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Utilization of SSR Markers for Seed Purity Testing in Popular Maize Hybrids

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A B S T R A C T

Microsatellite markers can be used for fingerprinting of hybrids, assessing variation within parental lines and testing the genetic purity of hybrid seed lot. In this study 75 simple sequence repeat (SSR) markers well distributed on all the 10 chromosomes were employed for fingerprinting of six popular maize hybrids and their parental lines. Seven SSR markers were found to be polymorphic and produced unique fingerprints for the different hybrids. These markers can be used as referral markers for unambiguous identification and protection of the hybrids.

Introduction

Maize (*Zea mays* L.) is one of the most important food, feed and industrial crops globally. It is predominantly a cross-pollinating species, a feature that has contributed to its broad morphological variability and geographical adaptability. Economically, the most important types of maize are grown for grain or fodder and silage production. FAO predicts that an additional 60Mt of maize grain will be needed from the annual global harvest by 2030. The demand for maize as an animal feed will continue to grow faster than the demand for its use as a human food, particularly in Asia, where a doubling of production is expected from the present level of 165 Mt to almost 400 Mt in 2030 (Paliwal *et al.*, 2000).

There is an increasing trend towards production of hybrid maize varieties, which offer increased yield, wide adaptability and reliability in performance and quality. However production and distribution of high quality hybrid seeds is fundamental for potential crop yield. It is estimated that the yield per hectare will decrease about 135 kg if the maize hybrid seed purity drop 1% (Liu *et al.*, 2000). Genetic purity testing of seeds (i.e.,

the percentage of contamination by seeds or genetic material of other varieties or species) contributes to overall seed quality. Determining the genetic purity of hybrid seed is an essential requirement for its commercial use, since there is always a chance of contamination in the hybrid seed production plot because of pollen shedders, out crossing and physical mixtures during the subsequent handling of the harvested material.

Farmers also often complain about low quality seed and seed mixtures. Moreover the characterization of genetic stocks and varieties is mandatory for the purpose of registration with the competent authority and for granting Plant Breeder's Rights under the criteria of distinctness, uniformity and stability (DUS).

Conventional characterization of hybrids based on specific morphological and agronomic data is time consuming, restricted to a few characteristics, influenced by environmental condition and inefficient. Protein markers, seed storage proteins and isozymes have also been used to estimate genetic purity as in sunflower (Alireza 2014), maize (Dou *et al.,* 2010) and in some flowering plants (Sinha *et al.,* 2012).

Molecular marker assisted identification with high power of genetic resolutions has emerged as a robust technique for cultivar fingerprinting, identify profiling, estimating and comparing genetic similarity and variety protection. Among available DNA markers systems, PCR based co-dominant SSRs (also known commonly as microsatellites) are preferred for genotyping because of their reproducibility, abundance and amenability to high throughput screening. The SSR markers are of great importance for rapid assessment of hybrid and parental line seed purity (Dongre *et al.,* 2011, 2012; Pallavi *et al.,* 2011; Reddy *et al.,* 2011, 2015). These markers allow the early identification of true interspecific hybrids for further evaluation and crossing, and simultaneously, enable the early disposal of non-hybrids, thus delivering substantial savings in time and resources. In recent years, many SSR markers have been developed and are publicly available (http://www.maizegdb.org/ssr.php) based on their target sequences among different maize germplasm accessions.

The primary objective of the present study was to develop the DNA fingerprints for the six popular maize hybrids and their parental lines, and to establish the basis for identification and monitoring of seed purity for these hybrids.

Materials and Methods

Plant material

For the purpose of molecular identification, six maize hybrids HQPM-1, HQPM-4, HQPM-5, HQPM-7, HM-2 and HM-4 released for commercial cultivation in different parts of India and their parental lines were selected. The F_1 seeds of these hybrids and their parental lines (Table 1) were obtained from Maize Section, Department of Genetics and Plant Breeding, Chaudhary Charan Singh Haryana Agricultural University, Hisar. Seeds of the above mentioned hybrids were grown in greenhouse. A random sample of 400 seeds of each of the hybrid representing the commercial F_1 seed lot was used for testing their genetic purity.

