Genetic purity analysis in maize (*Zea mays* L.) hybrid varieties; MI maize hybrid 1 and MI maize hybrid 2 using SSR markers

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Abstract

Genetic purity is an important criteria for quality assurance of hybrid seed production. Grow-Out Test (GOT) is the usual practice to confirm hybrid purity based on morphological differences, which is time consuming, laborious, costly and highly influenced by the environment. The main objective of the present study was to identify informative simple sequence repeat (SSR) markers capable of distinguishing purity of maize hybrids, MI maize hybrid 1 and MI maize hybrid 2. The two hybrids, along with their parents were used in this study. Sixteen simple sequence repeat SSR primers representing maize genome were tested to identify putative markers for purity testing. Among them, SSR marker UMC 1997, was polymorphic, co-dominant and could identify MI maize hybrid 1 discriminating parental lines CML 161 and CML 194. Similarly, phi 402893 and phi 076 showed polymorphism with co-dominant nature in MI maize hybrid 2 and discriminated the parental lines CML 451 and CLO 2450. For validation of selected primers for genetic purity analysis, fifty randomly selected seeds from each maize hybrid were tested with their parental lines. MI maize hybrid 1 showed 100% genetic purity with the selected primer UMC 1997 and MI maize hybrid 2 showed 100% genetic purity with the selected primers phi 076 and phi 402893. This study suggests that identified SSR markers are reliable for assessing genetic purity of selected hybrids, and the results will be useful in verifiying genetic purity of maize hybrid seeds with higher accuracy in a short period of time.

Keywords: Maize hybrids Microsatellite markers, Molecular characterization, Purity testing, Seed genetic purity,

Introduction

Maize (*Zea mays* L.) is one of the most important coarse grains in Sri Lanka and occupies the second highest extent of land next to rice. In year 2018, the extent of cultivation of maize in Sri Lanka was 70,895 ha and production was 270,041 t with an average yield of 3.81 t ha^{-1} (AgStat, 2019). Annual maize requirement is about 200,000 MT from which the country import was about 125,000 t at a cost of 1,169 million rupees, mainly to fulfill local consumption, production of Thriposha and other industries, 33,000 t, 8,000 t and 5,000 t, respectively (Agriculture Statistics, 2018).

Hybrids have higher yield potential than open pollinated varieties. Such varieties can be used to increase the productivity and production of maize. Maintaining genetic purity is an essential pre-requisite for commercialization of any hybrid (Sudharani *et al*., 2012). Genetic purity is one of the quality criteria required for successful hybrid seed production and identification of genetically distant parental combinations (Eminur *et al*., 2014). It is estimated that the yield per hectare will decrease by about 135 kg if the maize hybrid seed purity drop by 1%. Therefore, continuous testing of the genetic purity of hybrid seed is an essential requirement for its commercial use (Chaudhary *et al*., 2018).

Conventionally, characterization of hybrids is being done based on specific morphological and floral characters in plants grown until maturity through Grow Out Test (GOT), which is normally practiced by the Seed Certification Service of the Department of Agriculture, Sri Lanka. However, this method is time consuming, restricted to a few characteristics and influenced by environmental factors (Tiwari, 2020). Therefore, SSR markers are useful in the identification of hybrid, assessment of respective parents, and testing genetic purity of hybrids (Mawgood *et al*., 2006, Sudharani *et al*., 2012, Daniel *et al*., 2012, Awaludin *et al*., 2013).

Molecular markers based on DNA polymorphism can be used to identify hybrids. Microsatellite markers are used to identify the parent of a hybrid with high probability (Mawgood *et al*., 2006). Microsatellite markers have been proven to be preferred molecular markers for purity identification due to their high efficiency, reproducibility and simplicity (Bhat *et al*., 2017). Further, Chaudhary *et al*. (2018) stated that PCR based co-dominant SSRs are preferred for genotyping because of their reproducibility,

abundance and amenability to high throughput screening and are of great importance for rapid assessment of hybrid and parental line seed purity.

