Validation of cytoplasmic genetic male sterility in *rabi* sorghum hybrids and their parents using diagnostic set of microsatellite markers

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Abstract

Sorghum is an important cereal crop where cytoplasmic male sterility is exploited for hybrid breeding. Presently, A_1 cytoplasm CGMS system has been well established because of the ease and stability of fertility restoration. Conventionally, genetic purity of hybrids is ensured by grow out test. In this context, DNA marker assisted selection technology offers an efficient alternative over traditionally grow out test especially in terms of time, environmental influence, space and cost-effective. Microsatellite markers are best suited for genetic purity assessment due to co-dominant nature.

The present investigation involved hybrid identification in sorghum through a set of robust microsatellite markers on the cytoplasmic genetic male sterility hybrids 'AKMS 30A × AKRB 335-3', 'AKMS 30A × AKRB 431' and 'AKMS 30A × AKRB 428' along with their parental lines 'AKMS 30A' (CMS line), 'AKRB 335-3', 'AKRB 431' and 'AKRB 428' (Restorers). Seventeen SSR primers were used for the study, out of which five primers Xtxp 406, Xtxp 297, Xtxp 211, Xnhsbm 1084 and SB 2386 linked to Rf1, Rf2, Rf5 and Rf6 (fertility restoration factors, respectively found polymorphic and were able to distinguish between the parental lines and hybrids. The structure software differentiates the genotypes in three subpopulation. The study revealed that marker assisted selection an efficient tool in the quality control of the commercial hybrid seeds for the genetic purity assessment and accelerate the testing of genetic purity of hybrids in seed industries in a short time.

Keywords: SSR Polymorphism, Genetic purity, Marker assisted selection.

Introduction

Sorghum (*Sorghum bicolor* L. Moench) is the fifth most important cereal crop in the world, cultivated globally for food, fodder, feed and fuel, popularly known 'King of Millet' or 'Great Millet' on account of its large grain size among millet and vast area under it and extensively used in hybrid seed production[9]. In Africa and South East Asia, sorghum is providing food of subsistence to over 500 million people[19]. Sorghum is also an emerging model for highly productive C_4 crops with a genome size ~730 Mb[21,38]).Worldwide, it is cultivated in about 42.5 M (million) hectare area with an annual production and an average productivity of 59.91 MMT (million metric tonnes) and 1.41 MT (metric tonnes) / hectare respectively whereas, in India, 5.58 M (million) hectare area under cultivation with production and productivity were 4.41 MMT (million metric tonnes) and 0.79 MT (metric tonnes) / hectare respectively[2].

Several hindrances are responsible for unstable production on account of *kharif* and *rabi* sorghum essentially including biotic and abiotic constraints coupled with the cultivation of traditional landraces or genotypes. The major limiting factor in *rabi* sorghum hybrid is the fertility restoration[22]. The presence of restorer genes that enable the production of fertile F_1 hybrids using cytoplasmic genetic male sterility (CGMS) approach is essential for the cost-effective production of hybrid sorghum seed[11]. Till date, total of nine different sources of sterile cytoplasm *viz*. A_1 (*milo*), A_2[32] A_3[24], A_4[37], Indian A_4 (A_4M, A_4VZM, A_4G)[25], A_5, A_6, 9E[36] and KS cytoplasms[30] have been reported in sorghum.

Amongst all nine CMS types, *milo* (A_1) CMS system has been extensively used in developing the hybrids for commercial cultivation in America, China, Australia and India. Till date, nearly all the released hybrids have *milo* (A_1) cytoplasm[27]. Restoration on *milo* (A_1) cytoplasm is quite easy as majority of the breeding lines are acting as restorers on this cytoplasm. Globally released sorghum hybrids were mostly relying on the A_1 cytoplasm[27].

