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GENETICS AND PLANT BREEDING

Assessment of SSR Specific Genetic Diversity for Fertility Restorer Gene (*rf*1) Among Various Sorghum (*Sorghum bicolour* (L.) Moench) Genotypes

Priyamvada Chauhan*[1], Pradeep Kumar Pandey[2] and Geeta Pandey[3]

[1]Assistant Professor, Agronomy, Lovely Professional University, Phagwara, 144411, Punjab, India
[2]Assistant Professor, Genetics and Plant Breeding, G.B.P.U.A.&T., Pantnagar, 263 145, Uttarakhand, India
[3]Ph.D scholar, Seed Science and Technology, G.B.P.U.A.&T., Pantnagar-263 145 U.S. Nagar, Uttarakhand, Pantnagar, 263 145, Uttarakhand, India

*Corresponding author: priyam09chauhan@gmail.com

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Abstract

The present study was carried out to evaluate the genetic variations for fertility restorer gene among various sorghum (*Sorghum bicolour* (L.) Moench) genotypes by SSR analysis. The experiment was conducted in UG Plant Biotechnology Lab of the Deptt. of Genetics and Plant Breeding, G.B. Pant University of Agriculture and Technology, Pantnagar (U.S. Nagar). A total of five SSR primers were used for the estimation of allelic diversity in thirty sorghum genotypes consisting, ten male sterile lines (female), ten maintainer lines and ten pollinator (male) lines. All primers were found to be polymorphic for all sorghum genotypes with polymorphism ranged from 80% (Xtxp250 and TS304T) to 100% (Drenshsbm 95 and TS050). Among all 34 alleles, the number of alleles per locus varied from 5 (Xtxp250 and TS050) to 10 (TS304T), with average of 6.8 alleles per locus. The PIC for all 5 primers varied from 0.45 (Drenshsbm 95) to 0.88 (TS304T), with a mean of 0.70. The genetic similarity (GS) was varied from 0.70 to 1.0 for all genotypes. Dendrogram revealed that male sterile lines ICSA264, SP55609A, ICSA702, $32A_2$ and ICSA293 showed more genetic diversity (30%) with restorers UPC2, HC260 and M35-1 and these combinations can be used as heterotic parents in improvement breeding program of sorghum.

Highlights

- Drenshsbm- 95 and TS050 exhibited 100% polymorphism with all thirty genotypes
- Restorers UPC2, HC260 and M35-1showed maximum diversity (30%) with male sterile lines ICSA264, SP55609A, ICSA702, $32A_2$ and ICSA293
- PIC value was maximum for primer TS304T (0.88)

Keywords: *Sorghum bicolor,* SSRs, allelic diversity, fertility restorer gene

Sorghum (*Sorghum bicolor* L. Moench) is the fifth most important cereal crop in the world after wheat, rice, maize and barley. It is a C4 crop which can be grown in environments that are too dry for cultivation of other cereals like maize (Rooney 2004) and is the staple food for millions of people in arid and semi-arid tropics of the world including India (Rakshit *et al.,* 2012b). In recent few years, sorghum has been emerged as a biofuel crop and is also growing for its rich stalk sugar content (Wang *et al.,* 2009). Sorghum breeding across the world is working on the development of high-yielding varieties and hybrids with better quality, disease resistance, drought tolerance and other useful agronomic traits (Klein *et al.,* 2008).

Male sterility is an important tool for the production of commercial hybrids in many crops including sorghum. The A1 cytoplasm has been used in

nearly all females in hybrid sorghum production. CMS plants of A1 cytoplasm have small pointed anthers and typically meiosis is normal but the microspores remain uninucleate and abort (Singh and Hadley 1961). Twenty one additional sources of cytoplasms that confer male sterility in sorghum have been described (Schertz *et al.,* 1989). Despite these discoveries, A1 cytoplasm is the primary CMS system used for hybrid seed production. In heterosis breeding, understanding genetic relationship among parental lines is of paramount importance and DNA markers such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) play an important role. Among different DNA markers, SSRs are most commonly used because they are hypervariable, co-dominant, robust, chromosome specific and multi-allelic in nature (Rakshit *et al.,* 2012a).

