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Original Research Article

Genetic Diversity and Hybrid Purity Analysis of Pearl Millet Using Molecular Markers

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A B S T R A C T

Pearl millet [*Pennisetum glaucum* (L) R. Br.] is one of the widely grown cereal crops in the arid and semi-arid region of India. In present investigation, six pearl millet hybrids *viz.*, AHB-1666 (released hybrid) and AHB-1214, AHB-1157, AHB-1199, AHB-1231, AHB-1240 (results of desirable crosses and hybrid are under pipelines for release) were screened with eleven RAPD primers and ten SSR markers. All the primers found highly polymorphic (except Xpsmp-2248 and m13_Xpsmp2229) and reproducible, generated distinct and polymorphic banding patterns. These primers enabled the assessment of genetic diversity and hybrid purity. The OPA-1 identified five hybrids by producing two male parent specific amplicons and three female specific amplicons and OPD-20 produced male parent specific amplicons of size ~300bp and ~ 250bp in two hybrids AHB-1666A, AHB-1240 respectively and one female parent specific amplicons of size 1kb and 200bp in two hybrids AHB-1199 and AHB-1231 respectively. Xpsmp-012 was found to be useful in identifying hybrid AHB-1214 and AHB-1157 with their respective female parent by generating female specific amplicons of size 400bp and 200bp (for AHB-1214) and 200bp (for AHB-1157). Based on RAPD and SSR dendrogram the percent similarity using Dice coefficient amongst lines was ranged between 73-97% and 72-95% respectively. The genetic similarity values retrieved from RAPD data ranged from 0.60 to 0.95 whereas genetic distance values ranged from 0.02 to 0.39. The genetic similarity values retrieved from SSR data ranged from 0.52 to 0.95 with an average of 0.21. The PIC values of eleven polymorphic RAPD markers ranged from 0.18 to 0.24 with an average of 0.20. RAPD primer OPA-11, OPD-10 and OPM-7 were observed to be most informative with the PIC value of 0.24 which significantly determined genetic relatedness among hybrids and parental lines. The PIC values of ten SSR markers ranged from 0.00 to 0.32. Based on combined RAPD and SSR data, average of 65.62% polymorphism was observed on the basis of banding patterns of each genotype and genetic distance values ranged between 0.04 and 0.362 with an average of0.21. The graphical result of Principle Co-ordinate Analysis was confirmation of results of genetic diversity and purity of hybrid with its parental lines.

Keywords

Pennisetum glaucum, RAPD, SSR, Hybrid purity, Genetic diversity, Dendrogram

Introduction

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is a member of the Gramineae family, in some of the hottest, driest regions of India and Africa, pearl millet is the only cereal that can be grown and so plays a critical role in food security. As demands for feeding the rising world population grow, need for crop plants yield improvement is being more apparent.

The fingerprinting of pearlmillet hybrids and identification of their genetic relationships

are very important for plant improvement, variety registration system, DUS (distinctness, uniformity and stability) testing, seed purity testing and the protection of plant variety and breeders' rights. Accordingly, clear-cut identification of elite crop varieties and hybrids is essential for protection and prevention of unauthorized commercial use.

On the other hand, purity of hybrid seeds supplied to farmers must surpass 96%. Conventional characterization of hybrids based on specific morphological and agronomic data is time-consuming, restricted to a few characteristics, influenced by environ- mental condition and inefficient. Molecular marker assisted identification with high power of genetic resolutions has emerged as a robust technique for cultivar fingerprinting, identity profiling, estimating and comparing genetic similarity, and variety protection.

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).

Pearl millet exhibits a tremendous amount of diversity at both phenotypic and genotypic level in India. In addition, SSR fingerprints are generally highly discriminative and are often used to distinguish varieties, or even individuals, and reveal parentage and identity (Bruford *et al.*, 1998).

The objective of this study was to assess genetic diversity of hybrids, their parental lines and promising lines using molecular markers.

