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Full Length Research Paper

Genetic diversity analysis of pearl millet (*Pennisetum glauccum* [L.] R. Br.) accessions using molecular markers

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Random amplified polymorphic DNA (RAPD) analysis was applied to pearl millet genotypes in order to assess the degree of polymorphisms within and among genotypes and to investigate if this approach was suitable for genetic studies of pearl millet. 20 genotypes were evaluated using 30 different 10-mer primers of arbitrary sequence. Most of the primers did not reveal any polymorphism; however 12 primers revealed scorable polymorphism between genotypes of pearl millet and these can be further evaluated for use in genetic mapping. Pair-wise comparisons of unique and shared polymorphic amplification products were generated by Jaccard's similarity co-efficient. These similarity co-efficients were employed to construct a dendrogram showing phylogenetic relationships using unweighted paired group method with arithmetic averages (UPGMA). The UPGMA analysis indicated a higher similarity between genotype PT 2835/1 and PT 5552 and lowest similarity index was observed between PT 5554 and PT 2835/1. Analysis of RAPD data appears to be helpful in determining the genetic relationship among 20 pearl millet genotypes. The associations among the 20 genotypes were also examined with Principle components analysis (PCA) from Jaccard's similarity co-efficient and it is more informative to analyze the extreme genotypes.

Key words: Random amplified polymorphic DNA (RAPD), genetic diversity, pearl millet, principle component analysis (PCA)

INTRODUCTION

Pearl millet (*Pennisetum glauccum* (L.) R. Br.) is a summer annual grass originating from Africa, from where it was introduced into other regions of the world with diverse agro-climatic conditions that is, from the hot area of Africa (the tropical zone) to the hot area of temperate zones. Therefore a large number of diversity is found within and among pearl millet cultivars. Due to its highly out-crossing breeding behaviour, pearl millet was originated from several independent domestication events and wide range of stressful environmental conditions, in which it had been traditionally cultivated. Pearl millet exhibits a tremendous amount of diversity at both phenoltypic and genotypic levels (Poncet et al., 1998; Liu et al., 1994).

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Estimation of genetic diversity and identification of superior genotypes are some of the prime objectives of any crop improvement programme. Highly diverse genotypes or accessions can be utilized as parents in hybridization programmes to produce superior varieties/hybrids. Therefore there is a need to evaluate available genotypes for their genetic diversity.

In the early days, crop breeders used morphological markers for the assessment of genetic diversity and choosing parents for developing new cultivars. Morphological markers data are affected by the interaction of the genotype with the environment in which it is expressed. Moreover, due to the high out-crossing breeding nature and structure of genetic diversity in pearl millet species, the morphological data/markers are inadequate in providing reliable information for the calculation of genetic distance and pedigree studies. Thus, for genetic diversity assessment, molecular markers offer considerable ad-

vantages over morphological markers. DNA markers have been used to evaluate genetic diversity in different crop species (Cooke, 1995). Various molecular markers are being used for fingerprinting such as restriction fragment length polymorphisms (RFLP) (Dubreuil and Charcosset, 1998), random amplified polymorphic DNA (RAPD) (Williams et al., 1990), micro satellites (Smith et al., 2000) and amplified fragment length polymorphism (AFLP) (Agrawal et al., 1999).

A major milestone in applications of molecular markers in pearl millet was the creation of the first genetic map in 1993 using RFLP markers (Liu et al., 1994); though RFLP and AFLP are robust, they involve laborious protocols and use of radioactive chemicals. RAPD and SSR are preferred since they are polymerase chain reaction (PCR) based, rapid and do not involve radioactive chemicals. The RAPD procedure involves no blotting or hybridizing steps (Williams et al., 1990). RAPD markers offer many advantages such as higher frequency of polymorphism, rapidity (Fahima et al., 1999), technical simplicity, requirement of few nanograms (ng) of DNA, no requirement of prior information of the DNA sequence and feasibility of automation (Subudhi and Huang, 1999). RAPD is quite efficient in bringing out genetic diversity at DNA level (Jaya Prakash et al., 2006). Hence, the present study was undertaken with an objective to evaluate the genotypes for assessing the extent of variation among pearl millet species at molecular level using RAPD markers.