The objective of the present study was to identify SSR markers for testing of hybridity and genetic purity in recommended hybrid maize varieties; MI maize hybrid 1 and MI maize hybrid 2.

Materials and Methods

The experiment was conducted at the molecular biology laboratory of the Plant Genetic Resources Center, Department of Agriculture, Gannoruwa from May 2019 to March 2020. Breeder seeds of MI maize hybrid 1 and MI maize hybrid 2 along with their parents were collected from the Field Crops Research and Development Institute (FCRDI), Mahailluppallama (Table 1). The study was conducted in two stages; SSR marker survey for purity testing and validation of selected SSR markers to test the genetic purity of maize hybrid seed lots.

Table 1. Maize hybrids and their parental lines used in this study

Hybrid	Female parent	Male parent
MI maize hybrid 1	CML 161	CML 194
MI maize hybrid 2	CML 451	CLO 2450

SSR marker survey for purity testing

Genomic DNA was extracted from fresh leaves of 14 days old seedling of two hybrids and respective parents by using Cetyl-Tri-methyl Ammonium Bromide (CTAB) method (Doyle and Doyle, 1990) with some modifications. The supernatant obtained by adding chloroform: isoamyl alcohol (24:1) was transferred into a new microcentrifuge tube and nearly $1/10^{th}$ of 10% CTAB and an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) were added and shaken well for 20 minutes for purification of DNA from protein. Extracted DNA was quantified using Flourometer (Quantus™ Flourometer, Promega, USA). DNA was diluted in 0.01 M TE buffer to a concentration of 15 ng μL^{-1} for PCR analysis. Sixteen SSR markers, distributed in maize genome were selected for the study. The primer sequence information was obtained from the maize genetics and genomics database (www.maizegdb.org) (Table 2). Annealing temperatures for each primer were optimized before initiating the study.

Table 2. SSR markers used for the hybridity testing for MI maize hybrid 1 and 2

SSR locus	Forward primer (5' - 3')	Reverse primer (5' - 3')	Bin location	Annealing temperature (°C)
UMC 1023	CTTGTGCCACCACATGCAGTA	CAGTTTGGAACAGGGAAAAGTACG	6.00	60
UMC 1071	AGGAAGACACGAGAGACACCGTAG	GTGGTTGTCGAGTTCGTCGTATT	1.01	56
UMC 1997	GTTCCAATGAGATGAGACGATGTG	CTCCAAAAAGGCCGTCGTAGTA	8.06	56
UMC 1040	CATTCACTCTCTTGCCAACTTGA	AGTAAGAGTGGGATATTCTGGGAGTT	9.01	56
UMC 1260	CTTAAGCAGAGCTCAAAAACTGCC	TAAATTGTCAAGCGAGGTTTGGAT	5.00	60
UMC 1470	AAAAACCTCAATAGCCGTTTCACA	GATTCTTGTGTTGCATACTGGTGC	8.03	56
UMC 1746	ACACGAGCATCCTACATCCTCCTA	ACCTTGCCTGTCCTTCTTTCTCTT	3.00	60
UMC 2047	GACAGACATTCCTCGCTACCTGAT	CTGCTAGCTACCAAACATTCCGAT	1.09	56
UMC 1017	GAAGAGGTAAGGACGACGACGA	GCACCTGCAGTGAACGTCAGTA	4.01	60
phi 402893	GCCAAGCTCAGGGTCAAG	CACGAGCGTTATTCGCTGT	2.00	60
phi 076	TTCTTCCGCGGCTTCAATTTGACC	GCATCAGGACCCGCAGAGTC	4.11	60
phi 112	TGCCCTGCAGGTTCACATTGAGT	AGGAGTACGCTTGGATGCTCTTC	7.01	58
phi 041	TTGGCTCCCAGCGCCGCAAA	GATCCAGAGCGATTTGACGGCA	10.00	62
phi 083	CAAACATCAGCCAGAGACAAGGAC	ATTCATCGACGCGTCACAGTCTACT	2.04	58
phi 029	TTGTCTTTCTTCCTCCACAAGCAGCGAA	ATTTCCAGTTGCCACCGACGAAGAACTT	3.04	58
nc 130	GCACATGAAGATCCTGCTGA	TGTGGATGACGGTGATGC	5.00	60

The PCR reaction was performed in 15 µL of PCR mixture containing of 3 µL of template DNA (15 ng μL^{-1}), 1 X PCR buffer (Promega, USA), 2.5 mM $MgCl_2$ (Promega, USA), 0.2 mM dNTPs (Promega, USA), 1.3 µM forward and reverse primers (Integrated DNA Technologies, USA), 1U Taq DNA polymerase (Promega, USA). Final volume of the mixture was adjusted using sterile distilled water.