However, the fertility restoration on the non-*milo* cytoplasm is very cumbersome and very limited work has been done in this area[10,16,17]. Two major genes along with some modifier genes controlled restoration of fertility in A_1 cytoplasm[16,17] with selection of complete fertility restoration in the restorers and complete sterility in the CMS parents[11]. In the present investigation *rabi* sorghum hybrid 'AKMS 30A × AKRB 335-3','AKMS 30A × AKRB 431' and 'AKMS 30A × AKRB 428' has been developed using CMS line 'AKMS 30A (A-line) and restorer lines 'AKRB 335-3', 'AKRB 431' and 'AKRB 428' at the Biotechnology center, Dr. PDKV, Akola.

Grow-out test (GoT) is the conventional method to ensure the genetic purity of the hybrids to minimize the considerable losses of the hybrid yield in the seed industries[39]. The major limitation factor in GoT is that it is extremely cumbersome, time consuming, requires field trials and cannot be routinely performed prior the onset of flowering[5,40]. In the given context an alternative approach used for the hybridity assessment is the molecular markers. Marker assisted selection offers an efficient and accurate system for cultivar identification and for genetic purity assessment at seedling stage[40]. Hybrid identification in crop species through molecular characterization is an effective approach to increase speed of backcrossing conversion, thus reducing time taken to produce crop varieties with desirable characteristics[1,8].

The utilization of the simple sequence repeats (SSRs) for DNA fingerprinting is mostly preferable because of its reproducibility and co-dominant nature[6,40]. Keeping this view, a series of diagnostic set of SSRs identified in the present investigation were employed for the molecular characterization of the CGMS based hybrids along with their parental lines for the fertility restoration trait to facilitate marker-assisted selection.

Material and Methods

Experimental layout and material: Parents and hybrids were sown during *rabi* - 2016 at the experimental field of Sorghum Research Unit (SRU), Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra state, India, using randomized block design. The soil was medium black with uniform topography and precise drainage system. The row to row and plant to plant spacing was of 45×15 cm and dibbling method used for sowing purpose. During the flowering, 3-4 irrigations were provided as per requirement and the material was harvested as per maturity of the genotypes. The sorghum genotypes are mentioned in table 1.

Genomic DNA extraction: Cetyl Trimethyl Ammonium Bromide (CTAB) method was used for the DNA extraction[20] using 0.5 g leaf sample of 10 days old seedling and relative purity of DNA was estimated with nanophotometer (Implen). The ultimate concentration of each DNA sample was adjusted to 30 ng/µl and was used for the molecular profiling.

PCR Analysis: Seventeen SSR primers enlisted in table 2 linked to different fertility restoration factors *Rf*1, *Rf*2, *Rf*3, *Rf*5 and *Rf*6 were employed for the molecular characterization of parental lines along with their hybrids. PCR reactions were performed in Eppendorf thermal cycler with a 20 µl reaction volume for SSR primers containing 2 µl of 10 X PCR buffer, 0.4 µl of 10 mM dNTPs, 0.25 µl of 5 U of Taq DNA polymerase and 0.75 µl of 10 pM each of forward and reverse primers. A total of 1 µl of genomic DNA (80 ng/ µl) was used for PCR reaction and volume was making up with nuclease free water (15.8 µl). A touch down PCR programme was followed for SSR amplification (modified from Bohra et al[7]).

The initial denaturation was followed for 5 m at 95ºC followed by denaturation at 94ºC for 30 sec, annealing temperature was set up at 56ºC (1ºC is reduced after each cycle) for 20 sec and finally extension for 30 sec at 72ºC and the programme was set up for four cycles. The next 36 cycles were performed with 30 sec at 94ºC, 45 sec at 52ºC and 1 min at 72ºC and a final extension step for 10 m at 72ºC. The amplified products were then visualized under gel documentation system and amplicons were then scored for the presence or absence of heterozygosity.

Gel Electrophoresis and Documentation: The PCR products were resolved on 4% agarose gel and the allelic sizes of the amplified fragments were determined using 100 bp DNA ladder (Fermentas). Further, amplicons were visualized under AlfaImager.