MATERIALS AND METHODS

Genetic material

20 pearl millet genotypes were selected based on diverse morphological data recorded in the Millet Breeding station of the Tamil Nadu Agricultural University, Coimbatore, India.

DNA extraction

Total genomic DNA was extracted from 12 day-old seedlings as a protocol given by Gawal and Jarret (1991) with slight modifications. Fresh leaves (3-4g) were collected from each genotype and powdered in liquid nitrogen using a pestle and mortar. The resulting powder was transferred to a 50 ml centrifuge-tube and centrifuged for 1 h with 10.0 ml of pre-warmed (65°C) extraction buffer (100 mM tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl, 2% CTAB, and 1% 2 - mercaptoethanol). Proteins were then extracted with one volume of chloroform:isoamyl alcohol (24:1). Nucleic acids were precipitated with 1 volume of cold isopropanal, then washed with 70% ethanol, vaccum-dried and finally resuspended in TE buffer (10 mM Tris HCl (pH 8.0), 0.1 mM EDTA) containing RNAase A (40 ng ml^{-1}), and incubated for 30 min at 37°C for RNA degradation. Degraded RNA was then removed with 1 volume of chloroform:isoamyl alcohol (24:1), then DNA was precipitated and resuspended as mentioned above. DNA concentration was determined using the UV spectrophotometer at 260 and 280 nm (Sambrook et al., 1989).

DNA amplification

A set of 30 random decamer oligonucleotides purchased from Operon Technologies Inc. (Alameda Calif., USA) were used as single primers for the amplification of RAPD sequences. The conditions reported by Williams et al. (1990) for creating RAPD markers by PCR were optimized for use in pearl millet template DNA. Amplification reactions of 25 µl containing 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.0 mM $MgCl_2$, 0.1 mM each dNTPs, 0.4 µmol of 1 oligonucleotide decamer primer, Ca 25 ng of genomic DNA and 1 unit of *Taq* DNA polymerase were conducted in a thermocycler model 9600 from Perkin Elmer Cetus (Norwalk, Connecticut, USA) programmed for 40 cycles and each cycle consists of denaturation (94°C for 15 sec), annealing (35°C for 30 sec) and primer extension (72°C for 60 sec). After the 40^{th} cycle, 1 extra primer extension step was allowed for 7 min at 72°C. Additionally, different amplification conditions were tested for varied levels of annealing temperatures (37, 42 and 52°C) and number of cycles (45 and 55 cycles). Amplification products were separated on 1.8% agarose gels containing ethidium bromide (0.2 µg ml^{-1}) and visualized under UV light and then documented in a gel documentation system model (Alpha EaseFc Alpha Imager 2200, Alpha Innotech Corp., California, USA).

RAPD data analysis

The amplified products were scored as 1 for presence and 0 for the absence of bands, respectively. The polymorphism percentage was calculated as per the method suggested by Blair et al. (1999). Polymorphism (%) = total number of bands - number of monomorphic bands/total number of bands × 100. The scores were used to create a data matrix to analyze genetic relationship using the NTSYS-pc program version 2.02 (Exeter Software, New York, USA) as described by Rohlf (1990). A dendrogram was constructed based on Jaccard's similarity coefficient (Jaccard, 1908) using the marker data for all the pearl millet accessions following the unweighted paired group method with arithmetic averages (UPGMA) (Sokal and Michener, 1958). Using SAHN clustering, similarity coefficient values were computed from binary matrix. These values were used to analyze the principal components.

RESULTS AND DISCUSSION

The PCR has proved to be a powerful tool for the identifycation of polymorphism in many crops. In the present investigation, RAPD showed a high level of polymorphism and a high number of clearly amplified bands. A high level of polymorphism was in accordance with the research of He et al. (1992), Jochi and Nguyen (1993), Autunes et al. (1997), Kale and Munjal (2005) and Jaya Prakash et al. (2006).

