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Comparative Study between Morphological and Microsatellite Markers for Genetic Purity Testing of Maize (*Zea mays* L.) F_1 Hybrids

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ABSTRACT: The genetic purity of seeds is the chief determinant to exploit the varietal or hybrid potential. Despite the serious attempts of many public and private sector institutes in the development of high yielding hybrids, farmers fail to realize the assured yield potential due to lack of genetic purity of procured seeds. In this study, we aimed to study and compare the efficiency of different methods of genetic purity testing *i.e.*, Grow-out Test (GOT) and Simple Sequence Repeat (SSRs) in maize hybrids MAH-14-5 and Hema. Ten unique morphological markers were identified between the hybrids MAH 14-5 and Hema and their parental lines. Twenty-five SSR markers were screened out of which seven showed polymorphism. Of these identified polymorphic markers, two *i.e.* Bnlg 1520 and Umc 1288 were uniquely polymorphic to MAH-14-5 and three *i.e.* Phi 053, Bnlg 1621 and Bnlg 1014 were uniquely polymorphic to Hema while two *i.e.* Bnlg 1185 and Umc 1594 showed common polymorphism to both Hema and MAH-14-5. Ten seed lots of Hema were tested with the identified morphological and microsatellite markers. The comparative analysis of lot testing confirmed the superiority of microsatellite markers over morphological markers in the precise detection of off-types. Hence, it is concluded that microsatellite marker analysis would be a better substitute for conventional GOT to test for seed genetic purity.

Keywords: Genetic purity, GOT, Maize, Microsatellite, SSR.

INTRODUCTION

Maize (*Zea mays* L., 2n = 20) is a potential cereal crop in the cropping systems of both developing and developed countries. Maize (*Zea mays* L.) is a dual-purpose crop that produces kernels for human consumption as well as fodder for livestock (Borkhatariya *et al.*, 2022). It belongs to the *Poaceae* family which includes rice, wheat, and millets. With the world average yield of 27.8 q/ ha, maize ranks first among the cereals. Even then, the FAO predicts that an additional 60 Mt of maize grain will be needed globally by 2030 [Food and Agriculture Organization, http://faostat.fao.org]. Despite the serious attempts of many public and private sector institutes in the development of high yielding hybrids, farmers fail to realize the assured yield potential due to lack of genetic purity of procured seeds. It is reported that yield per hectare will drop by 135 kg if the maize hybrid seed purity drops by 1% (Li *et al.*, 2000). Maize being an allogamous crop, there is a greater probability of crossing and added to it the chances of contamination remain high due to pollen shedders, outcrossing and physical mixtures during handling of harvested produce. Thus, genetic purity testing plays a pivotal role in assuring the quality of seeds and this puts light on why research on purity testing methods should be duly taken up in maize.

Quality seed is the chief determinant of future plant development and the master key to successful cultivation. *Seed quality* describes the potential performance of a seed lot and it encompasses both physical purity and genetic purity (Basu, 1995). Physical purity refers to the percentage of pure seeds present in the lot whereas genetic purity refers to trueness to its type or genuineness of the seeds. Complex interactions of genetic, environmental, physiological, biochemical, cytological and pathological factors influence the expression of seed quality (Elias, 2018).

Genetic purity of seeds can be assured by different methods beginning from conventional GOT to highly advanced sequencing. The conventional method of morphological examination is inadequate, tedious and ineffective (Pattanaik *et al.*, 2018). It is also highly influenced by the environmental factors and on the skill of the observer making the results less precise. So advanced techniques like protein, isozyme and DNA markers were introduced for purity testing. Of these, DNA markers like RFLP, RAPD, SSR, and SNP are highly effective. Comparatively, SSRs and SNPs are

co-dominant markers having higher significance in hybrid purity testing, varietal protection, cultivar fingerprinting, estimating and comparing the genetic similarity and identity profiling (Korir *et al.*, 2013).