Materials and Methods

Plant materials

The experiment was conducted at Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani (MS).The Pearl millet hybrids and their parental lines used in this study viz., A lines 88004A, 92444A, 99222A, 01777A, 99555A, 92888A, B lines (Hybrids) AHB-1666, AHB-1214 AHB-1157, AHB-1199, AHB-1231, AHB-1240. R lines (Male lines) AIB157R, 16428R, 16287R, 15043R, 16366R, 15157R. Promising lines PPC-6, ABPC-4, AIMP-92901.

RAPD analysis

Eleven random RAPD primers (Imperical Life Science) were selected for initial screening of the hybrids and parental lines of pearl millet OPA-1, OPA-11, OPA-12, OPC-20, OPD-10, OPD-18, OPD-20, OPM-7 OPM-16, OPM-20, OPZ -10.

PCR Reaction

The technique uses the repeat anchored primers of short oligonucleotide for DNA amplification by PCR. Amplification of genomic DNA tubes was then performed in thermal cycler (Eppendrof).

The components used for RAPD reaction mixture; Working concentration of genomic DNA (25ng per 25 µl reaction mixture), $MgCl_2$ (1.5 mM), dNTPs (0.2 mM) and *Taq* DNA polymerase (0.5U) were tried to obtain an optimum reaction mixture. The working concentration of primers (25pm /µl) were also tried for the complete amplification of the genomic DNA. A reaction mixture to a final volume of 25 µl was prepared. PCR cyclic parameters for RAPD markers; the annealing temperatures for eleven RAPD primers were optimized by using different

levels of temperature gradients. Four different levels of annealing temperature (35-38^0 C) of RAPD-PCR were set in RAPD fingerprint reaction.

PCR tubes were placed in thermal cycler (Eppendorf) for amplification of the genomic DNA as per the standardized conditions are initial denaturation at 94 °C for 4 min., denaturation at 94 °C for 45 sec., annealing temperature at 36 °C for 1 min., extension at 72 °C for 1 min and final extension at 72 °C for 10 min. for 40 cycles.

The amplified products were resolved on 1.5% agarose gel at 100 V for 1.5 h. 1.5% agarose gel was prepared by dissolving 1.5 g of agarose in 100 ml 1X TAE buffer. After cooling the solution to about 45^{0}C, 5µl ethidium bromide (10 mg/ml.) was added. After solidification 5µl of amplified products was mixed with 1µl of 6X gel loading dye and loaded into the wells.After electrophoresis, the gel was carefully taken out and photograph was taken on a Gel Documentation System (Alpha innotech).

SSR analysis

The SSR marker DNA fingerprint technique was used to assess genetic purity of 6 hybrids with their respective parents through genotyping. PCR reaction was performed with parental DNA by using 10 pair of Pearl Millet specific SSR markers (Xcump012, Xpsmp2208, Xpsmp2248, Xpsmp2236, Xpsmp2249, Xpsmp2273, Xicmp3027, Xctm25, m13_Xpsmp2229, Xicmp3032).

Ten SSR primers (Imperial Life Sciences) used in the present investigation are listed below in Table No.3.8.The sequence of pearl millet specific SSR marker was obtained from primers published in research papers (Supriya, 2010; Allnine *et al.,* 2008 and Khannan *et al.,* 2014).

Data scoring and analysis of RAPD and SSR markers

The amplified products generated from RAPD and SSR markers PCR reaction were resolved on agarose gel. Each amplification product was considered as RAPD bands and SSRbands were scored across all samples. Bands were scored as present (1) or absent (0). Missing and doubtful cases were scored as (9). Molecular weight of the bands was estimated using 1Kb DNA ladder for RAPD primers and 100bp DNA ladder for SSR primers (Banglore Genei) as standards.