To evaluate genetic diversity among genotypes, 20 pearl millet genotypes randomly selected from germplasm collection were used for this study. 30 oligonucleotides were screened to identify polymorphism markers, out of which 12 oligonucleotides have clear and reproducible banding patterns. To assess the reproducibility of the profiles the same template DNA was amplified in 3 different amplification reactions using the same primer. The results were highly comparable. Only strong and reproducible bands were considered for further analyses.

The 12 primers produced 99 polymorphic bands with an average of 8.25 polymorphic bands per primer (Table 1). Figure 1 (A and B) shows a sample of polymorphic

Table 1. Sequence of RAPD primers used for genetic divergence analysis of pearl millet, and results obtained

S/No.	Primer	Sequence (5'to 3')	No. of bands		Polymorphic (%)	GC content (%)
			Total	Polymorphic		
1	OPL -11	ACGATGAGCC	9	8	88.88	60
2	OPAL-20	AGGAGTCGGA	11	10	90.90	60
3	OPAL-08	GTCGCCCTCA	9	8	88.00	70
4	OPAL-15	AGGGGACACC	5	5	100.00	70
5	OPM -16	GTAACCAGCC	5	5	100.00	60
6	OPN-16	AAGCGACCTG	10	9	90.00	60
7	OPF-10	GGAAGCTTGG	9	9	100.00	60
8	OPF- 01	ACGGATCCTG	10	8	80.00	60
9	OPE-04	GTGACATGCC	11	11	100.00	60
10	OPE-09	CTTCACCCGA	10	10	100.00	60
11	OPE-18	GGACTGCAGA	11	11	100.00	60
12	OPAG-13	GGCTTGGCGA	6	5	83.33	70
Total			**106**	**99**	**93.4**	**62.5**

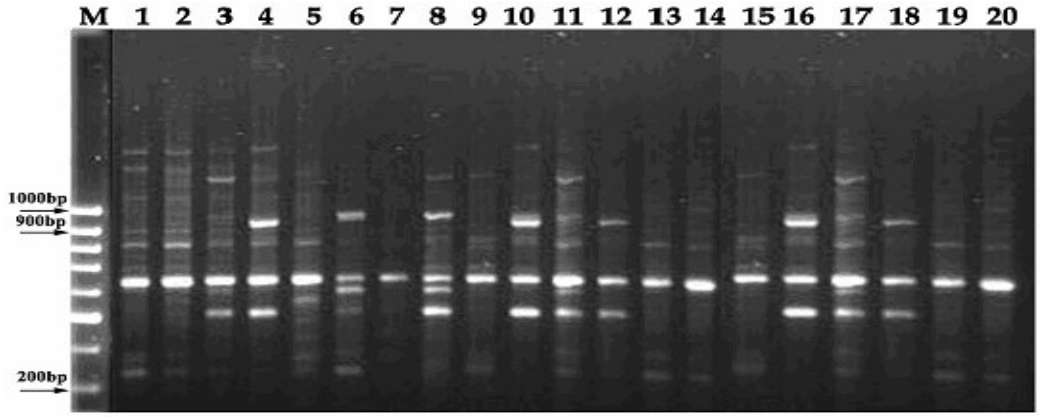

Figure 1A. RAPD banding profile of 20 pearl millet (accession primer OPE 04). The number representing accession code was shown in Table 2, and M is 100 bp DNA ladder