Population Structure: Population structure in the sorghum association panel of 1 CMS, 3 restorers and their corresponding hybrids were analyzed based on microsatellite data (5 SSR primer combinations) by use of the admixture model implemented in STRUCTURE V.2.3.4.

Results and Discussion

The commercial success of CGMS technology mostly relies on the genetic purity of hybrid seeds. CGMS system has been well documented in several crop species like rice[33], sorghum[28], sunflower[29], brassica[35] etc. Molecular markers offer an alternative approach to the traditional GoT method and these markers could be explored as powerful tools for the hybridity and genetic purity assessment[6]. SSRs are mostly employed because of its multiallelic and codominant nature and their reproducibility[31]. The microsatellite markers are co-dominant in nature and have good reproducibility with good banding pattern, therefore it is used for hybridity and hence purity testing[3,8].

The parents (AKMS 30A, AKRB 335-3, AKRB 431 and AKRB 428) were screened for the detection of marker polymorphism (Table 3). The scorable amplicons with expected product sizes were obtained. Among the seventeen SSRs screened, five SSRs viz. Xtxp 406 (*Rf*1), Xtxp 297 (*Rf*2), Xtxp 211 (*Rf*2), Xnhsbm 1084 (*Rf*5) and SB 2386 (*Rf*6) enabled the detection of the polymorphic fragments between parents. These SSRs were found polymorphic and are linked to fertility restoration gene *Rf*1, *Rf*2, *Rf*5 and *Rf*6, thus it is assume that these genes were present in the restorers and therefore restore fertility in the A1 cytoplasm based CMS lines AKMS 30A to yield good *rabi* sorghum hybrids with high heterotic value.

Polymorphic SSRs were further used for hybridity assessment of genotype 'AKMS 30A × AKRB 335-3, AKMS 30A × AKRB 431 and AKMS 30A × AKRB 428'. Recovery of both paternal- and maternal-specific amplicons

in the SSR profiles of both hybrids 'AKMS 30A × AKRB 335-3, AKMS 30A × AKRB 431 and AKMS 30A × AKRB 428' confirmed the true heterozygous nature of the hybrid (Fig. 1). However, parental lines were found 100% homozygous as no residual heterozygosity was detected which confirmed their purity and considered as true pure lines (Table 3). The molecular profiling of CGMS based hybrid and its parents in *rabi* sorghum using microsatellite primer have been shown in fig. 1.

Table 1
Genotypes used in the present investigation

S.N.	Genotypes	Source
	CMS (Cytoplasmic male sterile) line	
1	AKMS-30A	CMS line from Sorghum Research Unit, Dr. PDKV, Akola.
	Restorers	
2	AKRB-335-3	Restorer from Sorghum Research Unit, Dr. PDKV, Akola.
3	AKRB-431	Restorer from Sorghum Research Unit, Dr. PDKV, Akola.
4	AKRB 428	Restorer from Sorghum Research Unit, Dr. PDKV, Akola.