The Polymorphism Information Value (PIC) was calculated as,

Polymorphism Information Value (PIC) $=\Sigma (1-P^2i)/n$,

Where n is the number of band positions analyzed in the set of accessions and P^2i is the frequency of ith allele. Data analysis was performed using NTSYS-PC (Numerical Taxonomy System, Version 2.02, Rohlf, 1993). The SIMQUAL programme was used to calculate the DICE coefficient. Dendrogram was constructed using unweighted pair group method for arithmetic mean (UPGMA) based on DICE coefficient. The polymorphic percentage of the obtained bands were calculated by using following formula,

Polymorphism % = (No. of polymorphic bands/Total bands) × 100

Results and Discussion

Genetic relationship among hybrid and their parental lines based on RAPD fingerprint profile analysis

In a systemic genetic diversity and hybrid breeding programme, it is essential to

identify superior parent to exploit the genetic variability for better heterosis development. In consideration of limitation of conventional Grow-Out-Test (GOT), there is need of rapid and accurate assay for assessment of genetic diversity and purity of hybrid seed. Thus, present study was implemented to exploit the utility of molecular marker for assessment of purity of six hybrids along with three promising lines for genetic diversity pearl millet.

In present investigation an attempt was made to exploit the potential of RAPD markers for identifying and discriminating, a set of hybrids and their parental lines, along with three promising lines using 11 random primers in initial screening and all of the primers *viz.*,OPA-1, OPA-11, OPA-12, OPC-20, OPD10, OPD-18, OPD-20, OPM-7, OPM-16, OPM-20, and OPZ-10 generated distinct and good banding patterns and revealed to be highly polymorphic and reproducible. All polymorphic primers were considered for assessment of genetic diversity and hybrid purity.

The primer OPD-10 could produce maximum of 100 % polymorphism while primer OPZ-10 showed 30.00 % polymorphism and found to be least polymorphic. These 11informative random primers were used for final scoring and obtaining similarity matrix of three promising lines along with parental lines and hybrids of pearl millet.

The average number of bands per primer per genotype ranged between 3.53 and 7.96 with an average of 5.21 bands per primer per genotype. The RAPD analysis produced 1206 amplicons, out of which 807 bands were polymorphic. The overall average of 65.62% polymorphism was observed on the basis of banding patterns of each genotype; where in total 73 loci were produced, out of these 56 loci were polymorphic. RAPD primer OPA-01, OPA-11, OPD-10 and OPM-7 were observed to be most informative with the PIC value of 0.24 which significantly determined genetic relatedness among hybrids and parental lines (Fig. 1 and 2). The PIC values of eleven polymorphic RAPD markers ranged from 0.18 to 0.24 with an average of 0.20. Similarity matrix was constructed from RAPD data by using Dice similarity coefficient. Based on the RAPD marker analysis, the genetic similarity values ranged from 0.602 to 0.958 whereas genetic distance values ranged from 0.028 to 0.398 (Fig. 5).

Genetic relationship among hybrid and their parental lines based on SSR fingerprint profile analysis

The trait specific SSR markers genotyping data were used for assessment of genetic diversity, hybrid testing or assessing the purity of hybrids, and their association with parental lines. Highly specific and co-dominant nature of the microsatellites encouraged their utilization for genetic diversity and purity testing of the hybrids and their respective parental lines. The present investigation performed by using 10 pearl millet specific SSR primers. Eight out of ten were polymorphic while two were found monomorphic.

