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Molecular Profiling for Identification of *Zea mays* L. True Hybrids

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ABSTRACT

Background: *Zea mays* (L.), commonly known as maize or corn, is a diploid (2n= 2x= 20) grain crop which belongs to the family *Poaceae*. It is important to protect and prevent unauthorized commercial usage of hybrids at large scale, which can be best done through molecular profiling. The present study was operated with the purpose to obtain specific simple sequence repeat (SSR) markers which can identify maize hybrids from their parental lines.

Methods: For this work, seven maize hybrids *viz.* HM 5, HM 8, HM 9, HM 10, HM 11, HM 12 and HM 13 along with their ten respective parents were used. A total of 45 SSR primers, well distributed on all the 10 chromosomes of maize were used for polymerase chain reaction amplification.

Result: Molecular study using SSRs resulted in 12 polymorphic and 24 monomorphic markers. Three SSR primers *viz.* pumc2246, pumc1020 and pumc1040 could clearly distinguish different hybrids and their parents. The study will be useful in detecting unwanted seed mixing with the hybrid seeds.

Key words: Hybrids, Maize, Molecular profiling, Simple sequence repeats.

INTRODUCTION

Maize (*Zea mays* L.) is the third most significant cereal crop after rice and wheat. It is a multiple aspect crop, chiefly used for forage and food (Raj *et al*., 2019). It also serves as a basic raw material in industries like starch, oil, protein, alcoholic beverages, food sweeteners, seasonings, fuel, *etc*. It is cultivated on nearly 197 million hectares globally in about 166 countries across the globe and contributes around 37% of the total global grain production (Erenstein *et al*., 2022). USA is the largest producer of maize which contributes 30% of the total global production (https://www.fao.org/3/cb4477en/online/cb4477en.html). All India *Rabi* maize production was 9.9 MT for the year 2020-21 (Maize Outlook Report, 2021). Major maize consumption states in India are Karnataka, Andhra Pradesh, Punjab, Gujarat, Haryana, Telangana, Tamil Nadu, Bihar and West Bengal (Agricultural Market Intelligence Centre, PJTSAU, 2021-22).

Maize has good heterotic potential for total yield, seed quality, disease resistance and uniformity (Dou *et al.,* 2012). However, main constraints for its productivity are suboptimal plant density, inadequate fertilizer use and water supply, weed infestation, insect/pest attack and selection of unsuitable cultivars under a given set of environments. It has been noted that adoption of high yielding hybrids has not just improved grain yield and quality, but has also led to higher income per hectare as compared to conventional varieties of maize (Abbas, 2001). Further, modern maize hybrids have greater potential as compared with older hybrids (Tahir *et al.,* 2008). Since the yield potential of existing maize varieties is deteriorating day by day, so selection of maize hybrids is essential which offer increased yield, wide adaptability and reliability in performance and quality (Ali *et al.,* 2012).

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To introduce heterogeneous hybrids for production of hybrid seeds, it requires two distinct homogenous inbred lines which are crossed and selfed for several generations. But during this practice, it is frequently contaminated by crossed pollens from another variety or sometimes undesired selfing occurs (Hipi *et al*., 2013). For dealing with this issue, plentiful strength has been directed towards achieving the largest kernel set and sophisticated genetic

purity (Fonseca *et al*., 2002). Further, genetic purity during multiplication stages is prone to contamination due to physical admixtures, presence of pollen shedders, out crossing with foreign pollens *etc*. High genetic purity is an essential pre-requisite for the commercialization of hybrid seeds, which is ascertained by compulsory genetic purity test for certified seeds. Besides, success of any hybrid technology depends on the availability of quality seeds supplied in time at reasonable cost.

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 (Dou *et al.*, 2012). One of the challenges faced during such testing is rapid and accurate assessment of hybrid seeds before they are supplied to the farmers. If low genetic quality hybrid seeds are sown then it results in loss of productivity (Hipi *et al*., 2013). It was estimated that for every single per cent impurity in the hybrid seeds, the yield reduction is 100 kg per hectare (Bora *et al.*, 2016).

Conventional purity assessment in fields is conducted based on morphological characters. Morphological identification by grow-out test (GOT) at field level wastes so much time, limits to resources and can affect the data by different environmental conditions (Hipi *et al*., 2013). Compared with morphological variations, molecular and biochemical polymorphisms are generally considered more informative. However, same limitations apply for isozyme analysis also, where different conditions of environment may vary the accuracy of results. Further, selection of isozyme markers must be precise (Lucchese *et al.*, 1999).

