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Assessment of genetic diversity among pearl millet [*Pennisetum glaucum (*L.) R Br.] cultivars using SSR markers

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Abstract
DNA based markers have emerged as a robust genomic tool for the estimation of genetic diversity and cultivar identification. We are reporting genetic diversity among 20 commercially released pearl millet cultivars comprising of hybrids and open pollinated varieties. Twenty one polymorphic SSR primer pairs, selected after initial screening of 60 were used to study the diversity which amplified 64 alleles. The number of amplified alleles among the cultivars ranged from 2-6 per locus with a mean value of 3 alleles per locus. UPGMA cluster analysis differentiated all the cultivars and eighteen of them were found to be clustered into three major groups at similarity coefficient of 0.43 while two, JBV-3 and BAIF Bajra-1 remained ungrouped and were quite distinct from others. Interestingly, all the cultivars developed at IARI, New Delhi, were present in a subgroup within group I. Similarly, majority of hybrids developed at HAU, Hisar were grouped in the another subgroup within the group 1. BAIF Bajra 1 an exclusive forage purpose variety was genetically most diverged. Besides, a set of five polymorphic primers were found to differentiate all the cultivars. The results have demonstrated presence of moderate level of genetic diversity among the pearl millet cultivars.

Key words: Fodder crops, Genetic diversity, Molecular markers, Pearl millet, Pennisetum

Introduction

Pearl millet (*Pennisetum glaucum* (L) R. Br) is an important forage and food crop in arid and semiarid regions of India and Africa. In India, pearl millet is the fourth most important staple food crop after rice, wheat and sorghum with 8.90 million hectares of total acreage and a production of 6.51 million tonnes, during the year 2009-2010 (http://dacnet.nic.in/eands). 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 like maize or wheat would not survive. In the last two decade, quantum jump in pearl millet production in India was obtained owing to the development of high yielding genetically diverse hybrids. The pearl millet production increased from 3.5 m tonnes since 1960s to 9.5 m tonnes in 2010 which was mainly attributed to more than 60 hybrids released during 1995-2010 for various niche ecologies (http://icar.org.in/en/node/2919).

Genetic improvement of crop species mainly depends on the extent of variability present in their gene pool for economically important traits. Therefore, estimation of the genetic diversity and identification of superior genotypes constitutes first important step in crop improvement.

The first application of molecular marker in pearl millet was creation of genetic map using restriction fragment length polymorphism (RFLP) markers (Liu *et al.,* 1994). Thereafter, sequence independent PCR based markers like, RAPD, ISSR, AFLP and microsatellites probes were used for genetic diversity studies in pearl millet. (Kumar *et al.,* 2006; Yadav *et al.,* 2007; Govindaraj *et al.,* 2009). Meanwhile, SSR markers were also developed (Qi *et al*., 2004) and used for genetic diversity and gene mapping studies in pearl millet (Mariac *et al*., 2006; Yadav *et al*., 2010; Satyavati *et al*., 2009; Stich *et al*., 2010). Because of their advantages, SSR markers may be 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. Therefore, the present study was conducted with the objective to assess the utility of SSR markers in estimation of genetic diversity among Indian pearl millet

cultivars, which could be useful to breeders for selecting diverse parental lines for breeding high yielding cultivars.

Materials and Methods

Plant material: Twenty cultivars of pearl millet comprising of hybrids (12) and open pollinated varieties (8) represented by composites and synthetics, developed from various institutions in India during 1985 to 2009 were included in this study (Table 1).

DNA isolation: Total genomic DNA was extracted from leaves of 15 days old seedlings following the protocol of Sagahai Maroof *et al.* (1984) with minor modifications. DNA concentration of each sample was determined using a DNA fluorimeter (Hoefer, model DyNa Quant 200) and quality was checked on 0.8 % agarose gel. Aliquots from the isolated DNA were used to prepare working stocks of 10 ng/ìl for the polymerase chain reaction (PCR).