SSRs also called microsatellite markers are the tandem repeat motifs of 1–6 nucleotides that are present abundantly in the genome. The occurrence of SSRs may be due to the slippage of single-strand DNA, recombination of double-strand DNA, transfer of mobile elements (retrotransposons) and mismatches (Nadeem *et al*., 2018). Expressed sequence tag (EST) projects have prompted a vast amount of publicly available sequence data from plant species which can be mined for simple sequence repeats (SSRs). SSR markers are valuable because of their higher level of transferability to related species (Huang *et al.*, 2018) and they can often be relied on as anchor markers for comparative mapping and evolutionary studies. Satya Srii *et al*. (2021) showed that even SNP markers obtained through ddRADseq has potential to differentiate parental lines and hybrids of maize. However, there is no standardized SSR markers identified for the Indian maize hybrids like MAH 14-5 and Hema and its efficiency over prevailing grow –out test.

In this study, we made a comparative analysis between the efficiency of the Grow-out Test and SSR markers in detecting the genetic purity of maize hybrids.

MATERIALS AND METHODS

Characterization of hybrids and parental lines based on morphological markers

Plant Material. The two maize hybrids and their parental lines, MAH 14-5 (CAL 1443 × CML 451) and Hema (NAI-137 × MAI-105) were obtained from the College of Agriculture, V.C. Farm, Mandya (UAS, Bengaluru), India. The seedlings of the maize hybrids and its parents were raised in the fields of Seed Technology Research Unit, AICRP on National Seed Project, GKVK, UAS, Bangalore. 50 seeds in each of two hybrids and its parental lines were sown in ridges and furrows with 45cm spacing and plant to plant spacing of 10cm. Morphological observations for specifically chosen stable DUS (distinctness, uniformity, and stability) characters were taken according to the UPOV guidelines at different growth stages of the crop to identify the morphological markers between parental lines and hybrids and also between hybrids.

GOT analysis. A field GOT was carried out for validation of identified morphological markers. For this, ten seed lots of the hybrid Hema were obtained from seed stores, NSP, GKVK, UAS, Bangalore. MAH-14-5 hybrid was not validated due to the unavailability of hybrid seed lots commercially, as the hybrid is still in the variety release pipeline. Two replicates of 400 seeds were sown in each lot at the standard spacing. Measures like proper irrigation, fertilization, and insect management were carried out during the plant growth. Purity evaluations were made based on the morphological markers identified (Table 1). The mean percentage of hybrid purity for both GOT and SSR analysis was calculated as follows

$$\text{Hybrid purity (\%)} = \frac{\text{Total number of plants - Number of off - types}}{\text{Total number of plants}} \times 100$$

DNA extraction and quantification. The leaf material of twenty-one days old seedlings of two hybrids and their parental lines raised in the field were used for isolation of DNA by following the protocol of the CTAB method (Tan *et al*., 2013). The quality and quantity of isolated genomic DNA were checked using 0.8% agarose gel electrophoresis and Nanophotometer respectively. Finally, DNA was diluted with double distilled water to a concentration of 20ng/µl for PCR analysis.

SSR marker polymorphism analysis. A total of 25 pairs of SSR markers were used to examine the polymorphism in the bulk of DNA extracted from ten seedlings of each female and male parent of maize hybrids, MAH-14-5 and Hema. Sequences of all SSR markers were obtained from the public source: https://www.maizegdb.org/ssr.php. DNA amplification was carried out with a 15µl reaction mixture containing 2µl of template DNA, 1.5µl of PCR buffer, 1.5 µl of forward and reverse primers, 0.75µl of dNTPs, 0.45 µl of Taq-polymerase and 8.8 µl of sterile water. The PCR components used were obtained from Sigma Aldrich Ltd., Bengaluru, India. The reaction mixture was mixed thoroughly and then PCR tubes were loaded in a thermal cycler (BioRAD). The annealing temperature of primers was 58°C. Amplified DNA samples were then separated in a 3% (w/v) agarose gel along with a 100bp DNA ladder as the molecular standard. The gel was stained with Ethidium Bromide and electrophoresis was carried out. The gels were visualized under a UV transilluminator and documented.

Lot testing by SSR markers. Twenty-one days old leaves were collected from the lots sown for GOT. The DNA extracted from the ten Hema lots was tested with identified polymorphic markers *i.e.* Phi 053 and Bnlg 1014. The purity percent was calculated using the formula mentioned in the GOT analysis.