The fertility restoration in hybrids with A_1 cytoplasm depends on the nuclear background of the female and male parents. Depending on the parental lines, a single major fertility restorer gene was observed in some crosses while in other A_1 cytoplasm crosses two or more major genes (or multiple genes with minor effects) controlled fertility restoration (Schertz *et al.,* 1989). Molecular markers tightly linked to *rf1* loci have several applications in hybrid breeding of sorghum and their application permit the identification of genetically diverse lines for heterotic crosses without the need for test crosses. Therefore, the present study was undertaken to assess the genetic diversity for fertility restoration genes among different lines (A, B & R lines) of sorghum with molecular markers.

Materials and Methods

Collection of Plant Material

The present investigation was conducted in UG Plant Biotechnology Lab of the Dept. of Genetics and Plant Breeding, G.B. Pant University of Agriculture and Technology, Pantnagar (U.S. Nagar). The experimental materials consisted ten diverse CMS lines (female), ten maintainer lines and ten pollinators (male) lines. Thus, total of thirty sorghum lines were investigated using molecular markers (Table 1).

Table 1: Description of thirty lines of sorghum

Sl. No.	Genotypes	Line no.	Line type	Source
1	ICSA469	L1	Male sterile	ICRISAT
2	ICSB469	L2	Maintainer	ICRISAT
3	$MR750A_2$	L3	Male sterile	DSR, Hyderabad
4	$MR750B_2$	L4	Maintainer	DSR, Hyderabad
5	$11A_2$	L5	Male sterile	DSR, Hyderabad
6	$11B_2$	L6	Maintainer	DSR, Hyderabad
7	ICSA276	L7	Male sterile	ICRISAT
8	ICSB276	L8	Maintainer	ICRISAT
9	2219A	L9	Male sterile	DSR, Hyderabad
10	2219B	L10	Maintainer	DSR, Hyderabad
11	ICSA264	L11	Male sterile	ICRISAT
12	ICSB264	L12	Maintainer	ICRISAT
13	SP55609A	L13	Male sterile	ICRISAT
14	SP55609B	L14	Maintainer	ICRISAT
15	ICSA702	L15	Male sterile	ICRISAT
16	ICSB702	L16	Maintainer	ICRISAT
17	$32A_2$	L17	Male sterile	DSR, Hyderabad
18	$32B_2$	L18	Maintainer	DSR, Hyderabad
19	ICSA293	L19	Male sterile	ICRISAT
20	ICSB293	L20	Maintainer	ICRISAT
21	PC5	L21	Restorer	Pantnagar
22	UPC2	L22	Restorer	Pantnagar
23	PC8	L23	Restorer	Pantnagar
24	PC6	L24	Restorer	Pantnagar
25	CSV15	L25	Restorer	DSR, Hyderabad
26.	HC260	L26	Restorer	HAU, Hisar
27	JJ1041	L27	Restorer	Indore
28	CS3541	L28	Restorer	DSR, Hyderabad
29	SPV1616	L29	Restorer	DSR, Hyderabad
30	M35-1	L30	Restorer	Mahol

Isolation of Genomic DNA and Quantification

High molecular weight genomic DNA was extracted for molecular biology work including marker

detection technology. With the help of anion detergent (SDS), genomic DNA was extracted from fresh seedlings (eight days old) by the method described by Dellaporta *et al.,* (1989). For qualitative analysis of DNA, 1% agrose gel was prepared and electrophoresis was done at 50 volts for 3-4 hours. After that gel was visualized on an UV transilluminator.