Amplification: SSR primers were selected from previous studies on the development of SSR markers in pearl millet (Qi *et al.,* 2004; Senthilvel *et al.,* 2008). Each optimized PCR reaction mixture consisted of 40ng template DNA, 1X PCR buffer [10 mM Tris-HCl (pH.8.3), 50 mM KCl] 1.5 mM $MgCl_2$, one unit of *Taq* polymerase, 200 µM of each dNTP, (all chemicals from Sigma-Aldrich, USA) and 0.5 µM primers in a total volume of 25 ìl. Amplifications were performed in a thermocycler (MJ Research, Model PTC-200) using following conditions: a denaturation step of 5 min at 95°C followed by 40 cycles each composed of 1 min at 94°C, 1min at 54-55°C and 1 min at 72°C, and final extension step of 8 min at 72°C. The amplified fragments were separated on 3% metaphor agarose (Cambrex Biosciences, USA) gels using IX TAE buffer at 100 V for 2 hrs, stained with ethidium bromide and recorded under UV in gel documentation system.

Data analysis: SSR bands were scored as present (1) absent (0) for each cultivar and resulting data matrix was analyzed for genetic relationships among the cultivars using the software NTSYSpc2.1 (Rohlf, 1993). Jaccard's similarity coefficients were calculated for all pair wise comparisons among the cultivars. Based on the Jaccard's similarity values, UPGMA cluster analysis was performed to generate a dendrogram. Jaccard's similarity values were also used for the principal component analysis to generate a two dimensional diagram showing genetic association among the cultivars. Polymorphism information content (PIC) of SSR loci was calculated using the formula (PIC=1-"Pi^2 where Pi is the frequency of i^{th} allele), suggested by Anderson *et al.* (1993).

Results and Discussion

A total of 21 pair polymorphic SSR primers identified on the basis of initial screening of 60, were used for assessing genetic diversity among the pearl millet cultivars. These primers amplified a total of 64 alleles, which varied from 2 to 6 per locus, with a mean of 3.0 per locus. The overall polymorphism among the cultivars was 92%. A representative gel showing distribution of alleles for locus PSMP 2088, across the 20 cultivars of pearl millet is presented in Fig. 1. However, we have observed lower average number of alleles per locus as compared to those found in previous studies in pearl millet. For example, Kapila *et al.* (2008) found an average of 6.26 alleles per locus, in diverse set of maintainer lines. Similarly, Stich *et al.* (2010) also observed more number of alleles per locus, while characterizing diversity in germplasm lines. The lower number of alleles / locus recorded in this study could be explained on two accounts: 1) generally cultivars represent low genetic diversity in comparison to the germplasm lines and 2) we had used metaphor agarose, which has lower resolution power in comparison to polyacrylamide gel or capillary electrophoresis used in above studies. Further, majority of SSR primers were highly polymorphic. The high level of polymorphism revealed that cultivars included in this study were considerably genetically diverse. This was obvious as different parental lines have been used in development of these cultivars (Table 1).

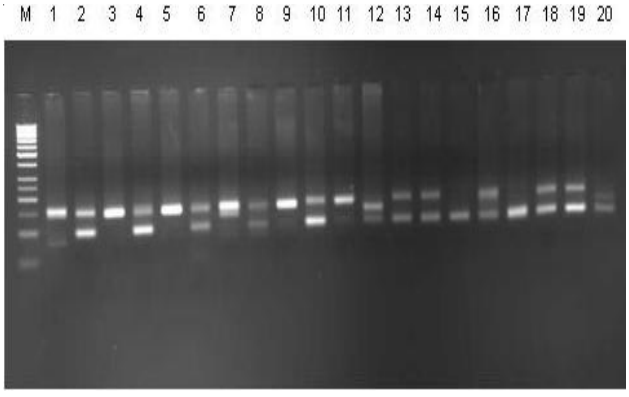


Fig. 1 Amplification profile of pearl millet cultivars using SSR locus PSMP 2088. Lane numbers corresponds to the serial number of the cultivars in Table 1. M= 50bp ladder