Nowadays, use of molecular markers-which reveals polymorphism at the DNA level-has been playing an increasing part in plant biotechnology and genetic studies (Kumar, 2013). Characterization by specific markers which can identify the male and female parents from selfed parental and outcrossed lines in F_1 can be effectively used for distinguishing the true hybrids (Mohan *et al.*, 2013). Amongst molecular markers, polymerase chain reaction (PCR) based simple sequence repeats (SSRs), also known as microsatellites have been most widely used, due to high degree of information provided by their large number of alleles per locus (Asma *et al*., 2016). Further, SSRs are co-dominant, can discriminate between closely related individuals efficiently and can verify hybrids also (Babaei *et al.,* 2007; Iqbal *et al.,* 2010). As SSRs are conserved between closely related species, therefore they could provide a marker database for cultivar identification (Zhang, 2005). The aim of the present study was to obtain SSR markers specific for male and female parents of maize hybrids which can help in convenient distinction of hybrids.

MATERIALS AND METHODS

Plant material

Seven maize hybrids *viz.* HM-5, HM-8, HM-9, HM-10, HM-11, HM-12 and HM-13 which are commercially cultivated in various parts of India along with their parental lines were used in the present study (Table 1). All these hybrids along with their parental lines were obtained from Maize Section, Department of Genetics and Plant Breeding, Regional Research Station, Uchani, Chaudhary Charan Singh Haryana Agricultural University, India. The seeds of the respective hybrids along with parents were grown in greenhouse of the Department of Molecular Biology and Biotechnology, College of Biotechnology, CCS HAU, Hisar during 2021-22.

DNA isolation and quantitative analysis

The young and fresh leaves from maize seedlings were used for extraction of DNA using cetyltrimethylammonium bromide (CTAB) method (Saghai-Maroof *et al.,* 1984) with some modifications. Quality and quantity of the isolated genomic DNA was estimated by 0.8 percent agarose gel electrophoresis. An uncut lambda DNA (50 ng/µl) was run as standard. DNA was diluted as 1:10 on the basis of nanodrop reading. The optimum concentration of DNA was 30 ng/µl for each PCR reaction.

PCR amplification

The SSR primer pairs were designed from the sequence information available in published data base (http://www.maizegdb.org/ssr.php). A total of 45 inconsistent SSR primers which were distributed on all the 10 maize chromosomes were used for PCR amplifications (Table 2). PCR was conducted in a reaction mixture of 10 µl consisting of 1 µl of 30 ng DNA, 1X PCR Buffer, 0.25 mM of each dNTPs, 10 µM of both forward and reverse primers and 1U *Taq* DNA polymerase.

The cyclic parameters used for PCR amplifications included initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 45 s, amplification at 46-65°C for 40 s, extension at 72°C for 45 s and final extension step at 72°C for 10 min. Amplified products were checked on ethidium bromide (10 mg/ml) stained agarose gel (2.5%) and photographed using gel documentation system (Benchtop UVP).

RESULTS AND DISCUSSION

With the rapid growth of population at global level, there is a high demand of maize for food and energy supplies. To tackle this increased demand, it is necessary to improve production and quality of maize through proficient breeding programs (Tester and Langridge, 2010). Hybrid maize

Table 1: Parents and their hybrids used in the present study.

Hybrids	Parent I	Parent II
HM-5	Hki1344	Hki1348-6-2
HM-8	Hki1105	Hki161
HM-9	Hki1105	Hki1128
HM-10	Hki193-2	Hki1128
HM-11	Hki1128	Hki163
HM-12	Hki1344	Hki1378
HM-13	Hki448-IPG	Hki 193-1

guarantees much more yields, improve value and decrease the cost of production. Hybrid vigor also called heterosis arises on two genetically distinct inbred lines as parents are crossed to produce a hybrid (Hi bred pioneer.inc) which results in large sized, robust and sturdy plants. Therefore, instant and precise estimation of hybrid seeds purity must be done to ascertain genetic purity before the seeds are supplied to the farmers.

Report Dallas (1998) was the first who gave the usefulness of DNA fingerprinting for cultivar identification.

Table 2: Simple sequence repeat markers used in the present study.