Table 2
***Rf* loci linked SSR markers used for parental genotyping**

S.N.	Marker	Gene	Forward sequence	Reverse sequence	Tm (F)	Tm (R)	Base pair (bp)
1	Xtxp18	*Rf*1	ACTGTCTAGAACAAGCTGCG[15]	TTGCTCTAGCTAGGCATTTC	57.3	55.3	231
2	Xtxp 250	*Rf*1	GCACATCCTCTAAAACTACTTAGT[4]	GAACAGGACGATGTGATAGAT	57.6	55.9	283
3	Xtxp 406	*Rf*1	GGCCTGAATCTCAGTGTTAAG[13,14]	AGTTGCCTGCTTCGACACTT	57.9	57.3	287
4	Xtxp 297	*Rf*2	GACCCATATGTGGTTTAGTCGCAAAG[34]	GCACAATCTTCGCCTAAATCAACACAAT	63.2	60.1	220
5	Xtxp 211	*Rf*2	TCAACGGCCAATGATTTCTAAC[4]	AGGTTGCGAATAAAAGGTAATGTG	56.5	57.6	221
6	Xtxp 50	*Rf*2	TGATGTTGTTACCCTTCTGG[4]	AGCCTATGTATGTGTTCGTCC	55.3	57.9	310
7	Xtxp 616	*Rf*2	GCATTTCTTTCCTGCAATGAC[12]	GCAGACAAGATCTCACCCAAG	55.9	59.8	280
8	Xtxp 304	*Rf*2	ACATAAAAGCCCCTCTTC[12]	CTTTCACACCCTTTATTCA	51.4	50.2	206
9	Xtxp 31	*Rf3*	TGCGAGGCTGCCCTACTAG[15]	TGGACGTACCTATTGGTGC	61.0	56.7	222
10	Xtxp 38	*Rf3*	ACAAACCGCGACGAAGTAAC[15]	ACAAGGCAAAGCACAAAGC	57.3	54.5	437
11	Xtxp 34	*Rf3*	TGGTTCGTATCCTTCTCTACAG[15]	CATATACCTCCTCGTCGCTC	58.4	59.4	360
12	Xnhsbm 1083	*Rf5*	TGACTGGTCAACAACGAGGA[11]	CTCTCCCGTGCATGTACTCA	57.3	59.4	219
13	Xnhsbm 1084	*Rf5*	CATTTCACATTCAAGGTCATGG[11]	ACATTTATGGGTGCGTGCTT	56.5	55.3	280
14	Xnhsbm 1085	*Rf5*	CGTGAATGAATGAACGAACG[11]	GAGAGCAGAGGGGTAACTGC	55.3	61.4	248
15	SB 2386	*Rf*6	GGCGGTAGGTGTAAAAAGGAAGGA[23]	GCATGCCCTACGACTCTTGTGTCT	62.7	64.4	169
16	Xnhsbm 1195	*Rf*6	CTAAAGGAACTCGGCGATTG[23]	GTCGTGTCCTTCGGCATTAT	57.3	57.3	255
17	Xnhsbm 1197	*Rf*6	CTGCAGAGGTCCTAGTGACAAA[23]	GAACGACTTATAATTTGAGCCAGA	60.3	57.6	262

Rf-Restoration of fertility genes, Tm (F)- Annealing temperature of forward primer, Tm (R)- Annealing temperature of reverse primer, bp- base pair

Table 3
SSR primers with their amplification, polymorphism and heterozygosity status

			Heterozygosity detected			Homozygous parents			
Primers	**Amplification status**	**Polymorphism status**	**AKMS 30A × AKRB 335-3**	**AKMS 30A × AKRB 431**	**AKMS 30A × AKRB 428**	**AKMS 30A**	**AKRB 335-3**	**AKRB 431**	**AKRB 428**
Xtxp18	A	M	-	-	-	-	-	-	-
Xtxp 250	A	M	-	-	-	-	-	-	-
Xtxp 406	A	P	+	+	+	-	-	-	-
Xtxp 297	A	P	+	+	+	-	-	-	-
Xtxp 211	A	P	+	+	+	-	-	-	-
Xtxp 50	A	M	-	-	-	-	-	-	-
Xtxp 616	A	M	-	-	-	-	-	-	-
Xtxp 304	A	M	-	-	-	-	-	-	-
Xtxp 31	A	M	-	-	-	-	-	-	-
Xtxp 38	A	M	-	-	-	-	-	-	-
Xtxp 34	A	M	-	-	-	-	-	-	-
Xnhsbm 1083	A	M	-	-	-	-	-	-	-
Xnhsbm 1084	A	P	+	+	+	-	-	-	-
Xnhsbm 1085	A	M	-	-	-	-	-	-	-
SB 2386	A	P	+	+	+	-	-	-	-
Xnhsbm 1195	A	M	-	-	-	-	-	-	-
Xnhsbm 1197	A	M	-	-	-	-	-	-	-