RESULTS AND DISCUSSION

Genetic purity assessment using GOT. The specific stable morphological characters were recorded according to UPOV guidelines and grouping of different maize genotypes (two hybrids along with their parental lines) for ten morphologically different characters are listed in Table 1 (Fig. 1). These ten identified morphological markers were used to distinguish off-types from true-to types. The mean percentage of purity of the replicated results of GOT obtained is listed in Table 2. The hybrid seed lots were

classified as substandard if the genetic purity percent is less than 95% as recommended by ISTA.

Table 1: Morphological characters used to identify the selfed/ off-types during grow-out test.

Morphological characters	CAL-1443	CML-451	MAH-14-5	NAI-137	MAI-105	Hema
First leaf : anthocyanin coloration of blade	Absent or very weak	Strong	Strong	Strong	Absent or very weak	Absent or very weak
Leaf: undulation of margin of blade	Intermediate	Absent or very weak	Strong	Strong	Intermediate	Intermediate
Leaf: angle between blade and stem	Medium	Medium	Large	Large	Medium	Medium
Leaf: curvature of blade	Strongly recurved	Absent or very slightly recurved	Strongly recurved	Strongly recurved	Moderately recurved	Moderately recurved
Stem: degree of zig-zag	Slight	Absent or very slight	Absent or very slight	Absent or very slight	Absent or very slight	Slight
Tassel: time of anthesis	Late	Late	Medium	Very early to early	Late	Very early
Tassel: anthocyanin coloration at base of glume	Medium	Medium	Medium	Strong	Medium	Strong
Tassel: anthocyanin coloration of glume excluding base	Absent or very weak	Absent or very weak	Absent or very weak	Absent or very weak	Absent or very weak	Strong
Tassel: anthocyanin coloration of anthers	Absent or very weak	Absent or very weak	Absent or very weak	Strong	Absent or very weak	Medium
Ear: anthocyanin coloration of silk	Strong	Medium	Medium	Strong	Weak	Medium

Table 2: Genetic purity percent of 10 hema seed lots tested by GOT and SSR.

Lot Number	GOT (Purity percent)	Status of lot	SSR (Purity percent)	Status of lot
1	92	Sub Standard	90	Sub Standard
2	95	Standard	92	Sub Standard
3	91	Sub Standard	88	Sub Standard
4	94	Sub Standard	91	Sub Standard
5	96	Standard	94	Sub Standard
6	92	Sub Standard	89	Sub Standard
7	95	Standard	93	Sub Standard
8	93	Sub Standard	90	Sub Standard
9	96	Standard	96	Standard
10	93	Sub Standard	91	Sub Standard

Identification of polymorphic SSR markers and genetic purity assessment. In the PCR-based assay, out of 25 selected marker pairs, seven showed clear polymorphism (Fig. 2, 3). The sequence information of the polymorphic markers is presented in (Table 3). Of these identified markers, two *i.e.* Bnlg 1520 and Umc 1288 were uniquely polymorphic to MAH-14-5 and three *i.e.* Phi 053, Bnlg 1621 and Bnlg 1014 were uniquely polymorphic to Hema while two *i.e.* Bnlg 1185 and Umc 1594 showed common polymorphism to both Hema and MAH-14-5. The details of the polymorphic markers and the specific band size for each hybrid and their parental lines are listed in Table 4. The identified polymorphic SSR markers (Phi 053 and Bnlg 1014) were utilized for testing the purity of the hybrid Hema seed lots. The mean percentage of purity of two SSR markers for ten lots obtained is listed in Table 2 (Fig. 4). The genetic purity obtained for ten Hema seed lots tested by GOT and SSR markers (Table 2) was tested with ANOVA single factor analysis using SPSS software. The ANOVA analysis showed that the results from testing by GOT were significantly different from SSR markers with $p<0.005$.

Table 3: Polymorphic microsatellite (SSR) markers and their sequence used in the study.

