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ALLELE SPECIFIC DIVERSITY USING FERTILITY RESTORATION RELATED SSR MARKERS IN SORGHUM [*SORGHUM BICOLOR* (L.) MOENCH]

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SUMMARY

A total of five SSR markers were used for the estimation of allelic diversity for fertility restorer gene in 17 sorghum genotypes consisting of five male sterile lines (female), four maintainer lines and eight pollinators (male) lines. The experiment was conducted in UG Plant Biotechnology Lab of the Department of Genetics & Plant Breeding, G. B. Pant University of Agriculture & Technology, Pantnagar (U. S. Nagar). All markers were found to be polymorphic for all sorghum genotypes. Polymorphism ranged from 50% (Xtxp250) to 100 per cent (Drenshsbm 95). Among all 22 alleles, the number of alleles per locus varied from minimum of 2 to maximum of 7, with average alleles of 4.4 per locus. The PIC for all five primers varied from 0.50 (Drenshsbm 95) to 0.87 (TS304T), with a mean of 0.73. The genetic similarity (GS) varied from 0.55 to 1.0 for all genotypes. Among all three clusters, cluster 3 was found to be most diverse having 0.472 allelic diversity for *rf1* gene. Dendrogram revealed that male sterile lines ICSA 467 and ICSA 276 showed more genetic diversity with restorers CS3541, PANTCHARI 5 and CSV 15 and these combinations can be used as heterotic parents in improvement breeding programme of sorghum.

Key words : *Sorghum bicolor,* SSRs, allelic diversity, fertility restorer gene

Sorghum [*Sorghum bicolor* (L.) Moench] is an important food, feed and fodder crop worldwide. It can tolerate drought, water-logging and saline-alkali soils. It is preferred over other non-leguminous fodder crops due to its high yielding capacity, better quality and utilization in various forms i. e. green fodder, stover, silage and hay. Besides food and fodder, it is also used for preparation of alcoholic beverages, fibres, sugar and syrup. Ethanol prepared from sweet sorghum is also appearing to be a viable source of non-conventional fuel (Dorofeev, 1992). Male sterility had been an important tool for the production of commercial hybrids in sorghum. The A_1 cytoplasm has been used in nearly all females in hybrid sorghum production. CMS plants of A_1 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, A_1 remains 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*., 2012). The fertility restoration in hybrids with A1 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 A1 cytoplasm crosses two or more major genes (or multiple genes with minor effects) controlled fertility restoration (Schertz *et al.*, 1989). Hence, the inheritance of fertility restoration in A_1 cytoplasm i.e. male sterile lines can be considered as complex although one or two major genes appear to operate in many crosses. Molecular markers tightly linked to *rf* loci have several

applications in hybrid breeding of sorghum and their application would permit the identification of genetically diverse lines for heterotic crosses without the need for test crosses. Therefore, the present study aims at identifying different lines (A, B and R lines) of sorghum with molecular markers and determination of polymorphism for *rf* gene among different lines of sorghum.

MATERIALS AND METHODS

The present investigation was conducted in UG Plant Biotechnology Lab of the Department of Genetics & Plant Breeding, G. B. Pant University of Agriculture & Technology, Pantnagar (U. S. Nagar). The experimental materials consisted of five diverse CMS lines (female), four maintainer lines and eight pollinators (male) lines. Thus, total of 17 sorghum lines were investigated using molecular markers (Table 1).

TABLE 1
Description of 17 lines of sorghum

S. No.	Genotypes	Line number	Source
A.	**Female lines**		
1.	ICSA 467	A	ICRISAT, Hyderabad
2.	ICSA 469	A^1	ICRISAT, Hyderabad
3.	ICSA 276	A^2	ICRISAT, Hyderabad
4.	11A2	A^3	DSR, Hyderabad
5.	MR750A2	A^4	ICRISAT, Hyderabad
B.	**Maintainer lines**	^{5}	
6.	ICSB 469	B	ICRISAT, Hyderabad
7.	ICSB 276	B^2	ICRISAT, Hyderabad
8.	11B2	B^3	DSR, Hyderabad
9.	MR750B2	B^4	ICRISAT, Hyderabad
C.	**Pollinators**	^{5}	
10.	M35-1	R	Mahol
11.	CS 3541	R^1	DSR, Hyderabad
12.	UPCHARI 2	R^2	G. B. P. U. A & T., Pantnagar
13.	JJ 1041	R^3	Indore
14.	CSV 17	R^4	Udaipur
15.	PANTCHARI 5	R^5	G. B. P. U. A & T., Pantnagar
16.	CSV 15	R^6	DSR, Hyderabad
17.	SPV 1616	R^7	DSR, Hyderabad
		^{8}	

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. SDS method described by Dellaporta *et al*. (1989) was used for DNA isolation. Qualitative analysis was done by agrose gel electrophoresis. For that 1 per cent agrose gel was prepared and electrophoresis was done at 50 volts for 3-4 h. After that gel was visualized on an UV transilluminator.