Primer	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
pumc1009	AGCAGCTCTGGTGATGGAAGAA	ATCCTAACAGGCGCATACCAGA
pumc1013	TAATGTGTCCATACGGTGGTGG	AGCTGGCTAGTCTCAGGCACTC
pumc1035	CTGGCATGATCACGCTATGTATG	TAACATCAGCAGGTTTGCTCATTC
pumc1064	GTGGGTTTTGTCTGTAGGGTGGTA	TCCATCCACTCGACTTAAGAGTCC
pumc1070	GGTCTCTCTATCGTCCGGTGAGTA	CCGGAGATGGGAAAGAAGATAAC
pumc1071	AGGAAGACACGAGAGACACCGTAG	GTGGTTGTCGAGTTCGTCGTATT
pumc1076	TTGGAAATCACCAATTGATATAGTTTG	TCTATTGCAAACGCCAAAAGTAGC
pumc1227	CAAGTTGGTGAGATGGATCTGTTG	GCTCCTGGGTCTTCCTCTCC
pumc1552	CTCGATAGCTCTGCTGCTTCCTC	CAACACCAGCCCTACCCAGA
pumc1823	AAAGCCTTACTGTTATTAGGCTAGGCA	AGAAAACCAGCCCCAGATGTTC
pumc2193	CCGAGGCATACGGACAATACC	GTAGGAGGACGGGTGCTGGT
pumc2246	AGGCTCCAGCTCTAGGGGAGT	GTGAACTGTGTAGCGTGGAGTTGT
pumc1458	CCAATAAACAAATCATCTCCCCCT	TGCTATGCTATGTACAGGGACAGG
pumc1931	TAGCCTGAGTCGTTACAATGTGCT	TAGGTCCACAAAATAAACAGGCAG
pumc1814	AGAAGAAGAGGAGGTTCCATGACC	GCATGTTTCCCTTTCACCTCC
pumc1886	GTTGACAGCACAAGTGCAAGAAA	GAGGTGGACATTGGACAACACC
pumc1228	TCCTCAAGGACCTGCTCCAC	ACCTATACAGACGGAGACGGGG
pumc1232	GGAATTACCACAATAAACTAAACTTGG	AGGCTCTAGCTACCTGGCTACGTT
pumc1561	TCTCTTCATCTCAGCATCTCTCCA	TATTGTGATGTGAGCTGCATTGG
pumc1669	ACGAGGGCTTCTTCTCTGAGC	GTTTCCTTCTTCATGCGACGAC
pumc1631	CATGAATAAAGATGGATGCTGGTG	GGAAAAACAAAGAAGCATAGTAGACAGC
pumc1853	TTATTATTAACACCTGCCTGCGCT	GCTAGCTAGGAAACATGGCTTGTC
pumc1901	CCTATAATAAGTTCACGCAAGTGGC	AAGTACCATTGCACATTTCTGGTG
pumc1002	AGCTAGCTATACACCGCCAGG	TCAGTTTGGAACAGGGAAAAGTA
pumc1020	CCTGGAGAGCCACTACAAGGAA	TCAGCCTGAGCTCACATCATCT
pumc1023	CTTGTGCCACCACATGCAGTA	CAGTTTGGAACAGGGAAAAGTACG
pumc1378	GAAGTCGCTGATGAGAACGTAACC	GCTAGCTAGTGTGAGTTCTTCCGC
pumc1577	TTTCCCTTCTTGGCAGGAGC	AAGAACTCCTTCAAGCTGCCG
pumc1139	TTTGTAATATGGCGCTCGAAAACT	GAAGACGCCTCCAAGATGGATAC
pumc1034	GTGTTTCCGTTTCGCTGATTTTAC	TCATCCATGTGACAGAGACGACTT
pumc1040	CATTCACTCTCTTGCCAACTTGA	AGTAAGAGTGGGATATTCTGGGAGTT
pumc2335	ATTCAGTTTGTAATTGTCGGTGGG	AATCACTATCATTACTGGCTGGGC
pumc2078	TTTTTGTGCTCGTCTGATTTCTTG	AGACCAATGCAACTCCTACAGACC
pumc1115	TGGAAGGGGATATCAGGATTTAGA	TGTGATGACCATGAATGTAAGCTG
pumc1239	ATCAACACACCTTTCGATTTCTGG	CGGTGATTAGTCGATGAAGAGTGA
pumc1545	GAAAACTGCATCAACAACAAGCTG	ATTGGTTGGTTCTTGCTTCCATTA
pumc1075	GAGAGATGACAGACACATCCTTGG	ACATTTATGATACCGGGAGTTGGA
pumc1018	GAACGGATATTGGAACCTGTGC	GTGCACGGTGTCGTACTTGAAC
pumc1105	ATTCCTGCATCATCATCCACTACA	GCCAACTGATCTGCTCTAGCTTC
pumc1066	ATGGAGCACGTCATCTCAATGG	AGCAGCAGCAACGTCTATGACACT
pumc1241	TGAAGCAAGTCACTGGTAAGAGCA	TGACACACCCATACTTCCAACAAG
pumc1480	AATGAAGGTGGATGTGCTGCTACT	CTTCCCCATCTCCTCTGAAGATT
pumc2042	GCAGTCTCTCCACTACCAGAGCAT	AACAGAGGAGTACGAGGAGGAGC
pumc1913	AAACAACTATCCATGTGGCTGACC	CGTTCAGTACAATTTGGCTCAGTG
pumc1084	GATAAAAAGGCAAGTGCAACAAGG	ATATCAACCAGAGGCTGGAACTTG