PCR amplification was performed using Applied Biosystems thermo cycler (model No.9902). Amplification profile used for PCR amplification comprised of 94 ºC for four minutes of initial denaturation, followed by 35 cycles of 94 ºC for one minute, primer annealing for one minute, extension at 72 ºC for two minutes and seven minutes at 72 ºC for the final extension. The annealing temperature was adjusted based on the optimized annealing temperature of each primer. Amplified products were confirmed by 1.5% agarose gel electrophoresis in 0.5 X TBE buffer. PCR products were analyzed by 8% non-denaturing polyacrylamide gel electrophoresis (PAGE) in 1X TBE buffer using consort vertical gel electrophoresis system (model no: E4300). The gels stained using ethidium bromide (0.5 µL mL^{-1}) and visualized under UV light using BIO-RAD gel documentation system with Quantity One software.

Scoring and analysis of data

Gel images were manually scored through visual observations and using Quantity One (version 4.6.3) software. Data analyses were done using Power Marker software (version 3.25) to obtain the diversity parameters; genotype number, allele number, gene diversity, heterozygosity, Polymorphic Information Content (PIC) and presence of polymorphism of the 16 SSR markers.

Validation of selected SSR markers for genetic purity testing of hybrid seeds

For the confirmation of selected SSR markers for genetic purity analysis of the two maize hybrids, a sample of 50 seeds were collected randomly from seed lots of each hybrid and from parental lines. The DNA isolated from 50 individual plants of the MI maize hybrid 1, MI maize hybrid 2 and their corresponding parents were subjected to PCR analysis using selected SSR markers, which have been identified for genetic purity testing of the MI maize hybrid 1 and 2.

Results and Discussion

Among the 16 primers studied, five primers UMC 1071, UMC 1997, UMC 1040, UMC 1260 and UMC 1746 were polymorphic for the MI maize hybrid 1 and its parental lines while the rest of primers were monomorphic. Out of the 16 primers studied, only eight primers UMC 1071, UMC 1997, UMC 1040, UMC 1260, UMC 1746, phi 402893, phi 076 and phi 029 were polymorphic for the MI maize hybrid 2 and its parental lines. The diversity parameters including genotype number, allele number, gene diversity, heterozygosity, PIC and presence of polymorphism of the 16 SSR markers for MI maize hybrid 1 and its parental lines, MI maize hybrid 2 and its parental lines are presented in Table 3 and Table 4, respectively.

Table 3. Genotype number, allele number, gene diversity, heterozygosity, PIC value and polymorphism of the SSR primers for MI maize hybrid 1 and its parental lines

SSR locus	Genotype no	Allele no	Gene diversity	Heterozygosity	PIC	Poly morphism
UMC 1023	1	1	0	0	00	−
UMC 1071	2	2	0.44	0	0.35	+
UMC 1997	3	2	0.5	0.33	0.38	+
UMC 1040	2	2	0.44	0.67	0.35	+
UMC 1260	2	2	0.44	0	0.35	+
UMC 1470	1	1	0	0	0	−
UMC 1746	2	2	0.44	0	0.35	+
UMC 2047	1	1	0	0	0	−
UMC 1017	1	1	0	0	0	−
phi 402893	1	1	0	0	0	−
phi 076	1	1	0	0	0	−
phi 112	1	1	0	0	0	−
phi 041	1	1	0	0	0	−
phi 083	1	1	0	0	0	−
phi 029	1	1	0	0	0	−
nc 130	1	1	0	0	0	−
Mean	1.4	1.3	0.15	0.07	0.11	

