	102-160	1	0.631
26	Xpsmp2248	(TG)10	2	177-209	6	-0.223
27	Xpsmp2090	(CT)12)	2	189-211	1	0.314
28	Xctm12		2	189-211	1	0.494
29	Xpsmp2081		4	250-260	4	0.669
30	Xpsmp2018		4	110-125	6	0.628

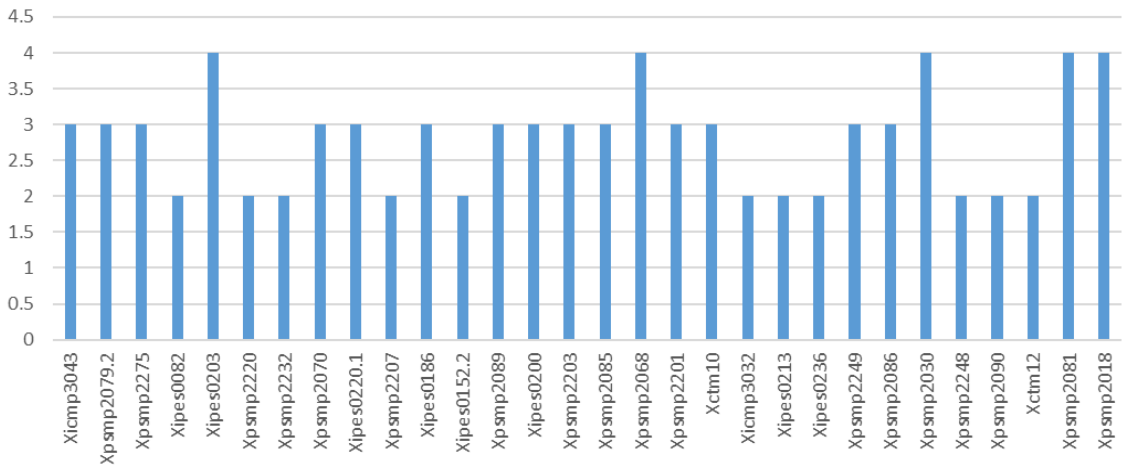

Fig. 1. Allele frequency of 30 SSR markers on 94 inbred lines.

cultivars (**Table 1**). Based on the PIC values of the most informative loci, it was possible to reduce the number of loci employed for cultivar discrimination significantly. In the present study, we employed only five primer pairs with high PIC values, that is, Xpsmp2079.2(0.76), Xpsmp2070(0.67), Xpsmp2081(0.67), Xpsmp2030(0.63), Xpsmp2086 (0.63) to differentiate all the pearl millets inbred lines. In pearl millet, the genetically uniform nature of the inbred lines is a matter of great concern. The current pearl millet breeding program in India is laying greater emphasis on broadening the genetic base of pearl millet cultivars by diversifying the source of restorer sterility in the cytoplasm and introducing genes conferring resistance to diseases, insects, and pests, as well as other economically essential traits from diverse germplasm lines and wild species of *Pennisetum* (Satyavathi *et al.,* 2013). Given this, the identified highly polymorphic SSR markers can be of immense use in differentiating parental lines, cultivars, and elite genotypes, thereby helping to select diverse lines that can be used as parents in breeding programs.

Neighbour-Joining tree constructed using DARwin grouped the 94 inbred lines into two major clusters (I and II),which were further separated into 13 sub-clusters (C1 to C13) as mentioned in **Table 3** and **Fig. 2.**

Among the 13 sub-clusters, cluster 1 was the most significant cluster with 16 genotypes, all of which were maintainer lines. Cluster 8 was the second largest with 15 genotypes, and these genotypes were restorer lines, whereas the Cluster 6was found to be the smallest cluster with one genotype. Cluster analysis showed a clear grouping of maintainer and restorer lines. This result is highly associated with pedigree and is based on the inbred type. A few deviations from the grouping of restorer and maintainer lines were observed, including PMB 140 (Cluster-9), PMB 130 (Cluster-10), PMB 131(Cluster-11), and PMR203(Cluster-5), as they were grouped into restorer lines. Although the clustering pattern of these lines deviated from the pedigree data, they showed similar morphological traits, including seed plant height, panicle length, and panicle shape in the present study. Therefore, the deviation may be because they share their parentage (Nepolean *et al.,* 2012; Semalaiyappan *et al.,* 2023). The remaining genotypes followed a prominent grouping pattern in the maintainer and restorer groups. Notably, it was very interesting that we can use these markers for future MAS projects. The grouping of the B and R lines is based on the history of breeding in pearl millet. These two groups represent a pool of favourable genes for increasing yields.