Molecular analysis

Plant DNA was isolated from seedlings using CTAB (Doyle and Doyle 1990) protocol as follows: about 0.1 g of young leaf tissue for each sample was homogenized in liquid nitrogen, and incubated at 60^0C for 30–45 min with 500 µL of CTAB buffer (1.0M Tris-HCL pH 8.0, 3M NaCl, 0.5M EDTA). Then 500µl

24: 1 of chloroform: isoamyl alcohol mixture was added and blended thoroughly for 5 min. After centrifugation (5 min, 13000 rpm), aqueous layer was pipetted into a new Eppendorf tube and an approximately equal volume of cold ethanol was added. After storage at -20 ^{0}C for 30–60 min, precipitated DNA was centrifuged, air dried and finally stored in TE buffer.

For fingerprinting, DNA from the bulk leaf samples of 2 to 5 individual plants was used. Quantification of DNA was accomplished by analyzing the DNA on 0.8 % agarose gel using diluted uncut lamda DNA as standard. DNA was diluted in TE buffer to a concentration of approximately 30ng/ µl for PCR analysis. The sequence information for the primer pairs was obtained from published sequence data (http://www.maizegdb.org/ ssr.php). A total of 75 hyper variable SSR primer pairs distributed across the 10 chromosomes were used for PCR amplification (Table 2).

PCR amplification

Seventy five SSR primer pairs were selected for this study. PCR was performed in a reaction mixture volume of 20 µL containing of 30 ng of template DNA, 1 x PCR buffer with 1.5 mM of $MgCl_2$, 0.2 mM of each dNTPs, 10 pmol of each primers and 1U of Taq DNA polymerase.

PCR was carried out in a Thermal Cycler programmed for 35 cycles of 95^{0}C (5 min), 94^{0}C (1 min) 56^{0}C (30 Sec.), 72^{0}C (1 min) then followed by final-extension at 72^{0}C for10 min. PCR products (10 µl) were used for electrophoresis and the amplicons were resolved on 2.5 % agarose gel stained with ethidium bromide at 1 µg/ ml, and visualized under UV in a gel documentation system and impurities were identified based on deviations in expected amplification pattern.

Results and Discussion

Characterization and identification of cultivars is crucial to varietal improvement, release and in seed production programme. It is mandatory to maintain the genetic purity of hybrid seed for the successful crop production. Unambiguous characteristic pattern of hybrids can be obtained using DNA markers and had been termed as DNA fingerprinting. The use of DNA markers to obtain genotype specific profiles had distinct advantages over morphological and biochemical methods. The morphological markers are influenced by the environmental conditions, labour intensive and time consuming. However, the biochemical markers such as isozyme and protein patterns are least influenced by the environment but exhibit limited polymorphism and often do not allow discrimination between closely related inbred lines (Lucchese *et al.,* 1999).

DNA markers overcome most of these disadvantages of morphological and biochemical markers that can be useful to distinguish varieties and off types. The usefulness of DNA fingerprinting technique for cultivar identification was demonstrated by Dallas (1988) for the first time in rice. The present study utilized the SSR marker techniques for identification of the maize hybrids along with their parental lines, demonstrating that this technique can be successfully applied to distinguish and identify the hybrids from its parental lines. SSR had much more polymorphism than most of other DNA markers, and is co-dominant and large in quantity. Therefore, the high polymorphic information content (PIC) of SSR had promoted the application of microsatellites as molecular markers in fingerprinting (Ashikawa *et al.,* 1999). In this study primer pairs of 75 SSRs associated with each hybrid and parental lines were assessed on 2.5 per cent agarose.

Fig.1 Polymorphic SSR marker profile confirm hybridity of HQPM1 with pumc1064; L: 100bp ladder, lane 1: HKI 193-1, lane 2: HKI 163, lane 3: HQPM1