Note: PIC- Polymorhpic Information Content, presence of polymorphism: + polymorphic,*

− monomorphic

Table 4. Genotype number, allele number, gene diversity, heterozygosity, PIC value and polymorphism of the SSR primers for MI maize hybrid 2 and its parental lines

SSR locus	Genotype no	Allele no	Gene diversity	Heterozygosity	PIC	Polymorphism
UMC 1023	1	1	0	0	00	−
UMC 1071	2	2	0.44	0.67	0.35	+
UMC 1997	2	2	0.44	0	0.35	+
UMC 1040	2	2	0.44	0	0.35	+
UMC 1260	2	2	0.44	0	0.35	+
UMC 1470	1	1	0	0	0	−
UMC 1746	2	2	0.44	0	0.35	+
UMC 2047	1	1	0	0	0	−
UMC 1017	1	1	0	0	0	−
phi 402893	3	2	0.5	0.33	0.38	+
phi 076	3	2	0.5	0.33	0.38	+
phi 112	1	1	0	0	0	−
phi 041	1	1	0	0	0	−
phi 083	1	1	0	0	0	−
phi 029	2	2	0	0	0	+
nc 130	1	1	0	0	0	−

*Note: * presence of polymorphism: + polymorphic − monomorphic*

For MI maize Hybrid 1 and its parents, genotype number ranged from one to three, while the allele number ranged from one to two, gene diversity ranged from zero to 0.5, heterozygosity ranged from zero to 0.67 and PIC value ranged from zero to 0.38. For Maize hybrid 2, diversity parameters showed similar ranges. However, polymorphic markers were different. In Hybrid 1, the maximum genotype number, gene diversity and PIC value produced by the SSR primer UMC 1997 (Table 2). It was selected to assess the purity of MI maize hybrid 1 while differentiate its parental genotypes. Similarly, the SSR primers phi 402893 and phi 076 were selected to identify and distinguish MI maize hybrid 2 and discriminate its parents.

Among the 16 SSR loci tested, UMC 1997 amplified an allele (fragment size of 120 bp) in MI maize hybrid 1 and its female parent (CML 161) but not in pollen parent (CML 194). Another allele (fragment size of 160 bp) was present in male parent

(CML194) and F_1 hybrid (MI maize hybrid 1) but not in female parent (Figure 1). Thus, it confirmed the presence of both female and male parent alleles in MI maize hybrid 1 as a result of crossing between the two parents, CML 161 and CML 194.

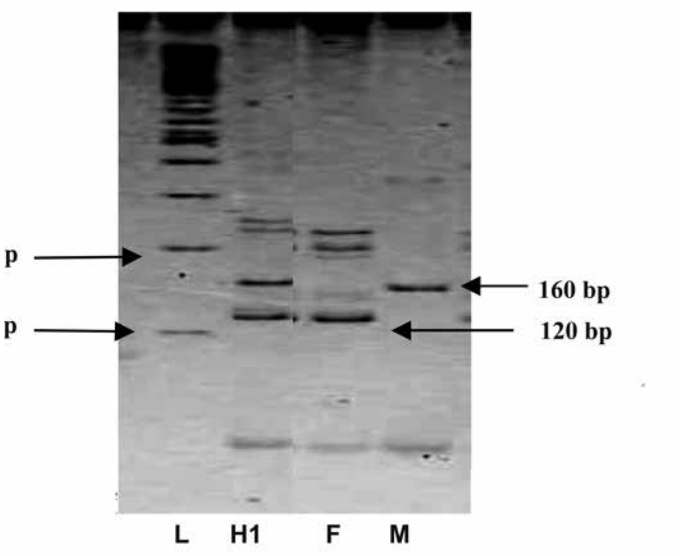

Figure 1. PCR profile of maize hybrid 1 and parents with UMC 1997
L= DNA Ladder (100bp), H1 = MI maize hybrid 1, F = female parent, M = male parent