The results obtained from PCoA were similar to the results of DARwin analysis (**Fig.3**). These results were used to interpret the phylogenetic relationship among genotypes in a scatter plot format. The first two principal components (PCs), explain 81.43% of the total variance in the data. PC1 is the most important PC, explaining 57.43% of the variance.PC2 is the second most important PC, explaining 24% of the variance.

Table 3. Clustering pattern of 94 pearl millet inbred lines based on DARwin analysis.

Clusters Group	Inbreds parents	No of inbreds	Main Group
Cluster-1	PMB107,PMB102,PMB103,PMB101,PMB132,PMB124,PMB133,PMB104,PMB106,PMB108,PMB109,PMB114,PMB116,PMB113,PMB111,PMB112	16	Maintainers
Cluster-2	PMB122,PMB129,PMB128,PMB126,PMB122,PMB118,PMB119,PMB123,PMB125,PMB121,PMB120,PMB134,PMB115,PMB117	14	Maintainers
Cluster-3	PMB141,PMB137,PMB139	3	Maintainers
Cluster-4	PMB145,PMB144,PMB143,PMB142,PMB136,PMB135,PMB138	7	Maintainers
Cluster-5	PMB105,PMR203	2	Maintainers
cluster-6	PMB110	1	Maintainers
cluster-7	PMR241,PMR240,PMR230	3	
Cluster-8	PMR234,PMR233,PMR238,PMR224,PMR218,PMR217,PMR235,PMR243,PMR236,PMR220,PMR242,PMR223,PMR225,PMR226,PMR229	15	Restorer
Cluster-9	PMR215,PMR214,,PMR213,PMR206,PMR204,PMR232,PMR216,PMR219	8	Restorer
Cluster-10	PMB140,PMR239	2	Restorer
Cluster-11	PMR228,PMR227,PMR226,PMR237,PMB130, PMR231,PMR202,PMR201	8	Restorer
Cluster-12	PMR210,PMR209,PMR249,PMR248,PMR247,PMR221,PMR246,PMR245,PMR11,PMR244,PMR208,PMB131	12	Restorer
Cluster-13	PMR207,PMR205,PMR212	3	Restorer
	TOTAL INBREDS	94	

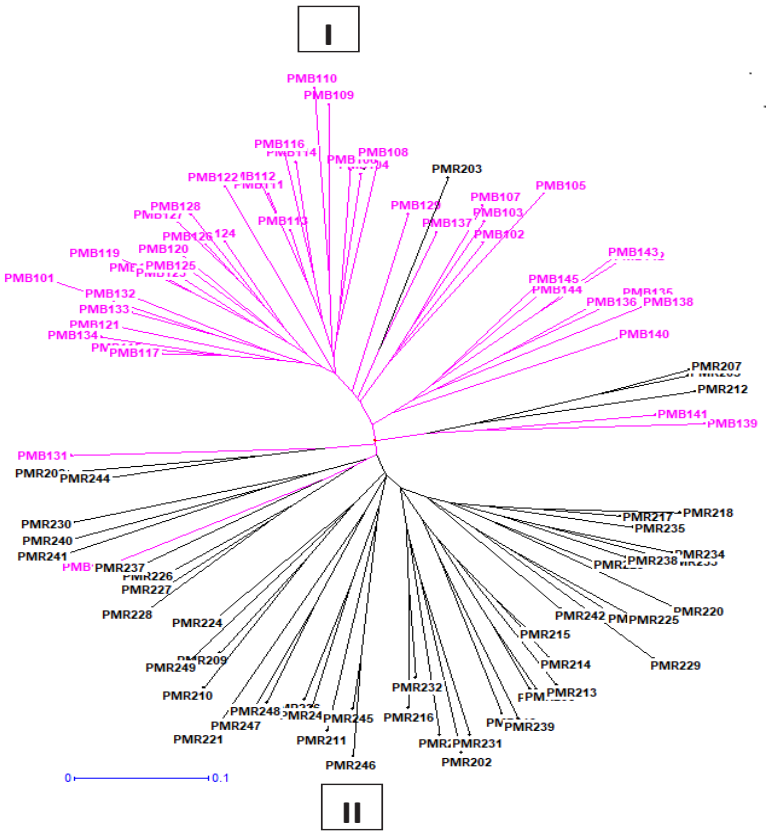

Fig. 2. Diversity analysis of 94 pearl millet inbred lines using DARwin. Set I comprises most of the B-lines and Set II comprises most of the R-lines