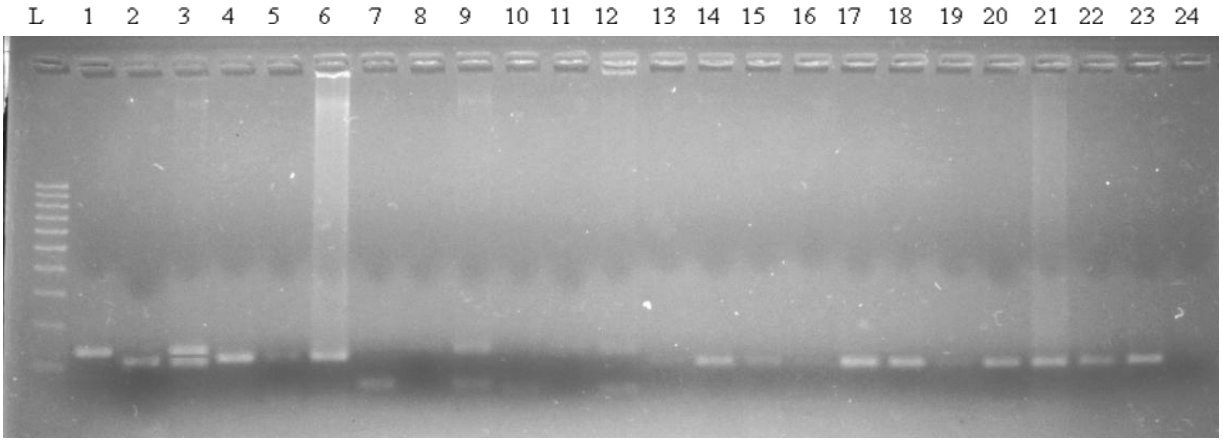

Fig.2 Polymorphic SSR marker profile confirm hybridity of HQPM4 and HQPM7 obtained with pumc1013; L: 100bp ladder, lane 4: HKI 193-2, lane 5: HKI 161, lane 6: HQPM4, lane 10: HKI 193-1, lane 11: HKI 161, lane 12: HQPM7

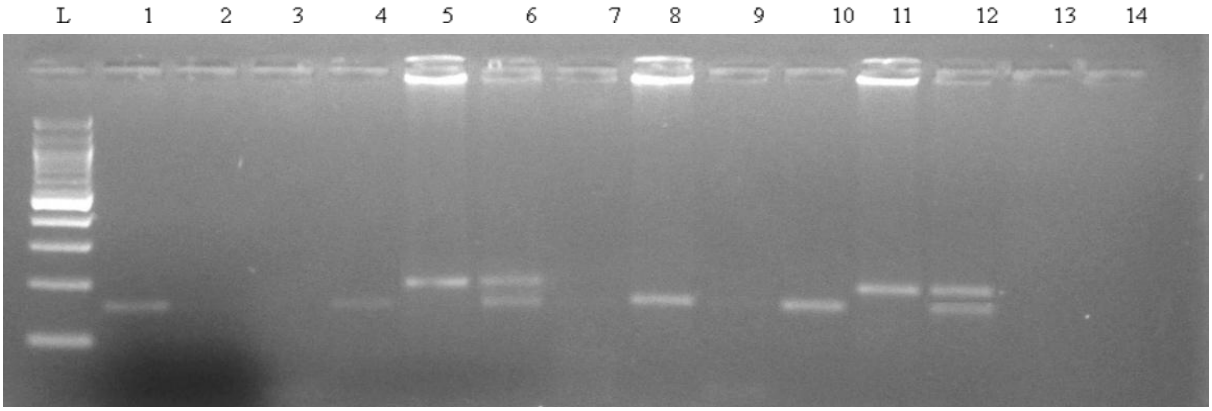

Fig.3 Polymorphic SSR marker profile confirm hybridity of HQPM4 and HQPM5 obtained with pumc1746; L: 100bp ladder, lane4: HKI163, lane5: HKI161, lane6: HQPM4 lane7: HKI163, lane8: HKI161, lane 9:HQPM5

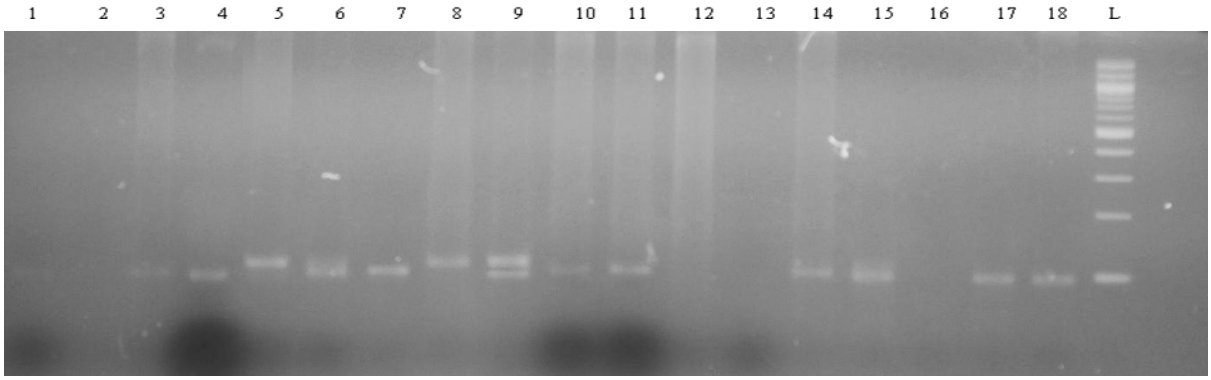

Fig.4 Polymorphic SSR marker profile confirm hybridity of HQPM 7 obtained with pumc1071; L: 100bp ladder, lane 10: HKI 193-1, lane 11: HKI 161, lane 12: HQPM7