The total number of polymorphic amplicons generated by 10 SSR primers among parents and hybrids were 319 out of which 119 were found to be polymorphic bands. Microsatellite data revealed the average 36.17% polymorphism on the basis of banding patterns of each genotype; wherein total 21alleles were produced; of these 11 were polymorphic. Eight SSR markers showed average polymorphism of 36.17% with 2 to 3 alleles (Fig. 3). The primer

Xcump-012, Xpsmp-2236 and Xpsmp2273 produced highly polymorphic patterns with 3 alleles among different hybrids and their parents. Primers Xpsmp-2208, Xpsmp-2249, Xicmp-3027, Xctm-25 and Xicmp-3032 produced 2 alleles. Remaining two primers Xpsmp-2248 and m13_Xpsmp2229 produced 1 allele each. The microsatellite primer Xpsmp-2208 was observed to be most informative with the PIC value of 0.32 which significantly determined genetic relatedness among hybrids and parental lines (Fig.4). The PIC values of eight polymorphic, SSR markers ranged from 0.00to 0.32 with an average of 0.18.

The genetic similarity was retrieved from microsatellite (SSR) data using Dice coefficient ranged from 0.52 to 0.95 with an average of 0.73 whereas genetic distance value ranged from 0.048 to 0.477 (Fig. 6), which were closer to those reported by Gupta *et al.,* (2015); Shaikh (2015), Arya *et al.,* (2014). Arya *et al.,* (2014) have reported the higher polymorphism *i.e.* 90.9% using SSR primers. About, 2 to 6 alleles (average of 3.4 alleles) and PIC values 0.28-0.61 were reported in sorghum hybrids and parental lines Shaikh (2015).

Hybrid confirmation based on RAPD/SSR fingerprint profile analysis

RAPD/SSR marker fingerprinting data was used for hybrid confirmation. Also, unique fragments for few of the genotypes represented considerable discrimination between the two genotypes for the given amplified loci by these random primers. The hybrid confirmation discussed here under was done by comparing banding patterns of hybrids with their respective parents as described by Shaikh T. R. (2015). Therefore, six types of marker patterns were considered (Table 1), which could help to identify purity of the hybrids.

Hybrid confirmation based on RAPD fingerprint profile analysis

Eleven RAPD primers *viz.*, OPA-1, OPA-11, OPA-12, OPC-20, OPD-10, OPD-18, OPD-20, OPM-7, OPM-16, OPM-20, and OPZ-10 found highly polymorphic and reproducible, generated distinct and polymorphic banding patterns. These primers enabled the assessment of genetic diversity and hybrid purity. OPA-1, OPA-12, OPD-10, OPD-18 and OPD-20 were found to be most informative for identification of true hybrid. OPA-1 identified five hybrids by producing two male parent specific amplicons and three female specific amplicons. OPD-20 produced male parent specific amplicons of size ~300bp and ~ 250bp in two hybrids AHB-1666A, HB-1240 respectively and one female parent specific amplicons of size 1kb and 200bp in two hybrids AHB-1199, AHB-1231 respectively.

Hybrid confirmation based on SSR fingerprint profile analysis

Eight SSR primers *viz.*, Xcump-012, Xpsmp-2208, Xpsmp-2236, Xpsmp-2249, Xpsmp-2273, Xicmp-3027, Xctm25 and Xicmp-3032 enabled to assess the purity of hybrid by generating 5 female parent specific polymorphic band and 3male parent specific polymorphic bands. Two SSR primers (Xpsmp-2248 and m13_Xpsmp-2229) generated monomorphic bands, which also proved the hybrid purity with its parents. The hybrid AHB-1666 and their parents were found homozygous with all SSR primers and it was revealed to be useful in assessing purity of hybrid AHB-1666 with its parents.

The purity of AHB-1214 could be assessed with Xcump-012 (produced two female parent specific alleles, ~200bp and ~400bp)

and Xicmp-3027produced (male parent specific allele, ~180bp) polymorphic allele pattern whereas hybrid AHB-1214 found to be homozygous with its parents when amplified with remaining microsatellites. The primer Xcump-012 was revealed to be useful in assessing purity of hybridAHB-1157 by producing female parent specific allele (~200bp), whereas AHB-1157 showed homozygous pattern with its parents when remaining microsatellites were used.