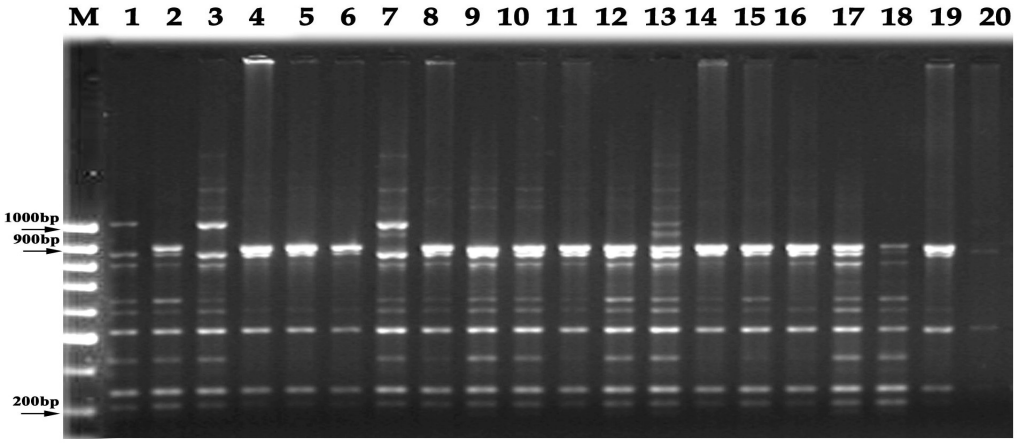

Figure 1B. RAPD banding profile of 20 pearl millet (accession primer OPE 18). The number representing accession code was shown in Table 2, and M is 100 bp DNA ladder.

Table 2. Similarity index for the 20 pearl millet accessions using RAPD banding profiles.

S/No.	Acc. No	PT 1796	PT 2582	PT 2610	PT 2835/1	PT 3488	PT 3657	PT 4219	PT 4377	PT 4572	PT 4896	PT 5005	PT 5010/2	PT 5541	PT 5552	PT 5554	PT 5604	PT 5625	PT 5744	PT 5765	PT 5864
1	PT1796	1.00																			
2	PT2582	0.61	1.00																		
3	PT2610	0.33	0.37	1.00																	
4	PT2835/1	0.35	0.35	0.37	1.00																
5	PT3488	0.43	0.51	0.28	0.42	1.00															
6	PT3657	0.47	0.50	0.39	0.44	0.39	1.00														
7	PT4219	0.40	0.38	0.39	0.34	0.52	0.31	1.00													
8	PT4377	0.39	0.39	0.40	0.31	0.32	0.41	0.32	1.00												
9	PT4572	0.43	0.45	0.38	0.38	0.46	0.37	0.38	0.38	1.00											
10	PT4896	0.52	0.55	0.36	0.41	0.49	0.59	0.33	0.43	0.43	1.00										
11	PT5005	0.55	0.45	0.36	0.31	0.40	0.48	0.39	0.53	0.40	0.51	1.00									
12	PT5010/2	0.37	0.54	0.56	0.26	0.36	0.43	0.46	0.47	0.38	0.39	0.50	1.00								
13	PT5541	0.39	0.41	0.59	0.35	0.34	0.36	0.41	0.33	0.47	0.29	0.43	0.52	1.00							
14	PT5552	0.39	0.41	0.38	0.82	0.48	0.45	0.41	0.32	0.49	0.43	0.34	0.33	0.43	1.00						
15	PT5554	0.46	0.59	0.44	0.23	0.37	0.29	0.35	0.32	0.35	0.40	0.32	0.52	0.36	0.28	1.00					
16	PT5604	0.40	0.39	0.25	0.38	0.61	0.32	0.54	0.37	0.39	0.39	0.47	0.37	0.35	0.46	0.28	1.00				
17	PT5625	0.38	0.39	0.43	0.38	0.43	0.48	0.48	0.50	0.35	0.48	0.46	0.41	0.29	0.33	0.27	0.42	1.00			
18	PT5744	0.41	0.42	0.36	0.54	0.48	0.39	0.47	0.44	0.44	0.40	0.42	0.38	0.38	0.58	0.32	0.66	0.47	1.00		
19	PT5765	0.63	0.63	0.32	0.39	0.48	0.54	0.35	0.46	0.48	0.57	0.49	0.40	0.40	0.46	0.35	0.48	0.37	0.44	1.00	
20	PT5864	0.66	0.52	0.41	0.35	0.36	0.46	0.34	0.39	0.34	0.53	0.51	0.41	0.37	0.33	0.5	0.33	0.42	0.37	0.52	1.00

bands generated by primers OPE 04 and OPE 18 respectively. The GC content of all the primers varied from 60-70% and this was in accordance with the findings made by Williams et al. (1990). The amplification products were used to estimate the genetic distance among the genotypes (Table 2) based on Jaccard's co-efficient following UPGMA method using SHAN's option of the NTSYSPC programme (Rohlf, 1990). The genetic distance ranged from 0.23 to 0.82. The genotype PT 5552 and PT 2835/1 were the most divergent among all the genotypes (82%). The least genetic distance among the geno-types was 23% (between the 2 pearl millet genotypes namely PT 5554 and PT2835/1). Based on the genetic distance, we are certain that there were no identified genotypes among these genotypes.