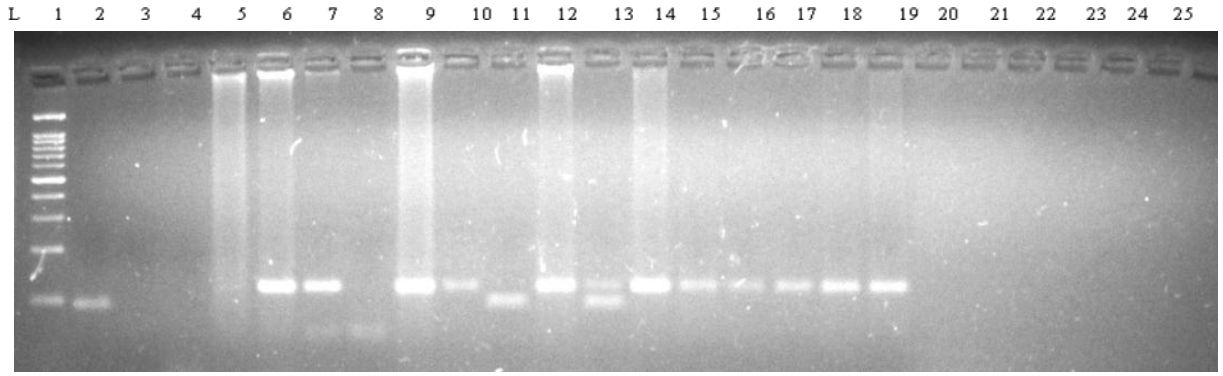

Fig.5 Polymorphic SSR marker profile confirm hybridity of HM2 with pumc1064; L: 100bp ladder, lane16: HKI 1352, lane17: HKI 1344, lane=18: HM2

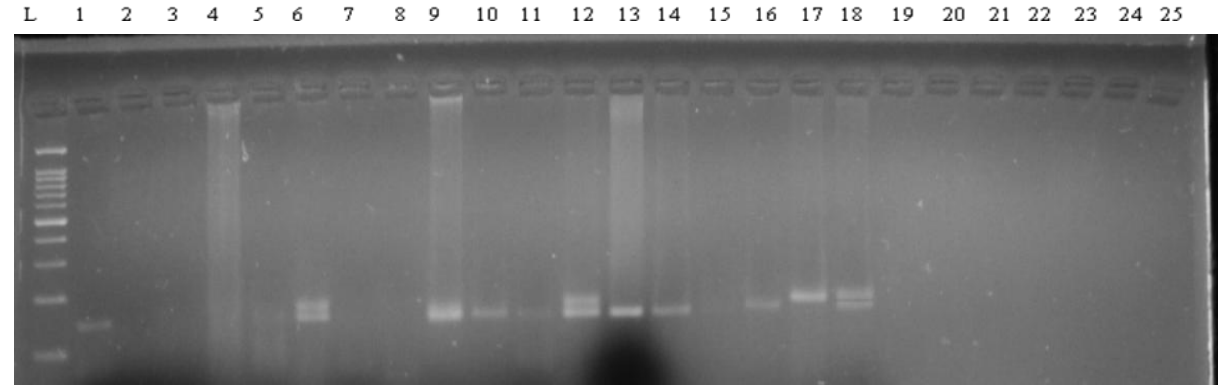

Fig.6 Polymorphic SSR marker profile confirm hybridity of HM 4 and HM 2 obtained with pumc1040; L: 100bp ladder, lane13: HKI 1105, lane14: HKI 323, lane15: HM4, lane16: HKI 1352, lane17: HKI 1344, lane18: HM2