The phi 402893 and phi 076 were able to generate complementary banding pattern between MI maize hybrid 2 and its corresponding parental lines CML 451 (Female) and CLO 2450 (Male) (Figures 2 and 3). The phi 402893 amplified an allele of 130 bp in F_1 hybrid and female parent (CML 451) but not in its male parent (CLO 2450). Further, phi 402893 also amplified another allele of 110 bp in male parent (CLO 2450). The same allele was expressed in F_1 hybrid, but not in the female parent (CML 451). These two alleles expressed in MI maize hybrid 2 revealed the heterozygous condition of this loci, thus confirming that this hybrid was produced from the cross combination of CML 451 and CL0 2450 (Figure 2).

Similarly, phi 076 loci amplified an allele of 190 bp only in male parent (CLO 2450) and F1 hybrid. On the other hand, the female parent (CML 451) generated an amplicon (220 bp) which was absent in the male parent. However, F_1 hybrid and MI maize

hybrid 2 showed the allele of both parents confirming the heterozygosity by generating two bands (Figure 3). Thus, it could be confirmed that these SSR markers, UMC 1997, phi 402893 and phi 076 can be identified as molecular tags for distinguishing purity of MI maize hybrid 1 and MI maize hybrid 2.

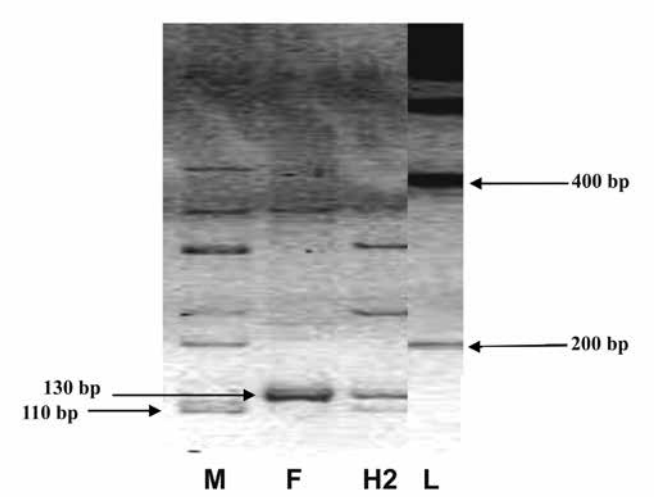

Figure 2. PCR profile of maize hybrid 2 and parents with phi 402893
M = male parent, F = female parent, H2 = MI maize hybrid 2, L = DNA Ladder (200 bp)

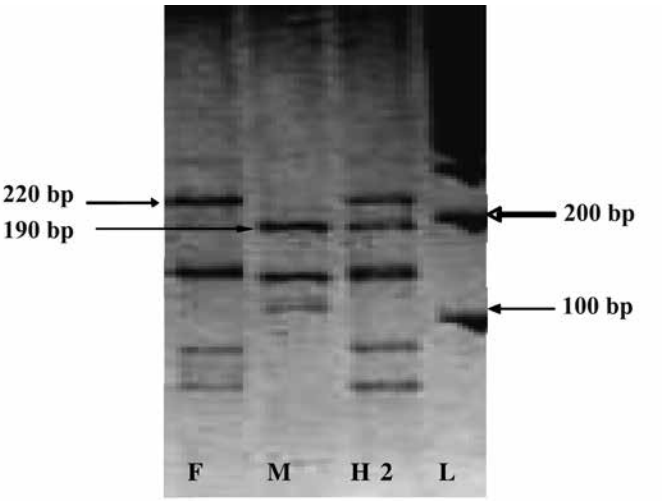

Figure 3. PCR profile of maize hybrid 2 and parents with phi 076
F = female parent, M = male parent, H2 = MI maize hybrid 2, L = DNA Ladder (100 bp)

The identified SSRs in F_1 hybrids showed complementary banding pattern of both the parents. MI maize hybrid 1 could be identified and distinguished by UMC 1997. MI maize hybrid 2 could be identified and distinguished by the phi 402893 and phi 076. The banding pattern of these two hybrids showed both the amplicons present in female as well as pollen parent, thus confirming the genuine crossing and heterozygote nature of the hybrids. Duplex PCR could be used to speed up the testing of genetic purity of MI maize hybrid 2 with phi 402893 and phi 076, as the PCR amplicon sizes of phi 402893 (110-130 bp) and phi 076 (190-220 bp) are distant and clearly distinguished. Duplex PCR would result more information with less sample, higher through put, cost effective and increased accuracy.