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 (24 ml each) and finally 1.0 ml of different DNA template was added in each tube. PCR amplification was performed in a final volume of 25 ml reaction set up containing 1 ml of DNA, 2 ml of dNTPs, 2.5 ml PCR buffer, 2.5 ml of forward primer, 2.5 ml of reverse primers and 0.4 ml of Taq DNA polymerase. 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 per cent 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 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 – (?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 17 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.

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 17 sorghum genotypes

SSR primers	Sequence	Chromo-some location	Allele size (bp)	Total number of	No. of polymorphic alleles	Per cent polymorphism	PIC
Drenshsbm- 95	F GTGGTTTGTTCCAGCCTTTG R GGGGGAGATGTGTTTCTACG	1	30	2	2	100	0.50
Xcup05	F GGAAGGTTTGCAAGAACAGG R CCAGCCCAACAAGTGCTATC	1	60	7	4	57	0.77
Xtxp250	F GCACATCCTCTAAAACTACTTAGT R GAACAGGACGATGTGATAGAT	1	140	4	2	50	0.78
TS304T	F ACATAAAAGCCCCTCTTC R CTTTCACACCCTTTATTCA	1	115	5	3	60	0.87
TS050	F TCGTGGATTTGCATTCCTTGAA R GAATGTGCCTTGTTTCTGTGCG	1	70	4	4	100	0.72
	Mean			4.4	3	73.4	0.73

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. Basied 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 AND DISCUSSION

Polymorphism of SSR Markers

A total of 5 SSR markers were used to assess the extent of genetic diversity for fertility restorer gene *rf*1 across the 17 sorghum genotypes. All markers were observed to be polymorphic with polymorphism varying from 50 (Xtxp250) to 100 per cent (Drenshsbm 95). A total of 22 alleles were detected across the 17 sorghum genotypes. The number of alleles per locus varied from minimum of 2 to maximum of 7, with average alleles of 4.4 per locus (Table 2). The polymorphic information content (PIC) values, derived from allelic diversity and frequency among the genotypes, were not uniform across the SSR loci tested. The PIC for all 5 primers varied from 0.50 (Drenshsbm 95) to 0.87 (TS304T), with a mean of 0.73. In the set of 17 genotypes, 10 loci showed null alleles. The frequency of genotypes revealing null alleles varied from a minimum of 1 (for primer Xcup05) to a maximum of 4 for primer TS304T.

Genetic Diversity Levels

The genetic similarity (GS) values among sorghum genotypes varied from 0.55 (between M 35-1 and SPV 1616, CS 3541 and SPV 1616) to 1.0 (between ICSA 469 and ICSB 469, ICSA 276 and ICSB 276, 11 A2 and 11 B2, MR 750A2 and MR750 B2). Between A and R lines, GS values ranged from 0.60 (between ICSA 469 and M 35-1, ICSA 469 and UPCHARI 2, ICSA 460 and JJ 1041, 11 A2 and M 35-1, 11 A2 and UPCHARI 2, 11 A2 and JJ 1041, MR 750A2 and SPV 1616) to 0.95 (MR 750A2 and UPCHARI 2).

Genetic Diversity Pattern

The UPGMA dendrogram based on SSR data (Fig. 1) revealed three clusters that were demarcated at a cut-off similarity coefficient level of 0.67, below which the similarity values narrowed conspicuously. The composition and size of clusters along with allelic diversity values among sorghum genotypes are given in Table 3. Cluster 1 included five genotypes consisting of male sterile lines ICSA 467 and ICSA 276. No diversity was observed between A and their corresponding B lines for allelic loci for *rf1* gene in this cluster. This cluster revealed lowest allelic diversity among genotypes of A and R lines. Cluster 2 comprised four genotypes including MR 750A2 male sterile line. A and its corresponding B line was found to be similar for *rf1* gene loci in this cluster and 0.236 allelic diversity was found among A and R lines. The largest cluster, cluster 3 comprised eight genotypes including ICSA 469 and 11