For hybrid purity estimation, SSRs are the most appropriate markers which are co-dominant and distinguish homozygotes and heterozygotes so that they can easily detect heterozygosity of the hybrids. These markers are currently used for purity identification in many crops.

In the present study 12 SSR markers *viz.* pumc2246, pumc1020, pumc1040, pumc1035, pumc1071, pumc1552, pumc1228, pumc1002, pumc1023, pumc1084, pumc1115 and pumc1064 were found to be highly polymorphic. These selected markers were used for testing seed genetic purity. Among these first 3 markers (pumc2246, pumc1020 and pumc1040) were able to produce polymorphic bands and were efficient to distinguish parental lines of the seven maize hybrids at allelic size of 100 bp to 200 bp (Table 3).

It is already proved that SSRs are authentic tools for hybrid identification or hybrid purity estimation and parentage verification in many crop species (Bohra *et al*., 2011). Utilizing such markers, true hybrids were identified in case of peanut (Busisiwe *et al*., 2015), mungbean (Sorajjapinun *et al*., 2012), rice (Hashemi *et al*., 2009), cotton (Dongre *et al*., 2011]) sugarcane (Zhang *et al*., 2009), maize (Sudharani *et al*., 2013), sunflower (Pallavi *et al*., 2011), oil palm (Thawaro *et al*., 2009), cabbage (Liu *et al*., 2019) and cassava (Mohan *et al*., 2013). SSR markers were used for detection of true hybrids, genetic purity testing, germplasm identification, cultivar fingerprinting, heterotic pattern in hybrids and parentage confirmation of hybrids (Darvhankar *et al*., 2019).

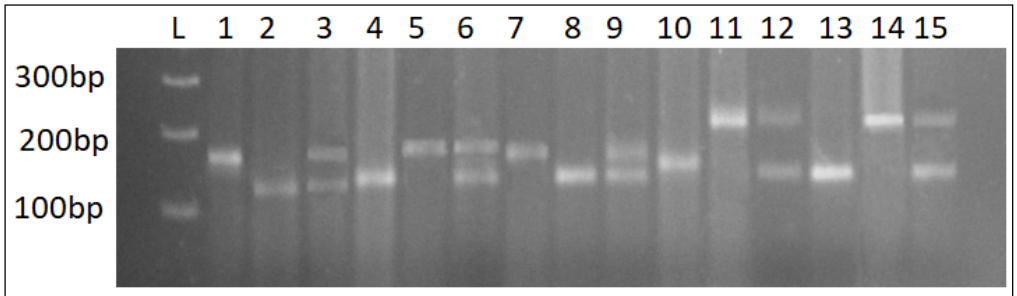
In the present investigation, marker pumc1040 showed the amplified product at 140 bp for male parents of hybrids HM-5 and HM-12. Also, this primer resolved the band position of amplicon at 100 bp and 110 bp for their female parents, respectively (Table 4). This concludes that the hybrid HM-5 was raised from the cross pollination of Hki1344

Table 3: Potential polymorphic simple sequence repeat markers which identified hybrids.

Polymorphic markers	Hybrids
pumc2246	HM-10, HM-13, HM-11
pumc1020	HM-5, HM-8, HM-9, HM-10, HM-11, HM-12, HM-13
pumc1040	HM-5, HM-12

Table 4: Allele size of different parents identified by simple sequence repeat markers.

