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Microsatellite markers for assessing genetic purity of hybrid seed in sorghum

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ABSTRACT Genetic purity of sorghum hybrid seed is tested conventionally through Grow-out-test (GOT) involving morphological descriptors, which is costly and time consuming. The utility of microsatellite (SSR) markers for analyzing the genetic purity involving three sorghum hybrids was verified. Field grown four-hundred hybrid plants were examined for purity involving morphological descriptors and with selected SSR markers. SSR assay confirmed the impure hybrids identified through GOT with banding pattern suggesting the impure plants could be from A x B seed and seed of R parent. Compared to GOT, levels of genetic purity of seed lots assessed with markers was slightly less indicating greater ability and precision of markers in identifying impure plants. The study showed that SSR markers are efficient to test genetic purity of sorghum hybrid seed, and can be safely employed to replace GOT for making quick and timely decisions for marketing of hybrid seeds in the following crop season.

Keywords: Sorghum, hybrids, genetic purity, SSRs, grow-out-test

Sorghum is one of the most important cereal crops in the semi-arid tropics catering to the needs of food, feed and fodder requirements. It is the second cereal crop after maize wherein heterosis was exploited commercially world over. Ever since the discovery of cytoplasmic male sterility system (CMS) in sorghum was reported [1], several commercial F1 hybrids have been developed by both public and private institutions for cultivation by farmers. The increased sorghum productivity in India or elsewhere has been largely attributed to the adoption of hybrids over a large area. In India, sorghum hybrids have recorded 20-25% grain yield advantage over high yielding open-pollinated varieties. The success of hybrids depends mainly on the production and timely supply of genetically pure homogenous quality seeds to farmers. Therefore, to ensure the benefit of growing hybrids, supply of genetically pure seed to farmers becomes paramount. In seed production plots, the seed production practices adopted by farmers provide opportunities for seed contamination leading to the presence of "off-types" in the hybrid seed lot. This is particularly so in a country like India wherein maximum hybrid seed production is done through contract farming involving a farmer or farmer groups whose education and technical skill levels are substantially low [2]. Thus, strict monitoring of hybrid seed production becomes necessary at several crop growth stages to ensure genetic purity.

Assessment of genetic purity of hybrid seed lot is an important step in hybrid seed production and ensuring quality seed supply in marketing chain. Conventionally, this is done following GOT ascertaining breeder designated morphological descriptors. This involves growing plants to maturity and evaluating several morphological characteristics to decide the purity of the hybrid seed. This is time consuming as it takes one full crop season and demands capital, labour and space. Therefore, it is necessary to adopt a method, which is quick, saves time, labour, capital and other resources, and can act as an alternative to GOT to take quick and timely decisions to market

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hybrid seed in the following crop season. For this, several workers have advocated the use of molecular markers in different crops such as rice, maize, cotton, [2-5]. So far, in sorghum, DNA markers have not been used for testing genetic purity of hybrid seed. Of the numerous DNA marker systems available at hand for purity test, microsatellites (SSRs) have been preferred in many crops due to their ease in use, abundance, ubiquitous, codominance, higher polymorphism levels etc. Therefore, the present study was undertaken to analyze the utility of SSR markers in assessing the genetic purity of three sorghum hybrids (CSH9, CSH13 and CSH14) as compared to the results of grow-out test carried out in the field.

MATERIALS AND METHODS

Plant material

Three commercial hybrids (CSH9, CSH13 and CSH14) and their parents were used in the study. The details of the hybrids with their parents are given in Table 1. Seeds of these three hybrids were obtained from the seed unit of the Directorate of Sorghum Research (DSR), Rajendranagar, Hyderabad, India.

Hybrid purity assessment: Grow-out-test

A random sample of 400 seeds representing the commercial seed lot of each of the three hybrids were taken for testing genetic purity both by conventional GOT as well as by SSR marker analysis. The experiment was conducted during the post-rainy season of 2009 at the research farm of DSR, Hyderabad. Each of the hybrids was grown separately in 25 rows with 3 m long rows. Plants were spaced at 15 cm in a row spaced at 45 cm between rows. The genetic purity of hybrids was assessed using the breeder defined morphological descriptors. All the plants (400) in each hybrid were tagged and examined throughout their growing season. Any plant, which deviated morphologically from its morphological descriptors, was considered as off-type (impure) plant. Based on the number of off-type plants, the genetic purity was calculated and expressed in percentage.