To better understand the genetic relationships among the 20 genotypes, we clustered them into different defined groups based on their genetic distances (Figure 2). This allowed us to separate the 20 genotypes into 8 distinct groups at a distance of 0.49. The first group composed of PT 5554, PT 1796 and PT 5864, which are separated from the others by a distance of 0.49. The second, fifth and sixth groups had only 1 genotype each, PT 2582, PT 4896 and PT 5625, respecttively. These geno-types considered as monogenotypic viz., showed great divergence from the rest of the genotypes and were not included in any of the other groups. The third group composed of PT 5005, PT 5010/2, PT 2610, PT 5541, PT 4572 and PT 5765. This indicates that these genotypes might have an ancestral relationship. Similarly, the fourth group had 2 genotypes namely PT 3657 and PT 4377, indicating that

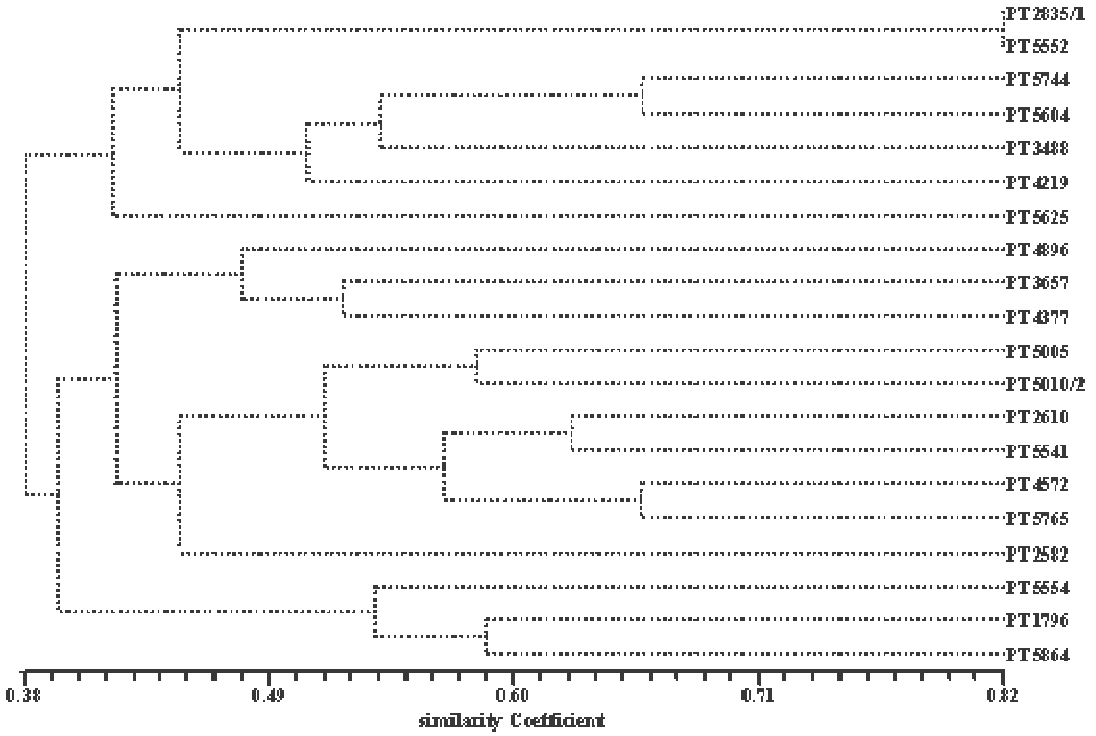

Figure 2. Dendrogram for the pearl millet genotypes based on the data from 12 RAPD primers