PCR Amplification

For PCR amplification, a master mix without DNA template was prepared for different tubes to reduce pipetting error. The master mix was then redistributed in each PCR tube (18 µl each) and finally 2.0 µl of different DNA template (50 ng/µl) was added in each tube. PCR amplification was performed in a final volume of 20 µl reaction set up containing 2 µl of DNA, 1.2µl of dNTPs, 2.0 µl PCR buffer, 0.5 µl of forward primer, 0.5 µl of primer reverse primers, 0.4 µl of Taq DNA polymerase and 13.4µl of double distilled water. The reaction conditions were as follows: initial denaturation (94°C for 5 min) followed by 35 cycles of denaturation (94°C for 1 min), annealing at 55°C for 2 min (temperature reduced by 1°C for each cycle) and primer extension (72°C for 2 min). This step was followed by final cycle of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 7 min. PCR amplified DNA fragments were resolved by submerged horizontal electrophoresis in 1.5% agarose gel and visualized by staining with ethidium bromide. After completion of electrophoresis, image of the gel was viewed and saved in a gel documentation system (Alpha Imager EC). SSR primers were selected on the basis of their close association with fertility restoration in sorghum genotypes as proved in previous studies. The details of primers are given in the Table 2.

Data Analysis

Each SSR band was scored as present (1), absent (0), or as a missing observation for each genotype. An accession was assigned a null allele for a microsatellite locus whenever an amplification product could not be detected for a particular genotype-marker combination. To measure the informativeness of the markers, the polymorphism information content (PIC) for each SSR locus was calculated according to the formula (Weir 1996): PIC = $1 - (\sum pi^2)$, where i is the total number of alleles detected for a SSR marker, and pi is the frequency of the i^{th} plus allele in the set of the 30 sorghum genotypes investigated. PIC is also an estimate of the discriminatory power of a SSR marker locus. The frequencies of null alleles were not included in the calculation of PIC values.

Genetic similarity (GS) between genotypes *i* and *j* was estimated by using Jaccard's coefficient, as described by Sneath and Sokal (1973). Markers with missing observations for genotype *i* and/or *j* were not included in the calculation of GSij. Basing on the genetic similarity matrix, an unweighted pair group method of arithmetic averages (UPGMA) cluster analysis was used to assess the pattern of diversity among the rice genotypes. All calculations were performed by using NTSYS software (Rohlf 2000).

RESULTS

Polymorphism of SSRs

A total of 5 SSR primers were used to assess the extent of genetic diversity for fertility restorer gene *rf*1 across the 30 sorghum genotypes. All 5 SSR primers generated polymorphic patterns. A total of 34 alleles were detected, per locus varied from 5 to 10, with a mean of 6.8 alleles per locus (Table2). The primers showed a high level of polymorphism, ranging from 80 (Xtxp250 and TS304T) to 100% (Drenshsbm 95 and TS050). The PIC values, derived from allelic diversity and frequency among the genotypes, were not uniform for all of the SSR loci tested. The PIC for all 5 primers varied from 0.45 (Drenshsbm 95) to 0.88 (TS304T), with a mean of 0.70. In the set of 30 genotypes, 3 loci showed null alleles for primer Drenshsbm 95.

Genetic Diversity Levels

The genetic similarity (GS) values among sorghum genotypes varied from 0.70 to 1.00 (among A- and R-lines) and 0.88 to 1.00 (among A- and B-lines). The UPGMA dendrogram based on SSR data (Fig.) revealed two major groups (A and B). Group A included fifteen sorghum genotypes consisted of six male sterile, six maintainer and three restorer lines. Group A was further divided into two major clusters (I and II). Cluster I consisted of one male sterile line (ICSA469) and its corresponding B-line (ICSB469)

Table 2: Characteristics of the SSR primers used and their chromosome location, product size, number of polymorphic alleles, and PIC values calculated for set of thirty sorghum genotypes