A-Amplified, M-Monomorphic, P-Polymorphic, + Presence of heterozygosity of particular marker, - Absence of heterozygosity, Hybrids- AKMS 30A × AKRB 335-3, AKMS 30A × AKRB 431 and AKMS 30A × AKRB 428, AKMS 30A- Female (CMS) line, AKRB 335-3, AKRB 431 and AKRB 428- Restorers

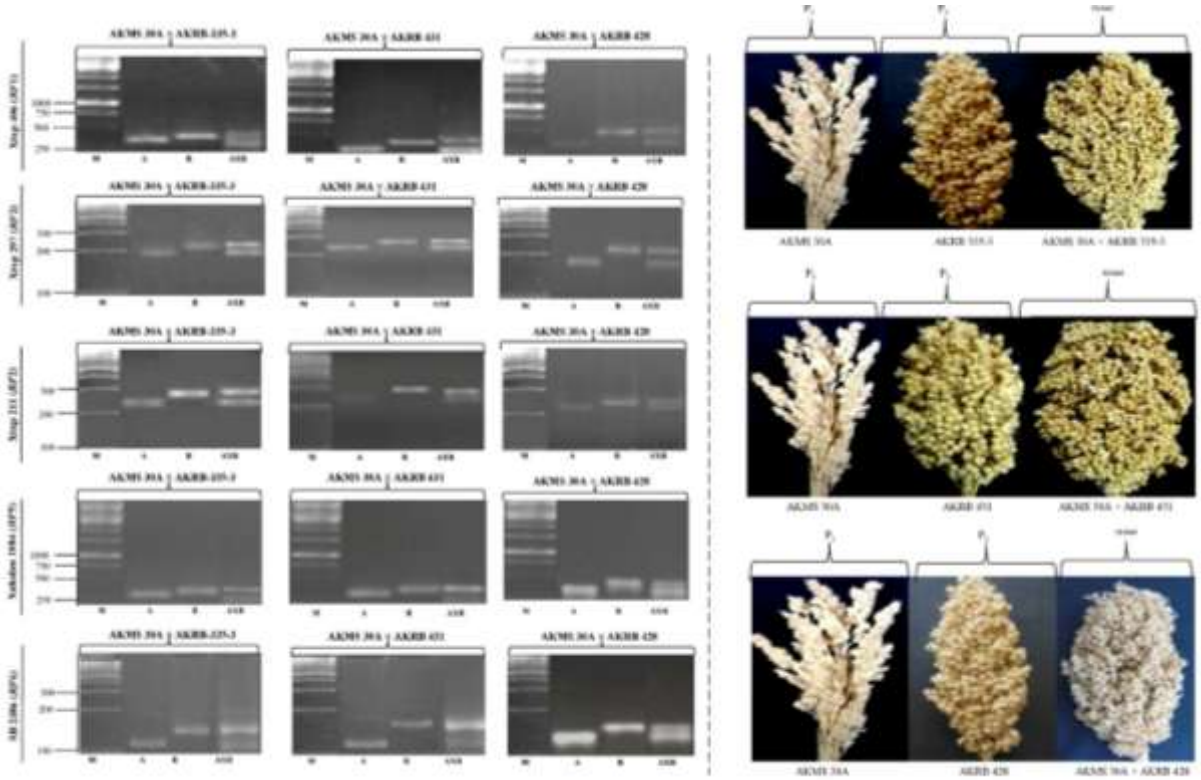

Fig. 1: *Rf* linked SSR based molecular profiling, A (AKMS 30A), R (AKRB 335-3 , AKRB 431 and AKRB 428) and AxR (AKMS 30A x AKRB 335-3, AKRMS 30A x AKRB 431 and AKMS 30A x AKRB 428). The name of each SSR markers has been indicated on the side of each gel image. M: marker 100 bp and 1kb DNA ladder (on extreme left) was chosen as standard. The right side image consist of hybrids along with their respective parents P_1 (AKMS 30A), P_2 (AKRB 335-3, AKRB 431 and AKRB 428) and hybrids (AKMS 30A x AKRB 335-3, AKMS 30A x AKRB 431 and AKMS 30A x AKRB 428)