Molecular analysis: DNA extraction

Genomic DNA from individual tagged F1 plants from each hybrid population was extracted using leaf tissue following CTAB method [6]. The DNA quantity of each sample estimated and DNA concentrations were normalized at 20-30 ng/µl. The DNA quality was evaluated by running 1µl of DNA on a 1% agarose gel.

Table 1. Pedigree details of the hybrids used in the study

S. No.	Hybrid	Female parent	Days to flowering*	Male parent	Days to flowering*	Hybrid features	Center
1.	CSH 9	296A	68	CS3541	64	Medium duration hybrid with grain yield potential of 39 q/ha and dry fodder of 98 q/ha.	DSR, Hyderabad
2.	CSH 13	296A	68	RS29	61	Dual-purpose hybrid with 39 q/ha grain yield and dry fodder of 144 q/ha potential.	DSR, Hyderabad
3.	CSH 14	AKMS14A	70	AKR150	74	Early duration hybrid with grain yield potential of 38 q/ha and dry fodder of 88 q/ha.	PDKV, Akola

* As per the variety release proposal

Table 2. Details of microsatellite markers used in the study

S. No.	Marker	LG	Forward primer	Reverse primer	Tm
1	Xtxp320	1	TAAACTAGACCATATAC TGCCATGATAA	GTGCAAATAAGGGCTAGAGTGTT	55
2	Xcup53	1	GCAGGAGTATAGGCAGAGGC	CGACATGACAAGCTCAAACG	55
3	msbCIR286	1	GCTTCTATACTCCCCTCCAC	TTTATGGTAGGATGCTCTGC	55
4	msbCIR306	1	ATACTCTCGTACTCGGCTCA	GCCACTCTTTACTTTTCTTCTG	56
5	Xcup63	2	GTAAAGGGCAAGGCAACAAG	GCCCTACAAAATCTGCAAGC	54
6	Xcup69	2	ACAGCACCAAGGTGAAGGAC	ATGTAGGGCACCAGCTTCAC	55
7	msbCIR238	2	AGAAGAAAAGGGGTAAGAGC	CGAGAAACAATTACATGAACC	55
8	Xisep310	2	TGCCTTGTGCCTTGTTTATCT	GGATCGATGCCTATCTCGTC	60
9	Xgap84	2	CGCTCTCGGGATGAATGA	TAACGGACCACTAACAAATGATT	55
10	Xcup11	3	TACCGCCATGTCATCATCAG	CGTATCGCAAGCTGTGTTTG	54
11	Xcup32	3	ACTACCACCAGGCACCACTC	GTACTTTTTCCCTGCCCTCC	55
12	Xtxp31	3	TGCGAGGCTGCCCTACTAG	TGGACGTACCTATTGGTGC	60
13	Xtxp59	3	GAAATCCACGATAGGGTAAGG	GACCCAGAATAGAAGAGAGG	55
14	Xgap10	4	GTGCCGCTTTGCTCGCA CTTCTC	TGCTATGTTGTTTGCTTCTCC	55
15	Xtxp343	4	CGATTGGACATAAGTGTTC	TATAAACATCAGCAGAGGTG	55
16	Xtxp60	4	GCTAGCTGACGCACGTCTCTG	TGCAACCGAGCGGTGACTA	55
17	Xtxp212	4	TTTCCCCTCTTTCTTGTGTC	CTCGGCGTCGTCGTA	55
18	Xisep228	4	GACATGGCCAGCTAAGAGGA	CCATGCAGTGATCGTTGTGT	60
19	Xtxp15	5	CACAAACACTAGTGCCTTATC	CATAGACACCTAGGCCATC	55
20	Xtxp65	5	CACGTCGTCACCAACCAA	GTTAAACGAAAGGGAAATGGC	55
21	Xtxp136	5	GCGAATAGCATCTTACAACA	ACTGATCATTGGCAGGAC	55
22	Xtxp145	6	GTTCCTCCTGCCATTACT	CTTCCGCACATCCAC	55
23	Xtxp176	6	TGGCGGACATCCTATT	GGAGAGCCCGTCACTT	55
24	Xtxp57	6	GGAACTTTTGACGGGTAGTGC	CGATCGTGATGTCCCAATC	55
25	Xisep441	6	CCCAACTAGGCATCCGTTTA	TTGGGTGTCAGCAATGAAGA	60
26	Xtxp40	7	CAGCAACTTGCACTTGTC	GGGAGCAATTTGGCACTAG	55
27	Xtxp278	7	GGGTTTCAACTCTAGC CTACCGAACTTCCT	ATGCCTCATCATGGTTCGT TTTGCTT	50
28	Xtxp312	7	CAGGAAAATACGAT CCGTGCCAAGT	GTGAACTATTCGGAAGA AGTTTGGAGGAAA	55
29	msbCIR283	7	TCCCTTCTGAGCTTGTAAAT	CAAGTCACTACCAAATGCAC	54
30	SbAGB02	7	CTCTGATATGTCGTTGTGCT	ATAGAGAGGATAGCTTAT AGCTCA	55
31	Xtxp354	8	TGGGCAGGGTATCTAACTGA	GCCTTTTTCTGAGCCTTGA	55
32	Xgap34	8	AACAGCAGTAATGCCACAC	TGACTTGGTAGAGAACT TGTCTTC	55
33	Xtxp47	8	CAATGGCTTGCACATGTCCTA	GGTGCGAGCTAGTTAAGTGGG	55
34	Xisep632	8	AGAGAGGAGGTCCCAAATGC	TTAAGGCCCAAACAAACTGG	60
35	Xtxp10	9	ATACTATCAAGAGGGGAGC	AGTACTAGCCACACGTCAC	50
36	Xtxp287	9	GCAAGCGAGCTGAC TTATGTAACGAGA	CAAAGTGCTACTAAAC CTATGCAGGGTGAA	55
37	Xtxp289	9	AAGTGGGGTGAAGAGATA	CTGCCTTTCCGACTC	55
38	Xcup2	9	GACGCAGCTTTGCTCCTATC	GTCCAACCAACCCACGTATC	54
39	Xtxp141	10	TGTATGGCCTAGCTTATCT	CAACAAGCCAACCTAAA	55
40	Xgap1	10	TCCTGTTTGACAAGC GCTTATA	AAACATCATACGAGC TCATCAATG	55
41	msbCIR248	10	GTTGGTCAGTGGTGGATAAA	ACTCCCATGTGCTGAATCT	56
42	SbAGA1	10	CGAACCATGATAAATGACTG	ATCCGTTTCACAAAAAAAGT	54