Marker	Hybrid	Allele size of parent I (bp)	Allele size of parent II (bp)
pumc2246	HM-10	120 (Hki193-2)	150 (Hki1128)
pumc2246	HM-11	150 (Hki1128)	170 (Hki163)
pumc2246	HM-13	130 (Hki448-IPG)	150 (Hki193-1)
pumc1020	HM-5	180 (Hki1344)	140 (Hki1348-6-2)
pumc1020	HM-8	130 (Hki1105)	180 (Hki161)
pumc1020	HM-9	130 (Hki1105)	170 (Hki1128)
pumc1020	HM-10	140 (Hki193-2)	170 (Hki1128)
pumc1020	HM-11	170 (Hki1128)	140 (Hki163)
pumc1020	HM-12	180 (Hki1344)	200 (Hki1378)
pumc1020	HM-13	120 (Hki448-IPG)	200 (Hki193-1)
pumc1040	HM-5	140 (Hki1344)	100 (Hki1348-6-2)
pumc1040	HM-12	140 (Hki1344)	110 (Hki1378)



L: 100 bp ladder, Lanes- 1: Hki1344, 2: Hki1348-6-2, 3: HM-5, 4: Hki193-2, 5: Hki1128, 6: HM-10, 7: Hki1128, 8: Hki163, 9: HM-11, 10: Hki1344, 11: Hki1378, 12: HM-12, 13: Hki448IPG, 14: Hki193-1, 15: HM-13.

Fig 1: Polymorphic simple sequence repeat marker (pumc1020) profile confirmed hybridity of maize hybrids (HM-5, HM-10, HM-11, HM-12 and HM-13).

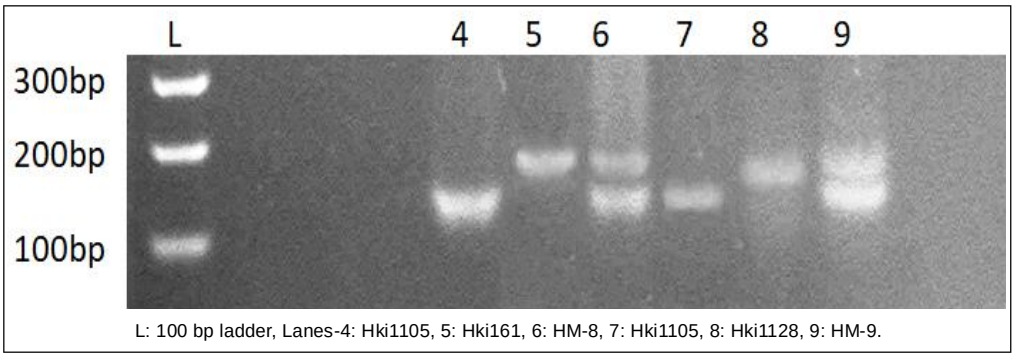


Fig 2: Polymorphic simple sequence repeat marker (pumc1020) profile confirmed hybridity of HM-8 and HM-9.

and Hki1348-6-2 while the hybrid HM-12 was raised from the cross pollination of Hki1344 and Hki1378 *i.e.* heterozygosity was confirmed from both the parents. SSR marker pumc2246 was able to distinguish hybrids HM-10, HM-11 and HM-13 where allelic sizes varied between 120 bp to 170 bp. On the other hand, pumc1020 could distinguish hybrids HM-5, HM-8, HM-9, HM-10, HM-11, HM-12 and HM-13 at allelic sizes between 120 bp to 200 bp (Fig 1, 2). SSR marker pumc1040 could distinguish hybrids HM-5 and HM-12 at allelic sizes between 100 bp to 140 bp.

Kovincic *et al.* (2023) also suggested SSR marker use for more accurate and time-efficient maize hybrids and parental lines genetic purity testing.

CONCLUSION

This study illustrated that SSR markers are rapid, valuable and results are mostly reproducible when compared with morphological data analysis presented by field survey. The identified SSR markers can be used for testing of hybrids for their genetic purity in regular means. This study will be helpful for hybrid maize industry to pick out the suitable combinations of markers for development of SSR markers meant for genetic purity evaluation. They are very convenient for identification of true hybrid seedlings at the early stage of growth and for precise genotyping of the progenies developed for gene mapping studies. The markers can also be utilized for optimizing genomic enabled prediction for maize hybrid breeding programs.

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Conflict of interest: None.

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