SSR primers	Sequence	Chromosome location	Product size (bp)	Total no. of alleles	No of polymorphic alleles	% Polymorphism	PIC
Drenshsbm-95	F GTGGTTTGTTCCAGCCTTTG R GGGGGAGATGTGTTTCTACG	1	~35	6	6	100	0.45
Xcup05	F GGAAGGTTTGCAAGAACAGG R CCAGCCCAACAAGTGCTATC	1	~64	8	7	87	0.84
Xtxp250	F GCACATCCTCTAAAACTACTTAGT R GAACAGGACGATGTGATAGAT	1	~142	5	4	80	0.64
TS304T	F ACATAAAAGCCCCTCTTC R CTTTCACACCCTTTATTCA	1	~116	10	8	80	0.88
TS050	F TCGTGGATTTGCATTCCTTGAA R GAATGTGCCTTGTTTCTGTGCG	1	~71	5	5	100	0.72
	Mean			6.8	6	89.4	0.70

with 100% genetic similarity. Cluster II separated in three subclusters, a, b and c. Subcluster a included five male sterile lines (ICSA264, SP55609A, ICSA702, $32A_2$ and ICSA293) and their corresponding B-lines (ICSB264, SP55609B, ICSB702, $32B_2$ and ICSB293).

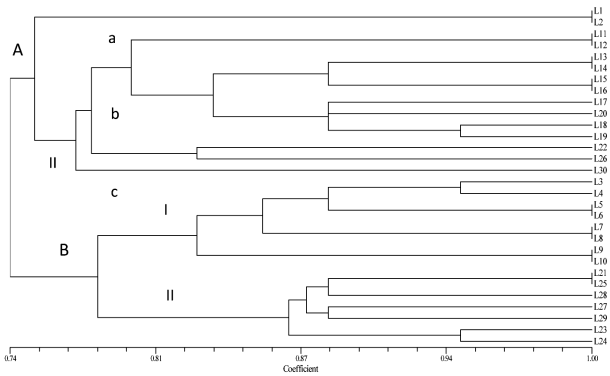

Fig 1: UPGMA Dendrogram of SSR specific genetic relationship within 30 different genotypes of sorghum

All male sterile lines showed 100% genetic similarity with their corresponding maintainer lines except $32A_2$ and ICSA293 which were 88% similar to corresponding B-lines for *rf1* gene. Subclusters, b and c enclosed three restorer genotypes (UPC2, HC260 and M35-1) which exhibited 79.3% similarity to each other. In this group, allelic diversity was recorded to be maximum in ICSA469 (28%) with all three restorers followed by ICSA264 (26%) and minimum in ICSA293 (20%). However, SP55609A, ICSA702 and $32A_2$ gave the allelic diversity of 22, 22 and 24%, respectively with all restorer lines for fertility restorer gene in this group.

Group B was divided into two clusters (I and II) which contained four male sterile lines ($MR750A_2$, $11A_2$, ICSA276 and 2219A) with their corresponding B-lines ($MR750B_2$, $11B_2$, ICSB276 and 2219B) in cluster I and seven restorer lines (PC5, PC8, PC6, CSV15, JJ1041, CS3541 and SPV1616) in cluster II. All male sterile lines showed 100% genetic similarity with their corresponding maintainer lines except $MR750A_2$ which was 94% similar to corresponding B-lines for *rf1* gene. Similarity indices were ranged from 82 to 100% among R-lines in this group. All male sterile lines exhibited 24 % genetic dissimilarity for restorer lines except $MR750A_2$ which showed 22% genetic dissimilarity with all the restores for fertility restorer gene.

DISCUSSION

The characterization and quantification of genetic diversity within closely related germplasm is of major importance due to its rational use of genetic resources. Analysis of genetic variation among genotypes is of fundamental interest to plant breeders, as it contributes immensely to selection, monitoring of germplasm, and also to prediction of genetic gain (Chakravarthy and Rambabu 2006). The A1 cytoplasm, one of approximately 21 known CMS systems for sorghum, remains the primary CMS system used for hybrid seed production (Schertz *et al.*, 1989). The inheritance of fertility restoration in A1 cytoplasm crosses is dependent on the parental lines involved through one or two major genes

appear to operate in many crosses (Schertz *et al.*, 1989).