SSR markers and polymerase chain reaction
Forty-two microsatellite markers covering sorghum 10 chromosomes were randomly selected to study polymorphism between the parents of the hybrids. The list of primers along with the annealing temperatures and expected amplicon size are given in Table 2. For testing the genetic purity of hybrid seed, one polymorphic SSR marker for each hybrid (SbAGB02 for CSH9, Xtxp343 for CSH13 and Xtxp15 for CSH14) was used. The PCR plate was labeled with respect to sample number and 2ml (~ 50-100 ng) of template DNA was added to the respective wells. The master mix consisted of 2 ml forward primer, 2 ml reverse primer, 0.5 ml dNTPs, 1.0 ml *Taq* DNA polymerase (M/s Bangalore Genei Pvt. Ltd.), 1.0 ml of 10x PCR buffer (Tris with 1.5 mM MgCl2) and 1.5 ml of sterile distilled water was added to make up the volume to 10 ml. The master mix (8.0 ml) was dispensed to the PCR plate with template DNA. PCR was carried out in a thermal cycler (M/s Applied Biosystem, USA). The PCR profile of 5 min at 94°C, 35 cycles of 30 sec at 94°C, 30 sec at 55°C, 1 min at 72°C and 7 min at 72°C for final extension was followed. After the completion of the PCR, the plate was stored at -20°C.

Genotyping and purity assessment
For hybrid seed purity assay, the amplicons were resolved on 4% agarose gels stained with ethidium bromide, and were visualized under UV in a gel documentation system (Alpha Imager, USA) and hybrid impurities were detected based on deviations in expected amplification pattern of A and R lines. The estimate of genetic purity in each of the three hybrids from marker analysis was compared with that of GOT.