Table 4
SSR marker alleles identified as molecular tags for sorghum hybrids

SSR markers	*RF* gene	CL	Primer sequence	Amplicon size (bp)								
				CMS line (AKMS 30A)	Restorer line (AKRB 335-3)	Hybrid AKMS 30A X AKRB 335-3	CMS line (AKMS 30A)	Restorer line (AKRB 431)	Hybrid AKMS 30A X AKRB 431	CMS line (AKMS 30A)	Restorer line (AKRB 428)	Hybrid AKMS 30A X AKRB 428
Xtxp 406	*RF1*	8	F:GGCCTGAATCTCAGTGTTAG R: TTGCCTGCTTCGACACTT	270	287	270/287	260	287	260/287	268	287	268/287
Xtxp 297	*RF2*	2	F:GACCCATATGTGGTTTAGTCGCAAAG R:GCACAATCTTCGCCTAAATCAACAAT	190	220	190/220	220	280	220/280	185	220	185/220
Xtxp 211	*RF2*	2	F:TCAACGGCCAATGATTTCTAAC R:AGGTTGCGAATAAAAGGTAATGTG	221	285	221/285	221	290	221/290	221	287	221/287
Xnhsbm 1084	*RF5*	5	F:CATTTCACATTCAAGGTCATGG R:ACATTTATGGGTGCGTGCTT	260	280	260/280	260	280	260/280	260	280	260/280
SB 2386	*RF6*	4	F:GGCGGTAGGTGTAAAAAGGAAGGA R:GCATGCCCTACGACTCTTGTGTCT	120	169	120/169	135	169	135/169	126	169	126/169

RF- fertility restoration gene, CL- Chromosome location, F - forward sequence of primers, R - Reverse sequence of primers, bp - base pairs (amplicons size)

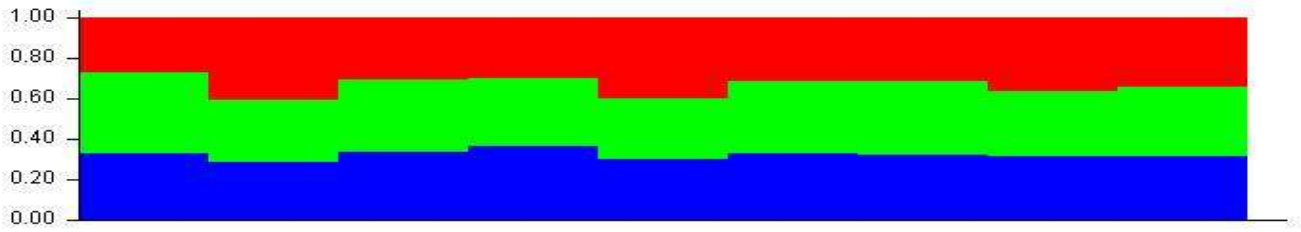

Fig. 2: STRUCTURE output of sorghum association panel with K=3 clusters based on 5 polymorphic SSR marker combinations. The first subpopulation (sp1) is labeled with blue color (CMS), second subpopulation (sp2) with green color (restorers) and third subpopulation (sp3) with red color (hybrids)