Sr. No.	Primer	Nucleotide sequence	
1.	Bnlg1621	F 5'-3'	CTCTTCGATCTTTAAGAGAGAGAGAG
		R 5'-3'	ACACGAGGCACTGGTACTAACG
2.	Umc1288	F 5'-3'	ATCCGGACAAATTGAACTTTCATC
		R 5'-3'	ATAGATTCAGTGTTGGACCGAGGA
3.	Umc1594	F 5'-3'	GCCAGGGGAGAAATAAAATAAAGC
		R 5'-3'	CACTGCAGGCCACACATACATA
4.	Phi053	F 5'-3'	CTGCCTCTCAGATTCAGAGATTGAC
		R 5'-3'	AACCCAACGTACTCCGGCAG
5.	Bngl1185	F 5'-3'	CGTGCCAGGCAGGTTAATTA
		R 5'-3'	GACTCGAGGACACCGATTTC
6.	Bnlg1520	F 5'-3'	TCCTCTTGCTCTCCATGTCC
		R 5'-3'	ACAGCTGCGTAGCTTCTTCC
7.	Bngl1014	F 5'-3'	CGTGCCAGGCAGGTTAATTA
		R 5'-3'	GACTCGAGGACACCGATTTC

Table 4: Summary of polymorphism and allele size in base pairs for different markers.

Primer	MAH-14-5			HEMA		
	F	M	H	F	M	H
Bnlg 1520	220	240	220,240	Monomorphic		
Umc 1288	220	200	220,200	Monomorphic		
Phi 053	Monomorphic			120	140	120,140
Bnlg 1621	Monomorphic			220	240	220,240
Bnlg 1014	Monomorphic			180	160	180,160
Bnlg 1185	120	100	120,100	100	110	100,110
Umc1594	140	120	140,120	140	130	140,130

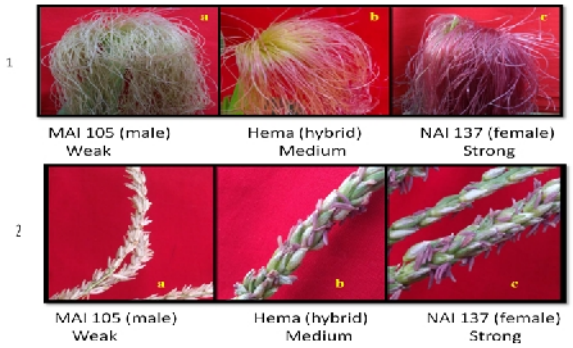

Fig. 1. Variability in morphological characters of maize hybrid Hema and its parents, 1- Anthocyanin coloration of silk; 2- anthocyanin coloration of anthers.

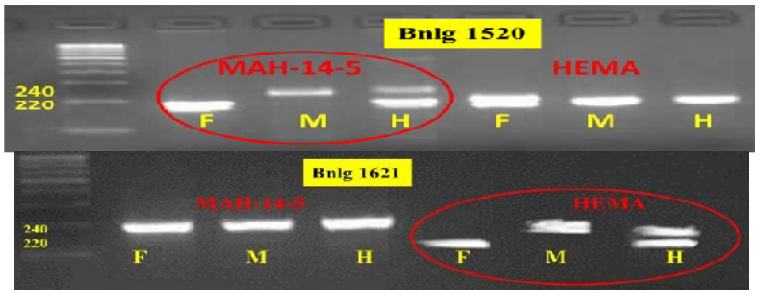

Fig. 2. Amplification profile of maize hybrid MAH 14-5 and Hema for unique co-dominant SSR markers Bnlg 1520 and Bnlg 162 respectively.

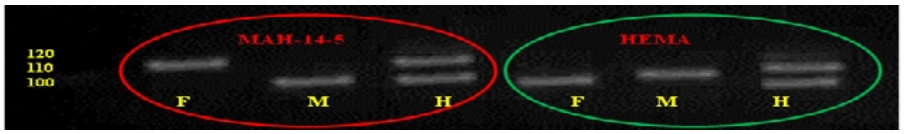

Fig. 3. Amplication profile of maize hybrids MAH 14-5 and Hema for polymorphic SSR marker Bnlg 1185.