RESULTS AND DISCUSSION

The commercial success of hybrid technology depends largely on the quality of hybrid seed supplied to farmers, especially that of genetic purity. Ever-growing popularity of hybrids and active involvement of public and private sectors in large-scale hybrid seed production demands strict quality control in terms of monitoring seed genetic purity at both parental and hybrid seed production stages to ensure the success of a hybrid.

Sorghum F1 hybrid seed is mass-produced using a three line system involving a cytoplasmic male sterile line (A-line), its isogenic maintainer line (B-line) and a hybrid specific restorer line (R-line). F1 hybrid seed is produced by open pollination of A-line with its specific R-line in isolated seed production plots in farmers' fields. Before hybrid seed is marketed, the seed needs to be tested for its genetic purity since any genetic contamination could result in significant economic loss to the farmers. In rice, it is estimated that 1% impure hybrid seed could lead to a loss of 100 kg ha-I of grain yield [7]. Therefore, establishment of genetic purity is an essential seed quality control requirement to avoid unacceptable impurity level in a hybrid seed lot prior to its marketing.

Purity of the cultivars is assessed conventionally by the GOT on a composite representative sample of the seed that is to be marketed [8]. As said, this method is time consuming, sensitive to environmental conditions. Further, there is also a possibility that adverse climatic conditions may damage and destroy GOT crop and make it difficult to collect the requisite data [9]. Unless certified for genetic and physical purity, hybrid seed lots are not permitted for marketing and cultivation. This may result in additional expenditure in storage and increased hybrid seed cost. Thus, there is a need to assess the genetic purity of cultivars using an assay that is both accurate and faster so that the seed produced in one season can be released for marketing and commercial cultivation in the ensuing crop season [9].

DNA-marker based methods have been recommended as alternative to GOT

which are helpful in rapid and large-scale purity screening of hybrid seed lots [10].

The instances of use of molecular markers for genetic purity assessment as a replacement to GOT are increasing in several crops. The use of SSR markers for assessing hybrid seed purity has been reported in agricultural crops like rice [2, 5, 9], maize [11], sunflower [12], safflower [10] and horticultural crops like tomato [13-14], cabbage [15-16] and melon [17]. However, use of DNA markers for hybrid purity test is not yet reported in sorghum. The present study is first of its kind reported to assess the relative potential of DNA markers in assessing the genetic purity of certified hybrid seed-lot of three commercial sorghum hybrids (CSH9, CSH13 and CSH14) compared with their GOT analysis.

Among 42 SSRs tested in the study, 17 (41%) markers were polymorphic between the parents of CSH9 and CSH13 while 14 (33%) were polymorphic between parents of CSH14 (Table 3). The polymorphic markers and allele size between five parental lines of three hybrids are presented in Table 4. Of these, to test genetic purity of hybrid seed lot, SbAGB02 was employed for CSH9, Xtxp343 for CSH13 and Xtxp15 for CSH14.

Using SSR markers, in the hybrid seed lot of CSH9, 23 off-types plant were identified while 33 and 19 plants were detected as off-types in CSH13 and CSH14, respectively. Generally, genetic impurity in hybrid seed production plots arises from two sources. One is the appearance of pollen shedders (B plants) in CMS rows, and the other is the mixing of restorer (R) seed with the hybrid

Table 3. Frequency of polymorphic SSR markers among three hybrids

A line	R line	Hybrid	Tested markers	Polymorphic markers	%
296A	CS3541	CSH9	42	17	41
296A	RS29	CSH13	42	17	41
14A	AKR150	CSH14	42	15	36

Table 4. Allele size (bp) of polymorphic markers in parents of three sorghum hybrids

S. No.	Marker	LG	CS3541	296A	RS29	AKMS14A	AKR150
1	msbCIR286	1	85	78	85	85	85
2	msbCIR238	2	140	122	140	140	136
3	Xgap84	2	194	185	196	196	196
4	Xcup11	3	176	174	172	176	172
5	Xcup32	3	167	163	152	152	161
6	Xgap10	4	297	250	296	246	292
7	Xtxp343	4	194	152	258	172	183
8	Xtxp15	5	208	238	223	231	223
9	Xtxp145	6	253	253	226	226	229
10	Xtxp312	7	138	157	238	152	157
11	SbAGB02	7	144	148	117	106	106
12	Xtxp354	8	173	170	179	166	170
13	Xtxp10	9	153	148	160	153	160
14	Xtxp287	9	333	367	367	333	344
15	Xtxp289	9	271	276	285	274	285
16	Xcup2	9	200	212	196	198	209
17	Xtxp141	10	179	188	182	179	176
18	SbAGA01	10	242	233	245	242	242

