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

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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.

Keywords
Hybrid, Maize, Molecular Markers, Identification, Seed purity

Abstract
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 60 Mt 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 F1 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 F1 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°C 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°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 lambda 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 MgCl2, 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°C (5 min), 94°C (1 min) 56°C (30 Sec.), 72°C (1 min) then followed by final-extension at 72°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

![Image of Fig.1](image1.png)

**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

![Image of Fig.2](image2.png)

**Fig.3** Polymorphic SSR marker profile confirm hybridity of HQPM4 and HQPM5 obtained with pumc1746; L: 100bp ladder, lane 4: HKI163, lane5: HKI161, lane6: HQPM4 lane7: HKI163, lane8: HKI161, lane 9: HQPM5

![Image of Fig.3](image3.png)
**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, lane 16: HKI 1352, lane 17: 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, lane 13: HKI 1105, lane 14: HKI 323, lane 15: HM4, lane 16: HKI 1352, lane 17: HKI 1344, lane 18: 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  | 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 | AGCAGCTCTGTTGATGGAAGAA          | ATCTCTAACAGGCGCATACCAG         |                                   | 1                   |
| 2      | p-umc1013 (GA)9       | TAAATGTGTCCATACGGTGTTG          | AGCTGCGTACTTCTGACGTCT          |                                   | 2                   |
| 3      | p-umc1021 (GT)14      | AGGCGTCTGAGACCTCCTATTG         | ACTTCCCAGACCTTACATCTT          |                                   | 1                   |
| 4      | p-umc1035 (CT)19      | CTGGCATATGACCGCTATTG           | TAAATCAGCAGGCTTGCTATTCC        |                                   | 1                   |
| 5      | p-umc1070 (TC)7       | GGGTCTCTATATGCTGTAATGTA        | CCGGAGATGAGAAAGAAGAAT          |                                   | 1                   |
| 6      | p-umc1071 (TACGA)5    | AGGAAGAAGAAGAAGGAGAAGTAG      | GTTGGGTGCTGAGCTGTTATTG         |                                   | 1                   |
| 7      | p-umc1082 (GA)16      | CCGACAGACATACAGCCACACC         | GTAGAGGAGGAGGACTATGGA          |                                   | 2                   |
| 8      | p-umc1227 (AGG)4      | CAAGTGGTGAGTGGGATGTG           | GCTCTCAGGAGGCTTTCCCCC          |                                   | 2                   |
| 9      | p-umc1552 (GAG)7      | CTCGATAGCTGCTGCTCTTCT          | GCTGCTTGGTCTGAGATGGTCT          |                                   | 2                   |
| 10     | p-umc1823 (TG)36      | AAAGCGTATGCTAAGTCGTAGGCA       | AGAAGAACAGACCGAGATGCTC         |                                   | 1                   |
| 11     | p-umc2186 (CGG)6      | CTTCCGGAAGACAGCAGCAC           | GAGTTTGCAGAGGCTGGTCTG          |                                   | 2                   |
| 12     | p-umc2193 (TCC)6      | CCGAGGCATAGGGACAACC           | GTAGAGGAGGAGGACTATGGA          |                                   | 2                   |
| 13     | p-umc2245 (CAA)7      | GCCTGCTATGACCTGCTGCAAG        | GCTGCTTGGTCTGAGATGGTCT          |                                   | 2                   |
| 14     | p-umc2246 (CCTCT)4    | AGGCGTCTGAGAGGAGGAG            | GTGAACTTGTTAGGCGAGTGTG         |                                   | 3                   |
| 15     | p-umc1057 (CGG)6      | GCCAGCTCCTAATACGCAAC           | GAACCCTCCAGATCACCTGAG          |                                   | 3                   |
| 16     | p-umc1183 (AG)15      | ATGTATITTTGGTCTGCTGAAAT        | GCATGTCACACACACACTCCCA         |                                   | 3                   |
| 17     | p-umc1458 (GCT)7      | CCAATACAACTATGCTGCTCCCT       | TGCTATGCTGACAGATGGGAGGAC       |                                   | 4                   |
| 18     | p-umc1746 (CAC)4      | AAGCAAGAAGAAGGAGGAGGAGC       | ATCTTGCTGAGCTGCTCTC            |                                   | 3                   |
| 19     | p-umc1793 (AT)6      | TGCACACCTCCTTACTGACCTACA      | CGTATAAGGTTTGGTCTGCT           |                                   | 3                   |