Table 1. List of varieties

variety	Parentage / Pedigree	Developer research centre(s)	Year of release
HHB 146	ICMA95222 X HTP 94/54	AICPMIP[a] CCSHAU, Hisar	2003
PUSA Composite 334	Bred from three selected lines and eight elight inbreds	IARI, NEW DELHI	2001
Pusa 605	841A X PPMI69	IARI, NEW DELHI	1999
CZP 9802	Bred by random mating 14 early maturing and high tillering FS progenies of high tillering population	CAZRI, Jodhpur	2003
PB 106	PSP41 X PP6	Proagro Hybrid	2001
Nandi 32	NMS7A X NMP24	New Nandi, Ahmadabad	1999
JBV 3	-	AICPMIP, Gwalior and ICRISAT	2001
GHB 558	ICMA94555 X J2290	AICPMIP -MRS[b], Jamnagar	2003
RHB 90	81A X RIB3135-18	AICPMIP -ARS[c], Durgapur	2000
HHB 117	HMS7A X H77/292	AICPMIP CCSHAU, Hisar	2004
HHB 67	843AX H77/833-2	AICPMIP CCSHAU, Hisar	1990
HHB 45	5141 A X H90 /4-5	AICPMIP CCSHAU, Hisar	1985
AVKB 19	Selection from material collected from Nagore, Rajasthan	IGFRI-RRS Avikanagar,	2006
MH 1236	95222A X J2454	AICPMIP MRS, Jamnagar	2006
MH 1234	ICMA9277A X J2454	AICPMIP MRS, Jamnagar	2006
PCB 164	Bred from 7 elite population and 27 inbred lines	AICPMIP PAU, Ludhiana	2007
ICMH 356	ICMA88004 X ICMR356	ICRISAT, Hyderabad	1993
Pusa Composite 266	Obtained by mixing eight lines	IARI, NEW DELHI	1997
Pusa Composite 443	-	IARI, NEW DELHI	2009
BAIF Bajra 1	-	BAIF, Research Foundation UruliKanchan	2008

[a]AICPMIP –All India coordinated pearl millet improvement programme, [b]MRS –Millets research station [c]ARS-Agriculture research station

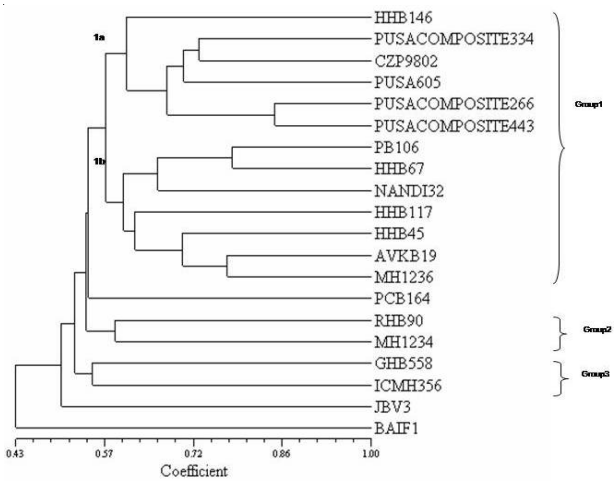
The PIC value of the SSR primers ranged from 0.05 to 0.77 with an average of 0.44 (Table 2). Based on the PIC values of the most informative loci, it was possible to greatly reduce the number of loci employed in cultivar discrimination. In the present study, by employing only 5 primer pairs with high PIC value *i.e.* PSMP2074, PSMP2013, PSMP2027, PSMP2084 and PSMP2229, all the pearl millets cultivars could be differentiated. Further, some SSR primers amplified unique band (s) to each cultivar. PSMP2088 amplified three bands (100 bp, 120 bp and 150 bp) in BAIF bajra 1 where as only two bands were present in other cultivars. Similarly, PSMP2084 amplified two bands (130 bp and 150 bp) only in the cultivar JVB-3. Combination of such primer pairs may serve diagnostic purposes such as analysis of genetic purity and cultivar protection.

Jaccard's similarity coefficient values for 20 cultivars ranged from 0.85 (between Pusa Composite 266 and Pusa Composite 4330) to 0.26 (between JBV-3, and BAIF Bajra 1) with an average of 0.52 indicating that a moderate level of genetic variation exists among the pearl millet cultivars used in this study. Yadav *et al.* (2007) reported average similarity 0.54 among 20 pearl millet genotypes comprising of commercially important hybrid, CMS and open pollinated varieties using ISSR markers.