The A line (AKMS-30A) produce 270bp, 190bp, 221bp, 260bp and 120bp amplicons size for the SSRs Xtxp 406, Xtxp 297, Xtxp 211, Xnhsbm 1084 and SB 2386 respectively whereas the corresponding restorer (AKRB 335-3) produced 287bp, 220bp, 285bp, 280bp and 169bp respectively. However, F_1 hybrid AKMS 30A x AKRB 335-3 amplified both the amplicons of A line and Restorer line. Similar amplicons were generated in case of hybrid AKMS 30A x AKRB 431, where A line (AKMS 30A) amplified fragments of 260bp, 220bp, 221bp, 260bp and 135bp, whereas, restorer line (AKRB 431) produced 287bp, 280bp, 290bp, 280bp and 169bp respectively and F_1 hybrid AKMS 30A x AKRB 431 exhibited alleles of both parents A line and Restorer line whereas amplicons generated in case of hybrid AKMS 30A x AKRB 428 as A line (AKMS 30A) amplified fragments of 268bp, 185bp, 221bp, 260bp and 126bp, whereas, restorer line (AKRB 428) produced 287bp, 220bp, 287bp, 280bp and 169bp respectively and F_1 hybrid AKMS 30A x AKRB 428 exhibited alleles of both parents A line and Restorer line, confirming the heterozygosity of hybrid for the respective markers with amplification of both amplicons of parents mentioned in table 4 and fig. 1.
These marker alleles have co-dominant effect. Moreover, no residual heterozygosity was detected in the parental lines using these *Rf* linked SSR markers thereby confirming their true pure-line nature harboring 100% homozygosity (Table 4 and Fig. 1). The SSR markers had both female and male specific amplicons and are useful in genetic purity testing and hybridity assessment.

Xtxp 406, Xtxp 297, Xtxp 211, Xnhsbm 1084 and SB 2386 linked to *Rf*1, *Rf*2, *Rf*5 and *Rf*6 and combination of all these four genes could distinguish three sorghum hybrids AKMS 30A x AKRB 3353-3, AKMS 30A x AKRB 431 and AKMS 30A x AKRB 428. Thus, PCR based DNA markers associated with *Rf* genes could be used as candidate tools to expedite the genetic purity and hybridity assessment of lines.

The population structure of the association panel consisting of 1 CMS, 3 restorers and their corresponding hybrids were

analyzed using STRUCTURE software to avoid false positive associations due to the population structure. By use of the Evanno method, three probable subpopulations were identified in the sorghum association panel using STRUCTURE V.2.3.4 (Fig. 2). CMS was grouped in the first subpopulation (sp1), restorers in second subpopulation (sp2), whereas, hybrids in third subpopulation (sp3). The mean value of the fixation index (Fst) for first subpopulation was 0.11, while Fst for the second subpopulation was 0.21 and Fst for third subpopulation was 0.37.

Arya et al[3] utilized ten sorghum hybrids along with their thirteen parental lines for molecular characterization of hybrids and found two diagnostic markers for the genetic purity testing of all the ten sorghum hybrids. For example, SSR profiles for fertility restoration locus were constructed for hybrid 296A x PVK809 using marker Xtxp 304 reported by Praveen et al[23] and it was found that *Rf*2 gene linked with primer Xtxp 304 is associated with fertility restoration trait in *rabi* sorghum hybrid and further used for the hybridity assessment.

Despite the availability of different CMS systems, hybrid vigor is exploited mainly using *milo* (A_1) cytoplasm because of ease and stability of fertility restoration[18,26].

Conclusion

Genetic purity testing is the major criteria for the purity assessment. Grow out test helps for the identification of the genetic purity but time consuming and cumbersome as compared to molecular markers. The identified polymorphic SSRs could be of use in future associated with CMS male fertility restoration and partial fertility which will allow breeders to use diversified parents for the hybrid breeding programme.

Microsatellite markers were used as powerful markers against parental lines and restorers to choose the good restorer (s) having best fertility and combining ability with other CMS lines to develop hybrids having high fertility restoration ability with high heterotic values.

Acknowledgement

We are grateful to all research scientists of biotechnology department for their cooperation and support during this investigation.

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(Received 12th September 2018, accepted 21st November 2018)