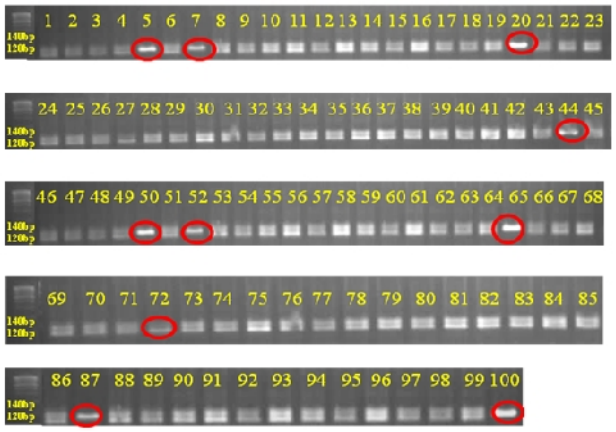

Fig. 4. Lot-1 tested by SSR marker Phi 053, 10 offtypes detected is marked in red circles.

The refinements genetically incorporated in crop plants by modern plant breeders are most efficiently maintained, multiplied and transmitted by seeds. Seeds, therefore, are the principal means for extending improvements effected in crops, from the breeding nursery to farmer's fields. So, the high-quality seed is a necessary input for farmers to achieve higher yields and profits (Sandra *et al.,* 2015). In this study, seed lots of the maize hybrid Hema were tested for genetic purity by both GOT and SSR markers. The purity percent observed by GOT and SSR are significantly different for 10 lots. The average genetic purity of the ten seed lots tested in replicates by GOT and SSR showed varying genetic purity of 93.7% and 91.4% respectively. It is noted that the GOT has exaggerated the purity percent at least by 2 percent. Similar results were observed in the study conducted by Keshavalu (2006)**.** This exaggeration would be a serious problem when a substandard seed lot passes the genetic purity testing due to the inefficiency of the method of testing. Table 2 clearly shows that most substandard seed lots passed standards while tested only by GOT. This exaggeration of results by GOT would be due to the environmental effects on morphological traits and the skill of the examiner. The recognition of true and false hybrids is not easy as morphological differences between them are not always apparent especially when parents are genetically similar, leading to potential inaccuracy (Ballester and Vicentem 1998; Dongre and Parkhiv 2005). Thus the assessment of genetic purity by morphological markers remains to be inferior to the molecular marker analysis due to its reduced efficiency in identifying the off-types (Akhare *et al.,* 2008; Staub *et al.,* 1996).

The time taken for assessment of genetic purity in GOT and SSR markers was 85-95 days and 48 hours respectively. The prolonged duration for GOT testing is because several of the striking distinguishing characters appear only during the flowering and post-flowering stage and we need to grow the crop for the entire duration (Pattanaik et al. 2018). Due to this reason, there is the unavailability of hybrid seeds for immediate cultivation which leads to the extra cost of storage and hence an overall increase in hybrid seed cost. SSR marker analysis, on the other hand, is quick as they are crop-stage independent and conducted in the laboratory. SSR marker analysis does not demand entire crop duration as the DNA can be isolated even from the seeds or from leaves obtained from earlier stages (usually 21-days old leaves).

The efficiency of GOT depends on the number and type of differentiating morphological traits. But, such highly differentiating traits don't appear if the parents are closely related. But, a single polymorphic SSR marker will be sufficient to test the lot (Yashitola *et al.,* 2002; Nandhakumar *et al.,* 2004). Also, SSR markers have better discriminatory power and can precisely detect even the residual heterozygosity (Nandhakumar *et al.,* 2004; Sundaram, 2008; Selvakumar *et al*., 2010). As most of the agriculturally important crop genomes are sequenced, it is not a problem to find an SSR marker for screening from the respective databases. The cross transferability of SSR markers also make it possible to use SSR markers even for orphaned crops whose genome information is not known.

CONCLUSIONS

We conclude that it is possible to differentiate maize hybrids more accurately and efficiently from their parental lines and off-types/selfed seeds using locus-specific allelic information through SSR markers. Molecular markers are much reliable, precise, and time saving compared to conventional markers. Due to the lesser time requirement for molecular marker analysis and its precision, farmers can be provided with high-quality seeds at the right time. So, it is suggested that a shift from morphological to molecular markers for genetic purity testing of seed lots would revolutionize the seed industry.

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Conflict of Interest. None.

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