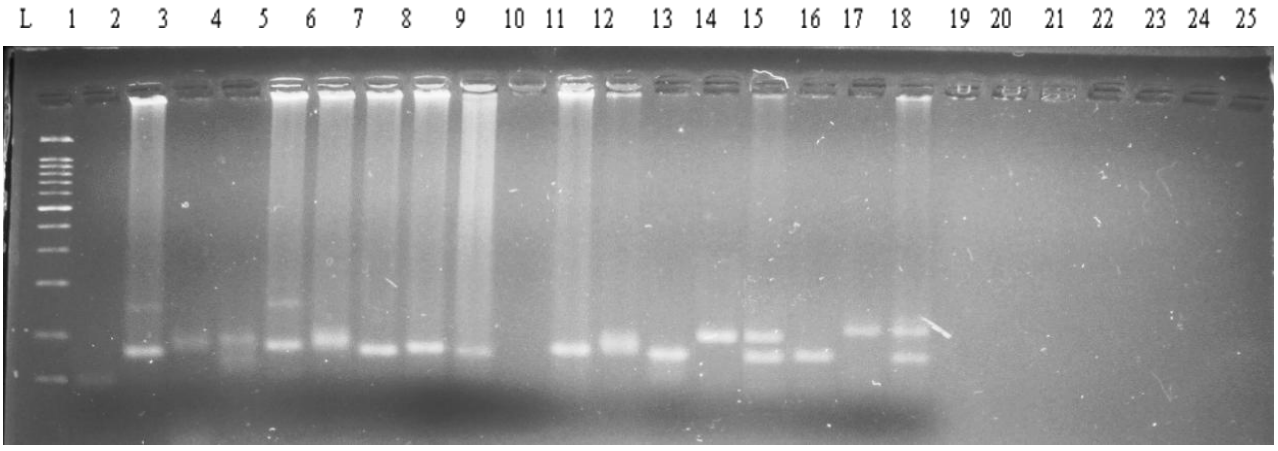

Table.1 Different hybrids along with their parental lines used in the present study

S. No.	Hybrid	Parent 1	Parent 2
1	HQPM1	Hki 193-1	Hki 163
2	HQPM4	Hki 193-2	Hki 161
3	HQPM5	Hki 163	Hki 161
4	HQPM7	Hki 193-1	Hki 161
5	HM2	Hki 1352	Hki 1344
6	HM4	Hki 1105	Hki 323