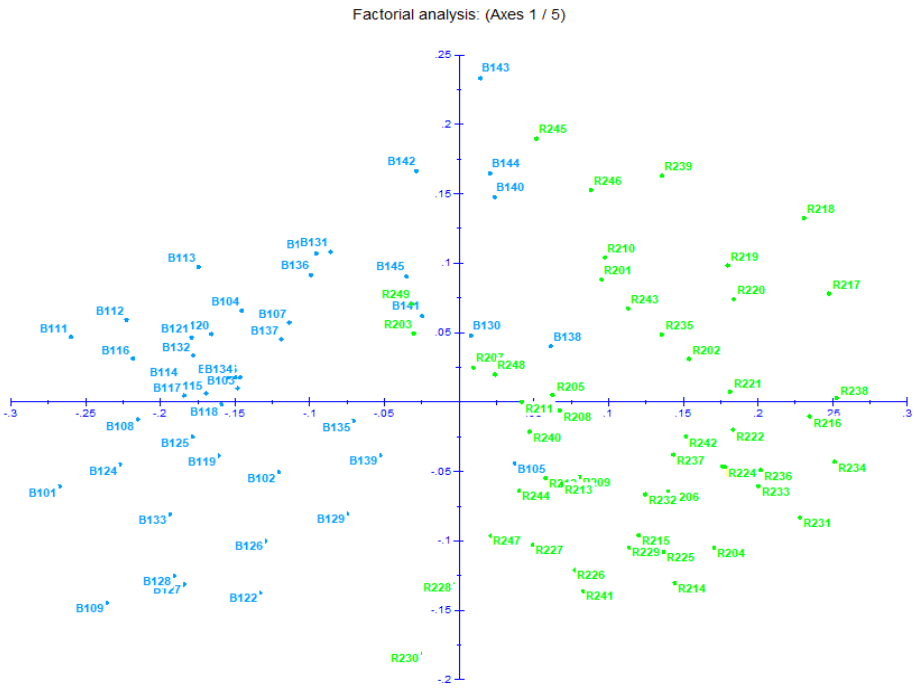

Fig. 3. PCoA analysis showing accessions grouped in a scatter plot

Sales appearance score (SA) is an important factor for the commercial success of a variety. It is calculated by evaluating all combination genotypes with a total score of overall impression and appearance, uniformity, and yield from 1 to 10, and then calculating the average score (Dobnik *et al.,* 2021). The score ranges from 1 to 10, with 1 being the lowest and 10 being the highest. The factors determining the score are plant standability, disease resistance, yield attributing traits such as panical length, girth, grain size, color, maturity and uniformity based on SA score. The consideration of measuring factors varies according to the variety developed for a particular trait. In the current study, the 45 inbred lines, including 19 B-lines and 27- R lines were found to possess a score 8, followed by 27 inbred lines containing 16 B-lines and 11 R-lines which were found to have a score of 7. The rest of the 21 inbred lines scored 6, where ten lines were B-lines, and 11 were R-lines (**Fig. 4**). We observed an excellent SA among the inbred lines. More than 45% of the lines were measured to have an SA of 8, and more than 30% had an SA of 7, whereas the rest possessed an SA of 6. This indicates that these selected inbred lines could form a potential heterotic group for hybrid development.