In the present study, the PCR based DNA markers were used to the analysis and identification of the parental line and the hybrids of maize sample to test the genetic purity. Comparison of traditional GOT and DNA-based PCR assay to estimate F_1 hybrid purity reveals that a GOT requires a complete season, is labour-intensive as well as sensitive to environmental changes and therefore is not totally a true-to-type method to assess genetic purity (Pattanaik *et al*., 2018). Due to above reasons, this limits the availability of hybrid seeds for immediate cultivation which leads to the extra cost of storage, and hence an overall increase in the hybrid seed cost. Verma *et al. (*2017) also proved that genetic purity assessment of indica rice hybrids, molecular marker-based genetic purity assessment may serve as a probable substitute to GOT, which is a cumbersome and time consuming task and would benefit the farmers and agencies involved in hybrid rice seed production and cultivation.

Validation of SSR markers for the genetic purity analysis of hybrid seeds

The genetic purity testing of 50 randomly selected individual seeds of MI maize hybrid 1 with SSR maker UMC 1997 showed similar banding pattern which reflected 100 percent purity in the selected seed lot. The SSR marker, UMC 1997 produced two distinguishable alleles in two parents, allele 1 was specific to male and allele 2 was specific to female parent and both alleles were present in MI maize hybrid 1 (Figure 4).

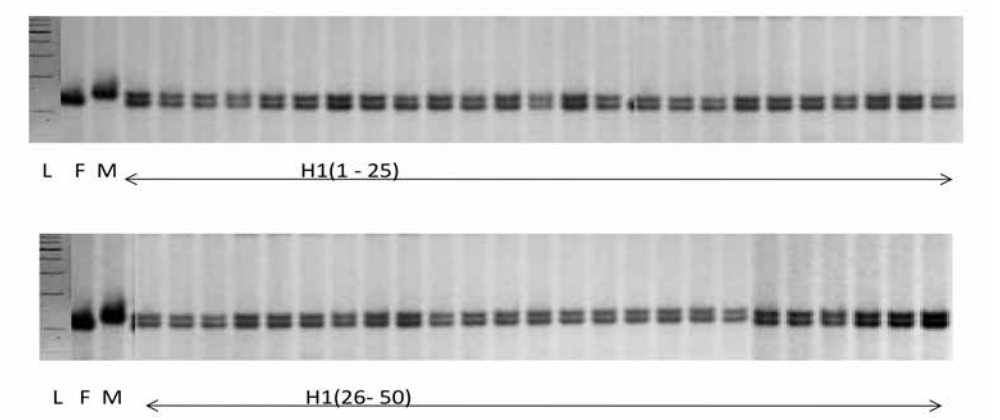

Figure 4. Testing the genetic purity of 50 randomly selected seeds from MI maize hybrid 1 using UMC 1997 showing 100% genetic purity

L = DNA Ladder (100 bp), 1- 50 = MI maize hybrid 1, M = male parent, F = female parent

MI maize hybrid 2 showed 100% genetic purity with SSR marker phi 076, which produced two polymorphic alleles in two parents. Alleles 1 and 2 were specific to female and male parent, respectively, and both alleles were present in MI maize hybrid 2 (Figure 5). This data suggested that no genotype mixing in the random sample collected from the seed lots of MI maize hybrid 2.