Table.2 SSR markers used in the study

S. No.	SSR	Repeat motif	Primer sequence (5′-3′) Forward	Primer sequence (5′-3′) Reverse	Chromosome location
1.	p-umc1009	*(GT)7(GA)7*	AGCAGCTCTGGTGATGGAAGAA	ATCCTAACAGGCGCATACCAGA	1
2.	p-umc1013	*(GA)9*	TAATGTGTCCATACGGTGGTGG	AGCTGGCTAGTCTCAGGCACTC	1
3.	p-umc1021	*(GT)14*	AGCCTCCTGAGACCTCTCGATT	ACTTCGCCACCTTACATTCTTGA	1
4.	p-umc1035	*(CT)19*	CTGGCATGATCACGCTATGTATG	TAACATCAGCAGGTTTGCTCATTC	1
5.	p-umc1070	*(TC)7*	GGTCTCTCTATCGTCCGGTGAGTA	CCGGAGATGGGAAAGAAGATAAC	1
6.	p-umc1071	*(TACGA)5*	AGGAAGACACGAGAGACACCGTAG	GTGGTTGTCGAGTTCGTCGTATT	1
7.	p-umc1082	*(GA)16*	CCGACCATGCATAAGGTCTAGG	GCCTGCATAGAGAGGTGGTATGAT	1
8.	p-umc2363	*(ATGT)4*	TTGACTCGAAAGACTTGTGAGCTG	GTGTGTCAGAAGGAGGACTGATGA	2
9.	p-umc1227	*(AGG)4*	CAAGTTGGTGAGATGGATCTGTTG	GCTCCTGGGTCTTCCTCTCC	2
10.	p-umc1552	*(GGA)7*	CTCGATAGCTCTGCTGCTTCCTC	CAACACCAGCCCTACCCAGA	2
11.	p-umc1823	*(TG)36*	AAAGCCTTACTGTTATTAGGCTAGGCA	AGAAAACCAGCCCCAGATGTTC	2
12.	p-umc2186	*(CGG)6*	CTCCCGCAGTCTATGAAGCTCAC	CTCCTCTTCCTCCTCCTCGTTGT	2
13.	p-umc2193	*(TCC)6*	CCGAGGCATACGGACAATACC	GTAGGAGGACGGGTGCTGGT	2
14.	p-umc2245	*(CAA)7*	GCCCTGTTATTGGAACAGTTTACG	CGTCGTCTTCGACATGTACTTCAC	2
15.	p-umc2246	*(CCTCCT)4*	AGGCTCCAGCTCTAGGGGAGT	GTGAACTGTGTAGCGTGGAGTTGT	2
16.	p-umc1057	*(CGG)6*	GCCACGCTCAACTACGACAAC	GAACCCCTCCACGTAGCTCAG	3
17.	p-umc1183	*(AG)15*	ATGTCATTTTTGGCTTCTCGAAAT	GCATGTACACAACACAACCTTTCA	3
18.	p-umc1458	*(GCT)5*	CCAATAAACAAATCATCTCCCCCT	TGCTATGCTATGTACAGGGACAGG	3
19.	p-umc1746	*(CAC)4*	ACACGAGCATCCTACATCCTCCTA	ACCTTGCCTGTCCTTCTTTCTCTT	3
20.	p-umc1793	*(AT)6*	TGCACACCCTTTATTGAATCATCA	CGTATAAGCTTTTTGGGGTCCTCT	3
21.	p-umc1814	*(CGA)4*	AGAAGAAGAGGAGGTTCCATGACC	GCATGTTTCCCTTTCACCTCC	3
22.	p-umc1886	*(CG)8*	GTTTGACAGCACAAGTGCAAGAAA	GAGGTGGACATTGGACAACACC	3
23.	p-umc1892	*(GA)8*	AGCTGCAAAAGCAAGTGAAACAAT	TCTCCTCGATCGTTGTATGTGTGT	3
24.	p-umc1228	*(CAG)8*	TCCTCAAGGACCTGCTCCAC	ACCTATACAGACGGAGACGGGG	4
25.	p-umc1232	*(ACAG)4*	GGAATTACCACAACAAACTAAACTTGG	AGGCTCTAGCTACCTGGCTACGTT	4
26.	p-umc1561	*(TTTA)4*	TCTCTTCATCTCAGCATCTCTCCA	TATTGTGATGTGAGCTGCATTGG	4
27.	p-umc1669	*(AGA)4*	ACGAGGGCTTCTTCTCTGAGC	GTTTCCTTCTTCATGCGACGAC	4
28.	p-umc1842	*(AG)16*	CCACCTGCACAACACACATAAAAC	GTGGTGGAGTCGCTAAGTCAGGT	4
29.	p-umc1758	*(CTT)5*	CTTCCTCCTCACCTCACCTCCTAT	GGTAGCCAATCCTTCCTTCCTATG	4
30.	p-umc2278	*(TCTC)4*	CTGACCTCCGTCATCAGCATC	ATCACGGACAAAGAAAATTGAAGC	4
31.	p-umc1834	*(AT)8*	AAGATAATGCAACCCTGGACAAAA	TTGCGGATCTGTTAAGGTGACATA	4
32.	p-umc1631	*(ATGT)8*	CATGAATAAAGATGGATGCTGGTG	GGAAAAACAAAGAAGCATAGTAGACAGC	4
33.	p-umc1086	*(CT)12*	CATGAAAGTTTTCCTGTGCAGATT	GGGCAACTTTAGAGGTCGATTTATT	4
34.	p-umc2291	*(CCT)5*	CTCGACGAGTTCAAGCGCTAC	AACTTCTCCTGGCGAGCATCT	5
35.	p-umc2292	*(CTGCCT)4*	AGCAGAAGAGGACAAACCAGATTC	ACTTCCGGCATGTCTTGTGTTT	5
36.	p-umc1853	*(GT)8*	TTATTATTAACACCTGCCTGCGCT	GCTAGCTAGGAAACATGGCTTGTC	5
37.	p-umc1423	*(CTAG)4*	TAGTATGGTCCATTGATGCTGGC	GAGCAGGCGGAGGATACTAGC	5
38.	p-umc1155	*(AG)20*	TCTTTTATTGTGCCCGTTGAGATT	CCTGAGGGTGATTTGTCTGTCTCT	5
39.	p-umc1491	*(AGA)5*	TAATAATCCCAAACCACCAAAAGG	GATTTGAGGCCATAGTGCTCCTTA	5
40.	p-umc1496	*(GCA)8*	GATTACAACCCACCGGAGTTACAG	GCTCTTCCTAGGTGCAGACAAAGA	5
41.	p-umc1679	*(AAG)5*	CACTGCTAAGCTGCTCCCTGTT	TGCTAACTAACCCTGACCCTCTCA	5
42.	p-umc2022	*(AGCG)4*	TTAGTCTAACCGAGTCCAACCAGTG	ACCAGCAGACGGAGAGCTTG	5
43.	p-umc1002	*(TA)10*	AGCTAGCTATACACCGCCAGG	TCAGTTTGGAACAGGGAAAAGTA	6
44.	p-umc1006	*(GA)19*	AATCGCTTACTTGTAACCCACTTG	AGTTTCCGAGCTGCTTTCTCT	6