| 20     | p-umc1814 (GA)16      | CAACACCCCTAATGCTGCTACCCA      | AGGCGTCTGCTGCTGCTGCT           |                                   | 3                   |
| 21     | p-umc1886 (CA)8      | GGTGCCAGCAGACCAAGGCTCATGAC    | GCTGCTTGGTCTGAGATGGTCT          |                                   | 2                   |
| 22     | p-umc1892 (GA)18      | AGCTGCAAAAGAAGAGAAGAAGAAT     | TCTCTCCATGCTGCTCTGCTG          |                                   | 3                   |
| 23     | p-umc1228 (CAG)8     | TCTCTCCACAGCCCTGCACTGCA       | ACCTATACACAGGAGGACGGG         |                                   | 4                   |
| 24     | p-umc1232 (ACAG)4    | GAAGTTTACCCAAACAAACTAATTGCTG  | AGGCGTCTGCTGCTGCTGCT           |                                   | 4                   |
| 25     | p-umc1561 (TTTA)4    | TCTCTCTGCTGCTGCTGCTCCCTCA    | TATTTTGAGTGAGGTGCTAGGG         |                                   | 4                   |
| 26     | p-umc1669 (AAG)7     | ACGAGGGCGCTTGGTCTGCTGAC       | GTCTGCTTGGTCTGAGATGGGAGG       |                                   | 4                   |
| 27     | p-umc1842 (AG)16      | CCACCTCCTCAACACACACAAAGAC     | GTGAGGAGCGATGCTAAGGCAAGG        |                                   | 4                   |
| 28     | p-umc1758 (CTT)5    | CTTCGCGCTTCAGCTGCTGCTATG     | GTTAGCGGCAATCTCCCTTCTCTAGG     |                                   | 3                   |
| 29     | p-umc2278 (TCT)4    | CTGACACCGTCTGCTGCTGCTCAG      | ATGTGCTGCTGCTGCTGCTG           |                                   | 3                   |
| 30     | p-umc1834 (AT)8      | AAAGAATGCAACCCCTGGCAAAGA      | TGGCGCGATGTTGAAGGGAATGGAGATA   |                                   | 4                   |
| 31     | p-umc1631 (ATG)8     | CATGAATTAAGAGTGGGATGTG        | GAGAAAACAAAGAAAGATGTAAGGACAG   |                                   | 4                   |
| 32     | p-umc1086 (CT)12     | CATGGAAGTTTTTCTGCTGCAAGTT    | GGGGACTATTAGGCGATAGGATTTT      |                                   | 4                   |
| 33     | p-umc2291 (CCT)5    | CTGCAGCAGGAGCTGCTCCTCCTAGC   | AACTCTCTGCTGCGACATCC          |                                   | 5                   |
| 34     | p-umc2292 (CTGCT)4   | AGCAGAGAAGGAGAAACACAGATC     | ACTGCTGTTATTCGTTGCTT           |                                   | 5                   |
| 35     | p-umc1853 (GT)8      | TTTATTTAATACACCTGCTGCTG        | GCTGCTGCTGCTGCTGCTG           |                                   | 5                   |
| 36     | p-umc1423 (CTAC)6    | TAGATGATGACCTGATGCTGAC       | GAGCGAGGGAGGAGTAGACTAGC        |                                   | 5                   |
| 37     | p-umc1155 (AG)20     | TCTTTATTTGCTGCTGCTGAGTT      | GCTGCTGCTGCTGCTGCTG           |                                   | 5                   |
| 38     | p-umc1491 (AGA)5     | TAATAATACCCCAACACCAAAAGG    | GATTTGCGGGCATACTGCTTCTGA      |                                   | 5                   |
| 39     | p-umc1496 (GCA)8     | GATTACCAACCCCTGCTGATTGAC     | GCTGCTGCTGCTGCTGCTG           |                                   | 5                   |
| 40     | p-umc1679 (AAG)5     | CACTGCTAAGCTGCTGCTGCTT      | TGCTAATACACCTGGACCTGCTCA       |                                   | 5                   |
| 41     | p-umc2022 (AGG)7     | TTAGCTGTTACAGCGTCAACCGT    | ACCAGCAGGAGGAGGCTT            |                                   | 5                   |
| 42     | p-umc1002 (TA)10     | AGCTACGTGTATACACCGGCAAG    | TCGAGTTTGAACAGGGGGAAGA        |                                   | 6                   |
| 43     | p-umc1006 (GA)19     | AATCCGCTACTTGTGAACCCATCTG   | AGTTCGCCAGCTGCTTCTC           |                                   | 6                   |
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                          | pumc1066                  |
| HQPM7  | 100 bp                          | 130 bp                          | pumc1013, pumc1066        |
| HM2    | 150bp                           | 170 bp                          | pumc1035                  |
| HM4    | 150 bp                          | 200 bp                          | 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 F1 hybrid

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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 (F1 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 F1 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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