The primers Xpsmp-2236 and Xicmp-3032 were revealed to be useful in assessing purity of hybrid AHB-1199 by producing male parent specific alleles (~400bp and~250bp) respectively, whereas AHB-1199 found to be homozygous with remaining microsatellites. The hybrid AHB-1231with primer Xicmp-3032 showed presence of female parent specific allele (~250bp), whereas AHB-1231 found to be homozygous with remaining microsatellites and it was revealed to be useful in assessing purity of hybrid AHB-1231 with its parents (female parent 99555A and male parent 16366R).

The hybrid AHB-1240 with primer Xcump-012 revealed presence of female parent specific allele (~200bp), whereas AHB-1240 found to be homozygous with remaining microsatellites and it was proved to be useful in assessing purity of hybrid AHB-1240 with its parents. The male parent specific SSR marker suggested direct introduction of character from male parent and confirm the identity of respective hybrid. Several male and female parent specific polymorphic SSR alleles were reported and potentially helped to assess purity of hybrid.

The six hybrids *viz.*, AH-1666, AHb-1214, AHB-1157, AHB-1199, AHB-1231 and AHB-1240 were homozygous with Xpsmp-2248 and m13_Xpsmp-2229 showing the presence of common alleles with female and male parent specific marker. These six hybrids were designated as pure hybrid. Any hybrid could show the presence of either female or male parent specific marker into which transfer of character either from male or female to that hybrid individual, which can also be confirmed true as hybrid. Eleven RAPD (OPA-1, OPA-11, OPA-12, OPC-20, OPD-10, OPD-18, OPD-20, OPM-7, OPM-16, OPM-20 and OPZ-10 and ten SSR markers Xcump-012, Xpsmp-2208, Xpsmp-2248, Xpsmp-2249, Xpsmp-2273, Xcimp-3027, Xctm-25, m13_Xpsmp2229 and Xicmp-3032 were found useful in assessment of hybrid purity in the present investigation.

Thus, the present study revealed that the PCR based molecular markers are likely to be promising for identification, registration and protection of commercial sample and will gain more and more influence on plant breeding in future and will speed up breeding processes considerably.

Table.1 Six types of RAPD markers observed in hybrids and their parents

Markers Type	Male (M)	Hybrid (H)	Female (F)	Remark
1	+	+	+	Good marker to confirm hybrid of its respective parents (male and/or female)
2	-	+	+	
3	+	+	-	
4	+	-	+	Good markers to identify self and off types
5	-	-	+	
6	+	-	-	