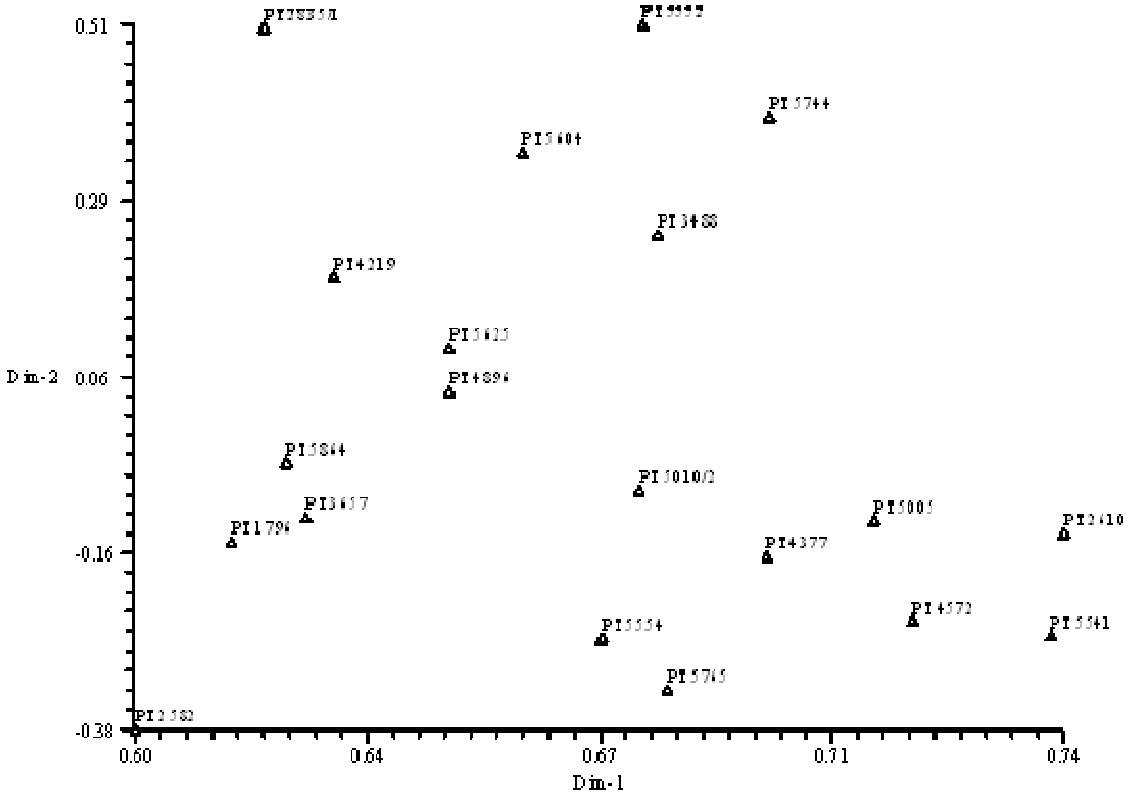

Figure 3. Association between the pearl millet genotypes on the basis of the first 2 principle components (PC1and PC2) obtained from PCA analysis of Jaccard's similarity coefficient based on RAPD data.