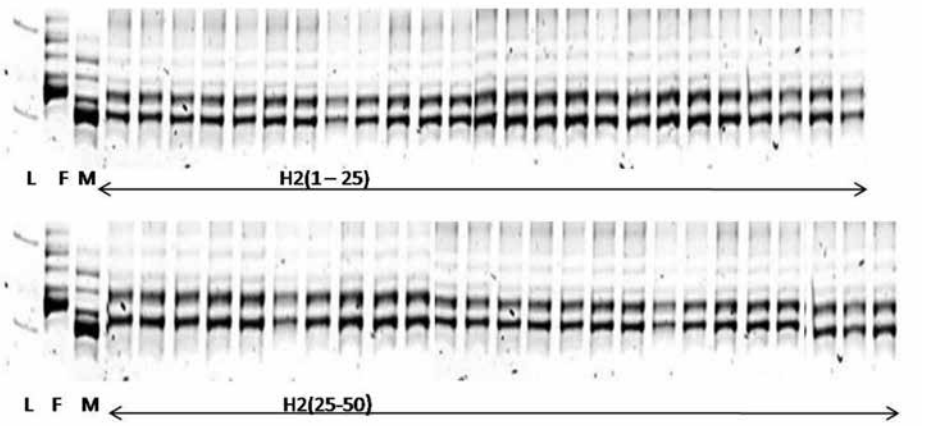

Figure 5. Testing the genetic purity of 50 randomly selected seeds from MI maize hybrid 2 using phi 076 showing 100 per cent genetic purity

L = DNA Ladder (200 bp), F = female parent, M = male parent, 1- 50 = MI maize hybrid 2

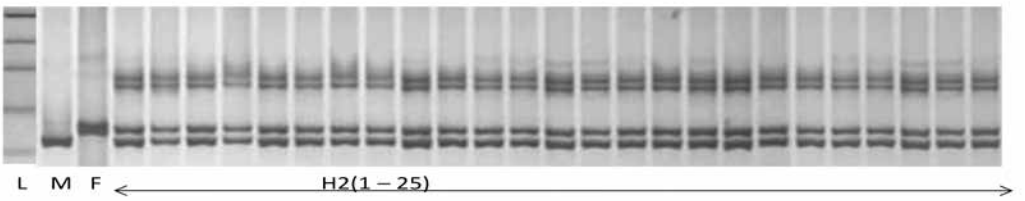

Figure 6. Testing the genetic purity of 25 randomly selected seeds from MI maize hybrid 2 using phi 402893 showing 100% genetic purity

L = DNA Ladder (200 bp), F= female parent, M = male parent, 1- 50 = MI maize hybrid 2

The assessment of genetic purity of hybrids and molecular fingerprinting of their parental lines based on informative SSR markers has been used in other crops such as rice (Bora *et al*., 2016; Cai *et al*., 2020), watermelon (Lu *et al.,* 2018), cotton (Selvakumar *et al.*, 2010), barley (Romdhane *et al*., 2018) and capsicum (Mongkolporn *et al.,* 2015). These reports highlight the utility and efficiency of SSR markers as precise and rapid molecular tool for genetic purity testing, fingerprinting and identification.

SSR-PCR analysis could be a useful tool for hybrid purity assessments, DNA fingerprinting for identification of species and protection of plant variety rights, and also for the genome mapping and gene tagging (Williams *et al.,* 1990). Genetic purity testing with molecular markers reduces the cost and the time associated with the selection of suitable plants for hybrid production and can be effectively adapted by the breeders (Tiwari *et al.*, 2020). Further SSR marker analysis would be beneficial to the seed industry for routine evaluation of genetic purity of cultivars due to its cost-effectiveness (Pattanaik *et al*., 2018).

Conclusion

Genetic purity analysis and differentiation of maize hybrids can be performed accurately and efficiently using molecular markers. The SSR marker UMC 1997 can be used to analyze the genetic purity of MI maize hybrid 1, and phi 402893 and phi 076 can be used for identification of purity of MI maize hybrid 2. The SSR markers identified through this study will be helpful for hybrid maize seed industry to select appropriate marker combinations and assess genetic purity of commercial maize hybrids.

Acknowledgement

Authors wish to express their sincere thanks to Mrs. W.M.R. Kumari, Assistant Director of Agriculture (Research), FCRDI, Mahailluppallama for providing seed materials and the staff of the molecular biology laboratory, Plant Genetic Resources Center for their cooperation given for this study.

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