Fig.1 Dendrogram generated by UPGMA analysis based on RAPD data

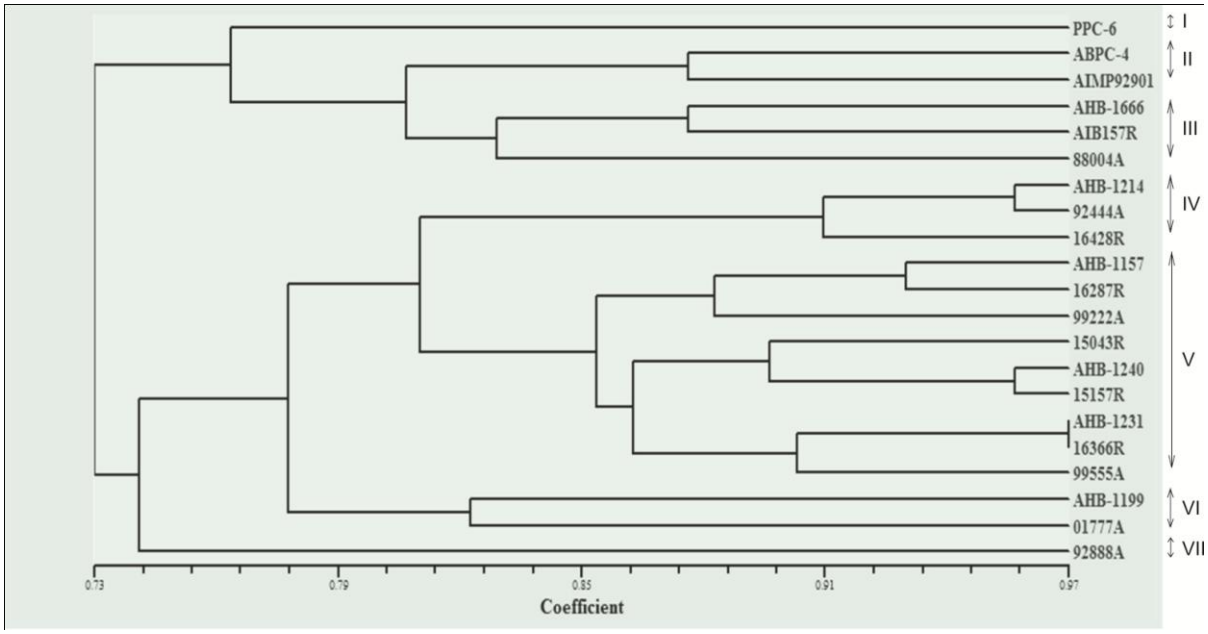
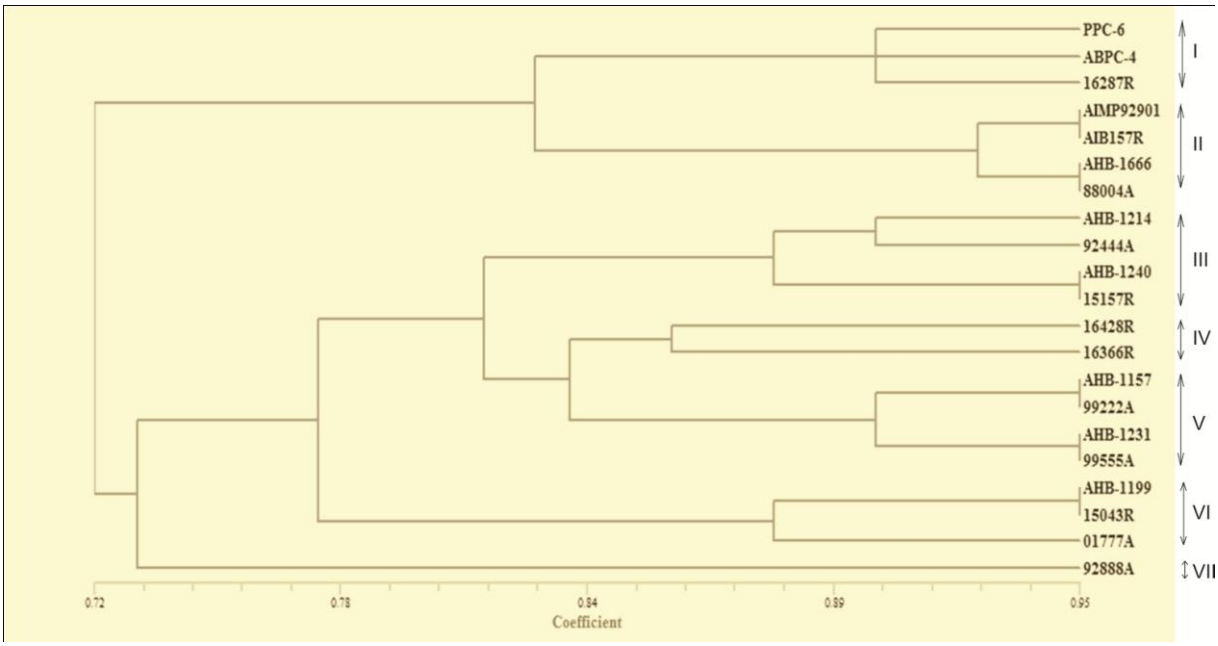


Fig.2 Dendrogram generated by UPGMA analysis based on SSR data



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