The correlation between sales appearance score and phenotyping data can be used to identify traits associated with high sales appearance. Hence, in the current study, correlation among traits was performed using R package cluster and Factoextra (R4.2.1). Plant height showed a positive correlation with all the traits studied, whereas the highest correlation for plant height was observed for internode distance (0.636) followed by SA (0.453). Panicle length was highly correlated with panicle girth (0.353) and negatively correlated with productive tillers (-0.025). In case of panicle girth, the highest correlation was found with SA (0.512) followed by leaf length (0.491). Productive tillers were found to be highly correlated with seed size (0.211) when compared to other traits. Leaf length was highly correlated with SA (0.549) followed by rust (0.393). Similarly, leaf width was found to be highly correlated with SA (0.172) followed by internode distance (0.131). Internode distance also correlated highly with SA (0.516), followed by rust (0.336). Seed colour was positively correlated with 1000seed weight (0.327), followed by seed size (0.287) and SA (0.233). Seed size was highly correlated with 1000seed weight (0.841) and negatively correlated with SA (-0.033). In case of 1000 seed weight, positive correlation was found for rust (0.319). Blast was found to positively correlate with rust (0.663) followed by SA (0.404). Rust was positively correlated with SA (0.438) (**Fig. 5**). Correlation analysis showed that six traits, namely plant height, panicle girth, leaf length, internode distance, blast, and rust, showed a strong positive correlation with SA; hence, they can be used for SA determination. In addition, as these lines also showed a positive correlation with rust and blast, they can serve as parental material for pearl millet disease-resistance breeding programs.

Our current diversity analysis and the use of prominent SSR markers will aid in comprehending the genetic relationships between and among the B and R line groups. This information can be used to establish new heterotic pools, segregate populations for trait mapping, and conduct association and genomic selection experiments. The crossing of germplasm lines from opposite heterotic groups can create heterotic populations and hybrid varieties. In contrast, crossing germplasm lines from within heterotic groups can lead to the synthesis and derivation of new inbred lines.

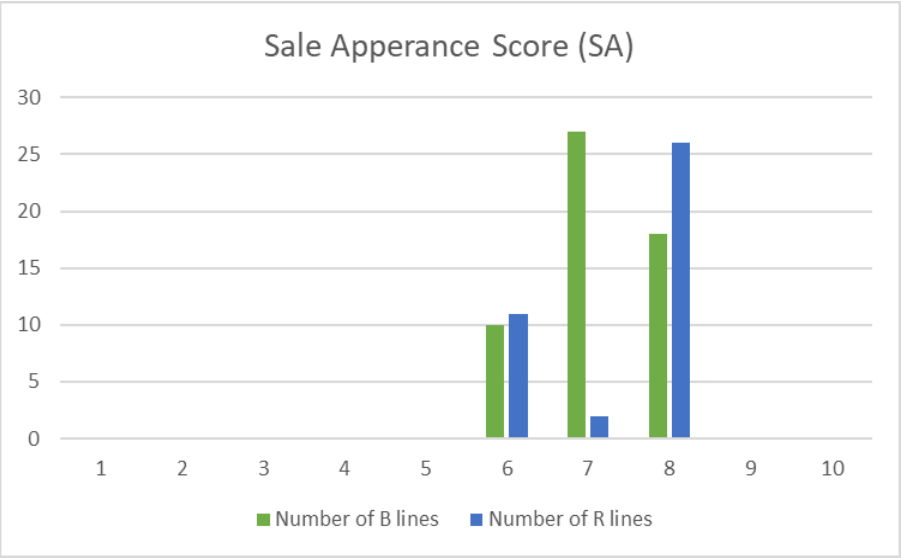

Fig. 4. Sales appearance score of 94 inbred lines
The scoring was done using 1 to 10 scale whereas the inbred lines score ranged from 6 to 8