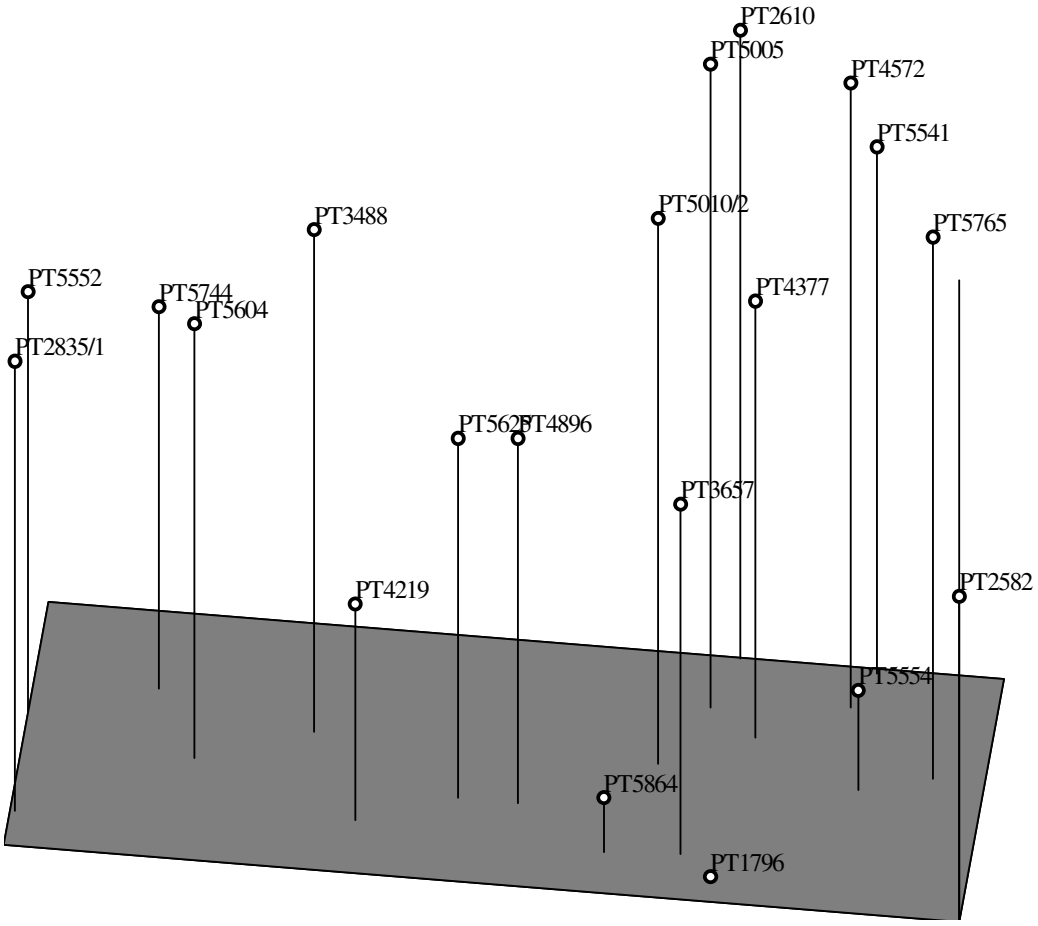

Figure 4. Pattern of relationship among the pearl millet genotypes revealed by PCA analysis based on RAPD data (three dimension)

these might have a common pedigree. The seventh group comprised of PT 5744, PT 5604, PT 3488 and PT 4219 while the eighth group composed of two entries (PT 2835/1 and PT 5552). It shows that these genotypes belong to same ancestor.

The main objectives of this preliminary study were to assess whether RAPD markers could be able to correctly disclose variability among pearl millet genotypes. Although most primers tested did not reveal any polymorphisms, 12 primers showed clear polymorphism among the pearl millet genotypes. Associations among the 20 genotypes were also examined with PCA (Figures 3 and 4); it was obtained from Jaccard's similarity co-efficient based on RAPD data sets. PCA analysis was more informative to study the extreme genotypes *viz.,* PT 2582 and PT 2835/1. PCA analysis scattered the extreme genotypes distantly in 2 dimensions.

To conclude, RAPD variations were more among the pearl millet genotypes. The genetic diversity analysis among the genotypes shows the genetic distance and similarity on the whole genome basis that is, difference in their genetic make up throughout the genome. If the genetic diversity in the germplasm is considerably less, measures should be taken to widen the available gene pool and germplasm and analyzed time to time to reveal genetic diversity. RAPD is quite efficient in bringing out genetic diversity at DNA level hence the wealth of information will help to select the markers which well distributed throughout the genome, can not only be exploited for current agricultural advance, but can also be used to get an insight into the genetic resources that can be employed in future hybridization programmes. In this study, the pearl millet genotypes PT 5625, PT 4896, and PT 2582 were found to be divergent from the rest of the genotypes, which can be used in hybridization programmes for the genetic improvement of pearl millet. However, characterization and quantification of genetic diversity has long been a major goal in germplasm conservation. The results showed that RAPD is an effective tool for pearl millet germplasm management.

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