A dendrogram was constructed using UPGMA cluster analysis based on the Jaccard's similarity coefficients. In this dendrogram, 18 cultivars were clustered into three major groups at a similarity coefficient 0.43 while two remained ungrouped (Fig. 2). Group I consisted of 13 cultivars, of which 12 cultivars could be further subdivided into two subgroups and one cultivar PCB 164 did not fall in any of the subgroup. Interestingly, all the cultivars developed from IARI, New Delhi were grouped in the subgroup I which revealed that IARI cultivars might have been derived from closely related set of parental lines. On the other hand, majority of the hybrids developed from HAU, Hisar were present in the subgroup II, suggesting that the parent lines used at the IARI, New Delhi would have been different from HAU, Hisar.

Grouping of the majority of the cultivars in the Group I can be explained on the ground that parental lines used in the development of the pearlmillet cultivars have been developed using parental lines derived either from Tift 23A1 or 5141A. BAIF Bajra 1 a forage purpose cultivar developed at BAIF, Research Foundation Urulikanchan was found to be genetically more diverse among the cultivars. Therefore, in general grouping of the cultivar appear to be consistent with respect to the institution from which they were developed. On this criterion the only exception was grouping of two cultivars, MH 1234 and MH 1236 developed from AICMP-MRS, Jamnagar, though both of them have common parent. It might be due to the quite divergent nature of the other parent used in the development of these two hybrids.

Fig. 2 Dendrogram based on UPGMA cluster analysis showing genetic relationship among pearl millet cultivars using 20 SSR markers.



Furthermore, Jaccard's coefficients matrix was subjected to PCA analysis for the two principal components. We found that cultivars groups identified by two dimensional PCA plot were consistent with those generated by UPGMA based dendrogram. Majority of the hybrids were clustered in the centre and the cultivars developed from IARI, New Delhi were grouped together. Likewise, BAIF bajra 1 which was genetically most diverse placed in the central left side of the plot.

In pearl millet, genetically uniform nature of the cultivar has been a matter of great concern. Current pearl millet breeding program in India is laying greater emphasis toward broadening the genetic base of pearl millet cultivars by diversifying the source of male sterility cytoplasm and introducing genes conferring resistance to diseases, insects and pests, and other economically important traits from the diverse germplasm lines and wild species of *Pennisetum* (Satyavati *et al.,* 2009). In view of this, identified highly polymorphic SSR markers can be of immense use in differentiating parental lines, cultivars and elite genotype, thereby helping selection of diverse lines, which can be used as a parent in breeding programs.

Table 2. List of the SSR primers and their characteristics

SSR Locus	SSR Motif	Size of the alleles (bp)	Number of alleles	Number of polymorphic alleles	PIC	Chromosome location (linkage group)
PSMP2013	(CT)19(GT)16	130-210	5	5	0.75	7
PSMP2018	(GT)30	160-210	4	3	0.46	6
PSMP2027	(GT)31	250-300	4	4	0.68	7
PSMP2074	(AC)11	140-270	5	5	0.77	4
PSMP2076	(AC)15	180-190	2	2	0.50	4
PSMP2084	(AC) 42	80-210	6	6	0.22	4
PSMP2088	(CA)24	100-200	3	3	0.58	2
PSMP2227	(GT)7	160-170	2	2	0.10	3
PSMP2229	(GT)5	200-220	3	3	0.66	5
PSMP2232	(TG)8	230-250	2	1	0.48	2
PSMP2237	(GT)8	180-200	3	3	0.51	2
PSMP2248	(TG)10	180-190	2	2	0.21	6
PSMP2270	(GA)26 imp*	300-310	2	1	0.26	6
PSMP2271	(GA)11	180-190	3	3	0.45	7
ICMP3013**	(AC)33	190-210	3	2	0.18	-
ICMP30019**	(CGTA)4	200-210	2	1	0.05	-
ICMP3021**	(CGTG)	180-190	3	3	0.28	-
ICMP3022**	(CGTG)5	190-210	2	2	0.50	-
CTM3	(CT)12	500-520	2	2	0.50	2
CTM10	(CT)22	190-200	3	3	0.56	3
CTM27	(CT)71	300-330	3	3	0.36	1

*imp – imperfect SSR repeat **marker not mapped to linkage group

In conclusion, we have utilized SSR markers for establishing genetic relationships among the Indian pearl millet cultivars. Moreover, as small set of SSR markers was able to distinguish all the cultivars, they may be used for testing the genetic purity of the seed lots of these cultivars.

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