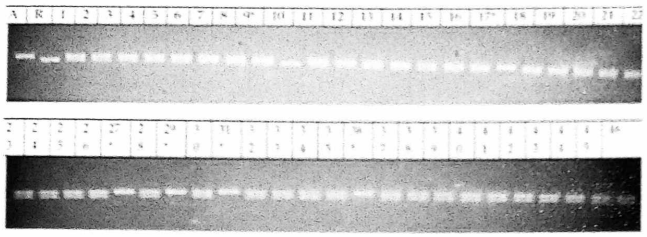

Fig.1. F_1 seedling assay for detecting genetic purity of CSH14 with Xtxp15. Data from a representative set of 46 samples along with their parents are shown. "A" is AKMS14A (female parent), "R" is AKR150 (Restorer parent) and Lanes 1-46 hybrid samples. The asterisk indicates off-types

seed (F1). In the three hybrid seed lots tested in the present study, both these genetic impurities were identified (Fig. 1). Since we used co-dominant SSR markers, pure hybrid plant is expected to exhibit both the alleles from A and R parents if a marker is polymorphic. Deviations from this (presence of single bands) indicate that the plant is genetically impure, and could be the result of A x B cross or mix of R seed. Of the 23 off-type plants in CSH9, 8 showed only "A" allele while 15 showed only "R" allele. Similarly, in CSH13, 14 plants showed "A" allele while 19 showed "R" allele, and in CSH14, 11 plants showed only "A" allele while 8 plants showed only "R" allele. In addition, detection of B lines in A line plots is very important in hybrid seed production since pollen shedders can pollinate A lines which genetically leads to the development of seed which when grown in next season as a hybrid seed results in sterile plants with no significant heterosis exhibited expected from A x R cross seed. Therefore, caution is taken to harvest the hybrid seeds more carefully by making separate harvests from A, B and R lines. Normally, restorer (R) seed is harvested first followed by the hybrid seed on the CMS (A) lines in a hybrid seed production plot [18].

Comparison of morphological and molecular data

Determining genetic purity of hybrid seed is an essential requirement for its commercial use. In certified seed of three hybrids, genetic purity ranged from 93-96% as assessed through GOT based on morphological characters (Table 5). The impure plants deviated for characters like plant height, flowering time, panicle architecture and fertility. The hybrid plants, categorized as off-type plants by GOT were also confirmed by marker analysis. In addition, use of markers detected few more off-type plants. Thus, compared to GOT, the level of the genetic purity of seed lots assessed with markers was slightly less but the difference was not significant. This shows greater ability of markers in identifying impure plants, and better precision in estimating hybrid purity. Of the three hybrids tested, CSH13 showed relatively less genetic purity. This is due to greater differences in flowering time between the two parents of this hybrid. Therefore, for achieving greater genetic purity in hybrid seed lot, it is always recommended to use A and R lines with same flowering time. This will help not only in achieving better seed yield, but also in ensuring ease in adoption of novel seed production packages to attain the desired genetic purity. The study also showed that the present protocols used in monitoring genetic purity following GOT satisfy

Table 5. Genetic purity in hybrid sorghum seed lots based on GOT and SSR markers

Hybrid	Total plants	GOT		SSR markers	
		Pure plants	% genetic purity	Pure plants	% genetic purity
CSH9	400	380	95	377	94
CSH13	400	370	93	367	92
CSH14	400	385	96	381	95

minimum seed standards for foundation and certified seed classes.

The study proved the utility of microsatellite markers in establishing genetic purity of commercial seed-lots of sorghum hybrids. The results obtained from GOT and marker analyses were comparable, and markers can safely be employed to replace the conventional GOT. As estimated in rice [9] use of markers in assessing hybrid seed purity allow immediate marketing of hybrid seed, thus saving cost on conduct of GOT, seed storage, and may bring down hybrid seed cost.

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