45.	p-umc1018	*(CT)7*	GAACGGATATTGGAACCTGTGC	GTGCACGGTGTCGTACTTGAAC	6
46.	p-umc1020	*(CT)8*	CCTGGAGAGCCACTACAAGGAA	TCAGCCTGAGCTCACATCATCT	6
47.	p-umc1023	*(AT)11*	CTTGTGCCACCACATGCAGTA	CAGTTTGGAACAGGGAAAAGTACG	6
48.	p-umc1083	*(GA)16*	CTTTCCTCTCTGGAGCGTGTATTG	ATATGTTGCAGAACCATCCAGGTC	6
49.	p-umc1114	*(AGAA)6*	CAATGTGTTATTGATTGTACACCGC	ACAGCAGGAGGCAGAGACTGAC	6
50.	p-umc1546	*(GAG)5*	CTGGTCTTGGCCTTGGACTTCT	GTCACAGCAAAGTCATCCTCCTCT	7
51.	p-umc1133	*ATAC*	ATTCGATCTAGGGTTTGGGTTCAG	GATGCAGTAGCATGCTGGATGTAG	6
52.	p-umc1066	*(GCCAGA)5*	ATGGAGCACGTCATCTCAATGG	AGCAGCAGCAACGTCTATGACACT	7
53.	p-umc1159	*(AG)8*	TTCCCATGTTCATTTCAGGTTCTT	TCATGGGTTTTGAGGCTGTATTTT	7
54.	p-umc1241	*(GTCTTTG)4*	TGAAGCAAGTCACTGGTAAGAGCA	TGACACACCCATACTTCCAACAAG	7
55.	p-umc1378	*(CGC)6*	GAAGTCGCTGATGAGAACGTAACC	GCTAGCTAGTGTGAGTTCTTCCGC	7
56.	p-umc1577	*(CTTGGC)4*	TTTCCCTTCTTGGCAGGAGC	AAGAACTCCTTCAAGCTGCCG	7
57.	p-umc1583	*(GAA)4*	AAAGGGCGACTTGTTTTTGTTTTT	GCCTGCTTTTGTGTATCTTAGGCA	7
58.	p-umc1545	*(AAGA)4*	GAAAACTGCATCAACAACAAGCTG	ATTGGTTGGTTCTTGCTTCCATTA	7
59.	p-umc2042	*(GCC)4*	GCAGTCTCTCCACTACCAGAGCAT	AACAGAGGAGTACGAGGAGGAGC	8
60.	p-umc1034	*GA(12)*	GTGTTTCCGTTTCGCTGATTTTAC	TCATCCATGTGACAGAGACGACTT	8
61.	p-umc1414	*(GCTA)4*	CGATCATCTCTCACTCTCTCGTCA	GTTGACGACGTCTGGCTCCT	8
62.	p-umc1786	*(TC)7*	ACCGTGACTTCCTCCTCATAACTG	CATTTTTCGCATTTAGGAAATCCA	8
63.	p-umc1868	*(AT)8*	CCATCATGGAGTTGCGGTTATTTA	CCCATAGAGTGCTTGAAATTGTTGA	8
64.	p-umc1064	*(CT)8*	GTGGGTTTTGTCTGTAGGGTGGTA	TCCATCCACTCGACTTAAGAGTCC	1
65.	p-umc1279	*(CCT)6*	GATGAGCTTGACGACGCCTG	CAATCCAATCCGTTGCAGGTC	9
66.	p-umc2335	*(TG)6*	ATTCAGTTTGTAATTGTCGGTGGG	AATCACTATCATTACTGGCTGGGC	9
67.	p-umc1809	*(GGA)5*	ACTCTTCTTCTTCCACCGGGAC	GTCGGACTCGAGAAACGACG	9
68.	p-umc1867	*(TC)8*	TGGTCTTCTTCGCCGCATTAT	ATAAGCTCGTTGATCTCCTCCTCC	9
69.	p-umc2084	*(CTAG)4*	ATCGCGACGAGTTAATTCAAACAT	TACGATGTCTTCAGTGTGACACCA	9
70.	p-umc1038	*(CT)15*	CGTCACACTCCTCTGCCACTT	GAGGATTCAGAACTCGACTCGG	10
71.	p-umc1054	*(CAG)6*	CCGTCTTCTTCAGGGTGTTCC	GTGGAGTTAGTAGGGTCGTTGCAC	10
72.	p-umc1077	*(CA)15(CGCA)12*	CAGCCACAGTGAGGCACATC	CAGAGACTCTCCATTATCCCTCCA	10
73.	p-umc1084	*(CT)23*	GATAAAAAGGCAAGTGCAACAAGG	ATATCAACCAGAGGCTGGAACTTG	10
74.	p-umc1115	*(AG)6*	TGGAAGGGGATATCAGGATTTAGA	TGTGATGACCATGAATGTAAGCTG	10
75.	p-umc1239	*(TG)6*	ATCAACACACCTTTCGATTTCTGG	CGGTGATTAGTCGATGAAGAGTGA	10