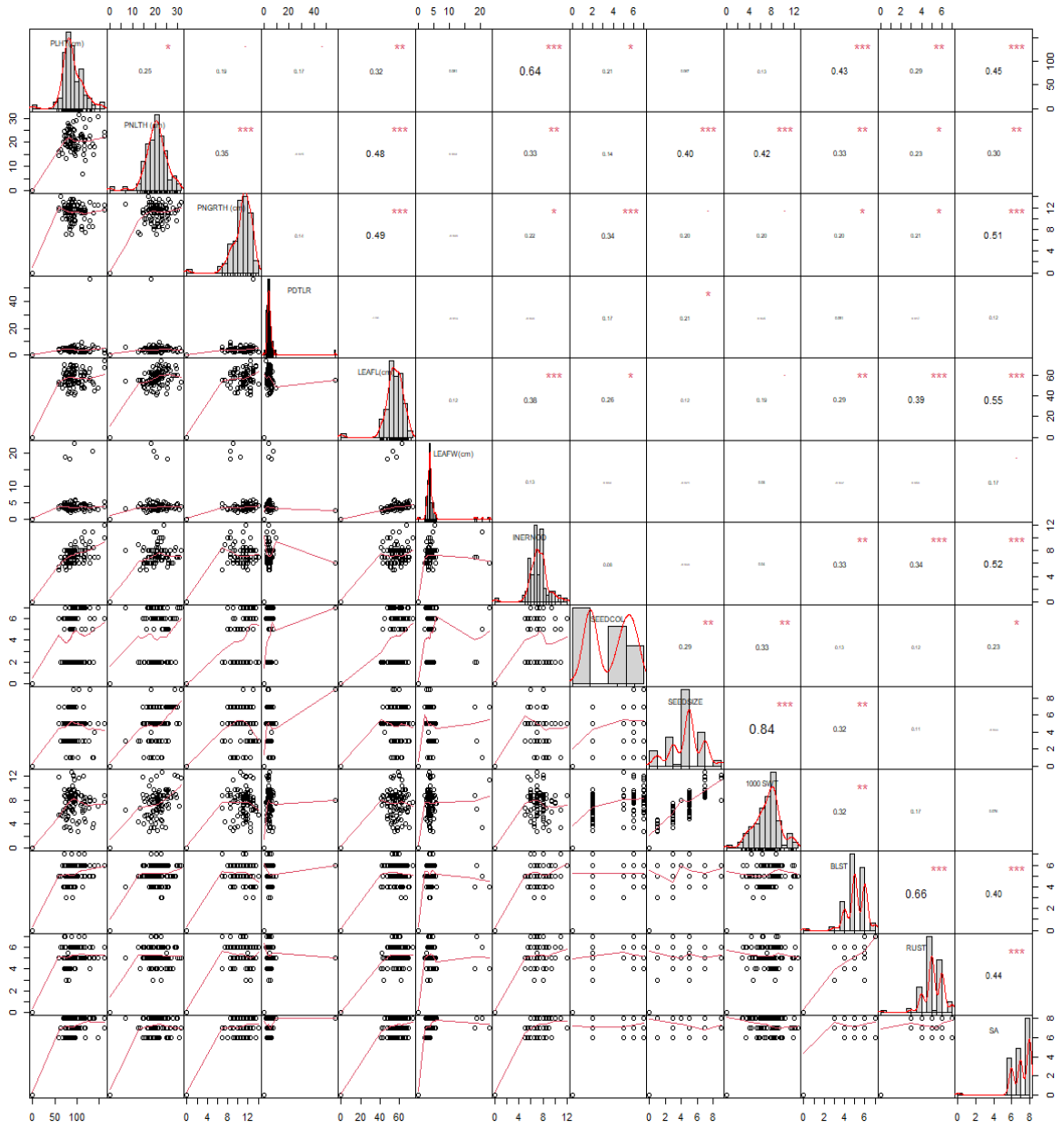

Fig. 5. Genotypic correlation analysis for12 qualitative and quantitative pearl millet inbred lines traits

PLHT(cm)-plant height in cm; PNLTH (cm)-panical length in cm; PNGRTH (cm)-panical girth in cm; PDTLR-productive tillers; LEAFL(cm)-leaf length in cm; LEAFW(cm)-leaf width in cm; INERNOD-internode distance; SEEDCOL-seed colour; SEEDSIZE-seed size; BLST-blast disease; RUST-rust disease; 1000SWT-1000 seed weight; SA-sales score.

In conclusion, we have utilized SSR markers for establishing genetic relationships among the Indian pearl millet cultivars. Moreover, a small set of SSR markers demonstrated a remarkable ability to distinguish all the cultivars, including both restorer and maintainer lines. Given this proficiency in SSR markers and germplasm, they can be effectively employed in Marker Assisted Breeding/ Selection projects and serve as valuable tools for assessing the genetic purity of seed lots associated with these cultivars. The genetic relationships identified among the restorer/inbred lines may be helpful in designing strategies to improve the use of available genetic variation in pearl millet breeding.

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