Evaluation of genetic diversity based on phenological and morphological characters varies among environments, and its evaluation requires growing plants to full maturity. In contrast, DNA-based molecular markers have proven a powerful tool in the assessment of genetic variation and of genetic relationship within and among species, characterized by environmental influence. Several diversity studies of sorghum have been made previously by many workers (Ritter *et al.*, 2007 and Ali *et al.*, 2008). However, few attempts have been made to study the diversity among parental lines to classify them based on heterotic groups with limited success (Menz *et al.*, 2004).

In the present study, the five SSR markers were used for genetic diversity studies generated a total of 34 alleles with an average of 6.8 alleles per primer (Tab 2). These results are in congruence with the earlier results of Smith *et al.* (2000), but lower than reported by Menz *et al.* (2004) and Muraya *et al.* (2011). Markers with PIC more than 0.5 are efficient in discriminating genotypes and extremely useful in detecting the polymorphism rate at a particular locus (DeWoody *et al.*, 1995). All five SSR primers recorded PIC values more than 0.5 suggesting the discriminating nature of these markers. Similar results showing high PIC values were reported by others (Smith *et al.*, 2000, Muraya *et al.*, 2011, Ashfaq and Khan 2013, Sonkar *et al.*, 2016, Chakraborty *et al.*, 2013).

Cluster analysis clearly classified the sorghum lines into two groups based on the male sterile lines and restorers, which are heterotic in nature. The distinct grouping of male sterile lines and restorers is largely due to the fact that separate breeding programmes are being followed for seed parents and their restorers, and more importantly separate gene pool are being maintained to maximize the level of heterosis (Rooney and Smith 2000). In each cluster, Cluster analyses gave a clear differentiation between A and R lines. Dendrogram revealed that male sterile lines ICSA264, SP55609A, ICSA702, $32A_2$ and ICSA293 showed more genetic diversity (30%) with restorers UPC2, HC260 and M35-1. So these combinations can be used as heterotic parents in breeding improvement program of sorghum. Unlike these findings Smith *et al.* (2000) and Menz *et al.* (2004) could not classify the genotypes based on the heterotic groups using SSR markers.

All the primers showed a high level of polymorphism in this study. Primers Drenshsbm and Xcup05 found to be 100 and 87% polymorphic, respectively. Reddy *et al.* (2011) reported that primers are associated with *rf*1 gene in A1 sorghum cytoplasm. There was found a clear polymorphism among the loci for fertility restorer genes of different sorghum genotypes. Reddy *et al.* (2011) also reported that Drenshsbm 95 primer have polymorphism with restoration of fertility gene in genotypes of sorghum. Primer Xtxp250 found to be 80% Polymorphic for fertility restorer gene in this study. Klein *et al.* (2001) reported that Xtxp250 primer is linked with QTLs for fertility restoration gene, *rf*1 in A1 sorghum genotypes and very useful for the identification of A1 sorghum genotypes. For primers TS304T and TS050, polymorphism was detected 80 and 100%, respectively. Delong *et al.* (2010) reported that these primers are associated with fertility restorer genes in sorghum. They recorded polymorphism among QTLs for all the genotypes and concluded that these primers are very useful for the identification of different lines in sorghum for the improvement of breeding programme.

Conclusion

All five primers used for genetic diversity analysis were found to be polymorphic for all 30 genotypes of sorghum. The UPGMA dendrogram revealed that almost all male sterile lines showed 100% similarity with their corresponding maintainer lines for fertility restorer gene. Restorer type genotypes were found to be genetically distant from each other and from male sterile lines. Hence these can be used as heterotic combinations for successful breeding programme. Genotypic diversity analysis suggested all 5 markers were able to determine the genetic relationship among all thirty genotypes for *rf1* gene in sorghum. Study would provide a common ground for sorghum accessions identification, breeding and improvement.

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