Table.3 SSRs identifying different hybrids along with the size of the amplified alleles

Hybrid	Size of the amplicon in Parent 1	Size of the amplicon in Parent 2	Hybrid Identifying SSR/(s)
HQPM1	130 bp	100 bp	pumc1064
HQPM4	180bp	200 bp	pumc1013
HQPM5	100 bp	130 bp	pumc1746
HQPM7	100 bp	130 bp	*pumc1071, pumc1066
HM2	150bp	170 bp	pumc1035
HM4	150 bp	200bp	pumc1040
***Both these SSRs produced same size of amplicons These SSRs can be used to differentiate hybrids from their parents**			

The PCR products of the DNA samples on the agarose did appeared and showed polymorphism among the hybrid and their parental lines. Among the 75 primers studied, eight primers viz., pumc1064, pumc1013, pumc1746, pumc1071, pumc1066, pumc1040, pumc1023 and pumc1035 showed polymorphism between the parental lines which were used for the production of maize hybrids and rest of primers showed monomorphic banding pattern (Table 3).

The SSR primer pumc1064 amplified a specific allele of size 130 bp in HQPM-1 and its female parent Hki193-1 but not in pollen parent Hki163. While the allele size of 100 bp was present in pollen parent. The same allele size of 100 bp has also appeared in F_1 hybrid

but not in female parent (Figure 1). Thus, it confirmed that the presence of both female and male parent alleles was observed as a resultant of crossing between two parents (F_1 hybrid). Similarly pumc1013 maker had also resulted in amplifying allele of size 180 bp in female parent (Hki193-2) which was absent in pollen parent (Hki 161). While, the pollen parent had an amplicon at 200 bp which was absent in female parent. However, the hybrid HQPM-4 exhibited both the alleles of the parents confirming the heterozygosity condition of the hybrid by having bands at 180 and 200 bp (Figure 2). The identified SSRs in F_1 hybrids showed complementary banding pattern of both the parents. Hybrid HQPM-5 could be identified and distinguished by the SSR marker pumc1746 (Figure 3), HQPM-7 by pumc1071 (Figure 4) and pumc1066, HM-2 by pumc1064 (Figure 5) and HM-4 by pumc1040 (Figure 6).

The banding pattern of all these hybrids showed both the amplicons present in female as well as pollen parent, thus confirming the genuine crossing and heterozygotic condition of the hybrid. The SSR markers identified had both female and male specific bands and are useful in genetic purity testing. The use of SSR markers for genetic purity testing has already been demonstrated in maize (Daniel *et al.,* 2012; Mrutu, 2015; Simon and Lovasz, 2016; Wu *et al.,* 2010); rice (Bora *et al.,* 2016; Galal *et al.,* 2014; Moorthy *et al.,* 2011; Sudharani *et al.,* 2013), pearl millet (Nagawade *et al.,* 2016), eggplant (Reddy *et al.,* 2015), soybean (Zhang *et al.,* 2014), cauliflower (Zhao *et al.,* 2012) and in sunflower (Pallavi *et al.,* 2011).

The present study showed that SSR markers are quick, effective and results are generally consistent with morphological analysis in the field study. Primers identified in the study could be utilized for routine genetic purity testing of these maize hybrids. The SSR marker information developed through this study will be of immense help for hybrid maize seed industry to select appropriate marker combinations and assess genetic purity of the crop.

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