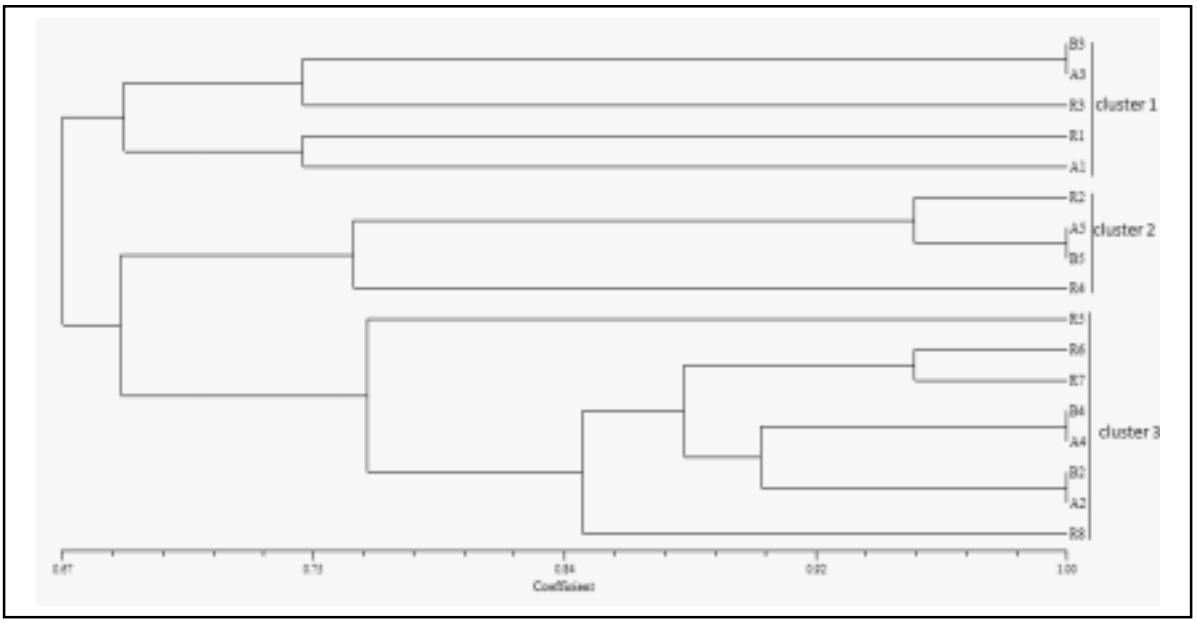

Fig. 1. The UPGMA dendrogram based on SSR data.

TABLE 3
Composition and size of clusters along with allelic diversity values among sorghum genotypes

Cluster number	No. of genotypes	Nature of genotypes (included male-sterile line)	Allelic diversity
1.	5	A_1 & A_2	0.219
2.	4	A_5	0.236
3.	8	A_2 & A_4	0.472

A2 male sterile lines, having 0.472 of allelic diversity and no diversity between A and their corresponding B lines for *rf1* gene.

The characterization and quantification of genetic diversity within closely related crop germplasm has long been a major goal, as it is essential for the rational use of genetic resources. Above and beyond, analysis of genetic variation among breeding materials 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). Deu *et al.* (2006) found that the bicolour race of sorghum had high genetic diversity and many rare alleles, which is not surprising considering that this race is considered to be the oldest and most widely distributed geographically due to its several uses (fodder, brooms and sweet stems). The A_1 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 A_1 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).

Estimation of genetic diversity based on phenological and morphological characters varies among environments, and its evaluation requires growing plants to full maturity. Also, the markers based on expressed gene products, proteins and enzymes or isozymes are also influenced by the environments and reveal a low level of polymorphism and low abundance (Ravi *et al.*, 2003). In contrast, DNA-based molecular markers have proven a powerful tool in the assessment of genetic variation and in the elucidation of genetic relationship within and among species, characterized by abundance and untouched by environmental influence. Several diversity studies of sorghum have been made previously by many workers (Tao *et al.*, 1993; Ahnert *et al.*, 1996; Ritter *et al.*, 2007; 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 used for genetic diversity studies generated a total of 22 alleles with an average of 4.4 alleles per primer (Table 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). 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; Agrama and Tuinstra, 2003; 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).

Cluster analysis clearly classified the sorghum lines into three 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 pools are being maintained to maximize the level of heterosis (Rooney and Smith, 2000). In each cluster, cluster analyses failed to give a clear differentiation between A and R lines. Dendrogram revealed that male sterile lines ICSA 467 and ICSA 276 showed more genetic diversity with restorers CS 3541, PANTCHARI 5 and CSV 15. So, these combinations can be used as heterotic parents in breeding improvement programme 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.

Menz *et al.* (2004) reported that different sorghum accessions showed a diverse genetic background in their study based on marker assisted selection. All the primers showed a high level of polymorphism in this study. Primers Drenshsbm and Xcup05 were found to be 100 and 57 per cent polymorphic, respectively. Reddy *et al.*, (2011) reported that primers were associated with *rf*1 gene in A_1 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 primer had polymorphism with restoration of fertility gene in genotypes of sorghum. Primer Xtxp250 was found to be 50 per cent Polymorphic for fertility restorer gene. Klein *et al.* (2001) reported that Xtxp250 primer was linked with QTLs for fertility restoration gene, *rf*1 in A_1 sorghum genotypes and very useful for the identification of A_1 sorghum genotypes. For primers TS304T and TS050, polymorphism was detected 60 and 100 per cent, respectively. Delong *et al.* (2010) reported that primers TS304T and TS050 were associated with fertility restorer genes in sorghum. They recorded polymorphism among QTLs for all the genotypes and concluded that these primers were very useful for the identification of different lines in sorghum for the improvement of breeding programme.

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