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Genetic Analysis of Maize *(Zea mays* L.*)* Hybrids Using Microsatellite Markers

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ABSTRACT

Genetic purity is one of the most important quality criteria required for successful hybrid seed production. In this study, molecular markers were used for assessing the genetic purity and diversity of three commercially important maize F_1 hybrids (Pasha, Frida and PG1661) and their parental inbred lines. Fifty Simple Sequence Repeats (SSRs) markers were used to analyze samples, also the efficiency of the markers were compared. Twenty three primer pairs among the fifty markers were able to detect polymorphism between the different types of hybrids with an average of 0.69 polymorphism information content (PIC) value. Genetic purity analyses revealed more than 98% homogeneity in the hybrid seeds. The hybrids were grouped into three main clusters. It can be concluded that, molecular markers are efficient to study the genetic purity and diversity in maize hybrids and microsatellites are more accurate marker-type because of their co-dominancy.

Keywords: Maize; Seed genetic purity; Genetic diversity; SSRs; Cluster analysis

Mısır *(Zea mays* L.*)* Hibritlerinin Mikrosatellit İşaretleyiciler Kullanılarak Genetik Analizleri

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ÖZET

Genetik saflık, hibrit tohum üretimi için gerekli olan önemli kalite kriterlerinden biridir. Bu çalışmada, moleküler işaretleyiciler ticari olarak önemli 3 mısır F_1 hibrit çeşidi (Pasha, Frida ve PG1661) ve kendilenmiş saf ebeveyn hatlarının genetik saflık ve çeşitlilik analizleri için kullanılmıştır. Örnekler, elli adet basit dizi tekrarları (SSR) işaretleyicileri kullanılarak analiz edilmiş ve işaretleyicilerin verimliliği karşılaştırılmıştır. Testlenen 50 işaretleyici içinden 23 primer çifti ortalama 0.69 polimorfizm bilgisi değeri ile değişik hibrit çeşitleri arasındaki farklılığı tespit etmiştir. Hibrit tohumların genetik saflık analizi, % 98'den yüksek homoloji oranı ile sonuçlanmıştır. Hibritler, 3 ana gruba toplamıştır. Bu çalışma ile moleküler işaretleyicilerin mısır hibritlerinin genetik saflık ve çeşitlilik analizleri için kullanılmasının verimli olduğu ve mikrosatellitlerin ko-dominantlık özellikleri nedeni ile doğru işaretleyiciler olduğu saptanmıştır.

Anahtar Kelimeler: Mısır; Tohum genetik saflığı; Genetik çeşitlilik; SSRs; Küme analizi

1. Introduction

Maize (*Zea mays* L.) is one of the most important cereal crops and has the highest production area worldwide followed by wheat and rice (FAO 2012). It can be consumed as boiled, roasted, vegetable directly by humans as well as being used for livestock feed. Maize cultivation has changed along with the revolution in genetics and maize breeding programs depend on characterization and genetic diversity among breeding material (i.e. inbred lines, hybrids, populations, landraces and races). Identification of genetically distant parental combinations provides best crop improvements for breeders. Also, it is essential to assess genetic purity of hybrids before seed marketing. Genetic purity is one of the quality criteria required for successful hybrid seed production.

Conventionally, purity of F_1 hybrids is assessed by grow-out test (GOT) at the field (Roos and Wianer, 1991). This test is time consuming and resource intensive. Also, it depends on morphological differences which are usually affected by environmental conditions. Isozyme analysis is an alternative method for seed purity testing but it is limited also by environmental conditions and requires accurate selection of isozymes (Lucchese et al 1999). Genetic purity can be determined based on agronomical, morphological, biochemical, and molecular analysis (Wang et al 1994; Dubreuil & Charcosset 1998; Koranyi 1989; Srdic et al 2007). However, molecular markers have advantages because they show very detailed genetic differences and are not affected by environmental factors. They involve fast and the techniques are reproducible (Pejic et al 1998; Warburton et al 2002).

Molecular marker technology provides effective, fast, accurate and appropriate tool for crop improvement. DNA markers such as RFLP (Restriction Fragment Length Polymorphism), SSR (Simple Sequence Repeats), CAPS (Cleaved Amplified Polymorphic Sequences), RAPD (Randomly Amplified Polymorphic DNA), ISSR (Inter Simple Sequence Repeats), AFLP (Amplified Fragment Length Polymorphism), SNPs (Single Nucleotide Polymorphisms) have been used for varietal identification, seed purity testing, genetic similarity analysis and marker-assisted selection of crops in many species (Ajmone-Marsan et al 1998; Bornet & Branchard, 2001; Dangel et al 2001; Powell et al 1996; Mammadov et al 2010). SSRs, also known as microsatellites, are repeated sequences of DNA(Gül-İnce et al 2011) and they can easily detect both parental alleles because of their codominancy.

The objective of the present study was to evaluate genetic purity and diversity among maize hybrids and their parental inbred lines by using microsatellites. Also, SSR marker efficiency was analyzed for further studies on maize.

2. Material and Methods

2.1. Plant materials

Seeds of three maize F_1 hybrids *cv.* Frida, Pasha and PG1661, that are of high commercial importance in Turkey, and their six parental inbred lines were examined in this study (Table 1). Ten seeds of each parental line were mixed and 94 seeds of each hybrid were used for genetic purity analysis. The seeds were randomly selected without bias.

2.2. Genetic purity analysis

A total of 50 SSR markers (Table 2) were screened to select the polymorphic markers. After parental survey of markers, the highly polymorphic ten markers were selected to analyze seed genetic purity of hybrids. The evaluated microsatellites were

Table 1- Agronomical characteristics of tested maize hybrids

Çizelge 1- Testlenen mısır hibritlerinin tarımsal karakteristik özellikleri

Hybrids	*Maturity*	*Plant height*	*Ear height*	*Grain colour*	*Cob colour*
PG1661	130 days	280-300 cm	120-125 cm	Yellow-orange	Red
Pasha	125 days	300-320 cm	120-140 cm	Yellow-orange	Red
Frida	120 days	280-300 cm	110-120 cm	Yellow-orange	Red

selected from maizeGDB website and the primers' information is given at Table 2.

DNAs were extracted according to CTAB method (Doyle & Doyle 1990). Seeds were homogenized by TissueLyser (Qiagen, Germany) and incubated at 65 ºC for 30 min. in CTAB buffer (2% CTAB; 1M Tris-HCl, pH 7.5; 0.5mM EDTA, pH 8.0; 5M NaCl; 2% β-mercaptoethanol). After centrifugation of Chloroform-isoamylalcohol (24:1) added tubes, the supernatant was transferred into a new tube and cold isopropanol was added. DNA was washed and precipitated with ethanol and resuspended in 50 µL TE buffer pH 8.0 (0.1mMTris-HCl; 0.1mM EDTA). The quality and quantity of isolated DNAs were measured by NanoDrop Spectrophometer (ThermoScientific, USA). The selected DNAs were also used for cluster analysis.

After dilutions of DNAs, PCR was carried out with 2 µl of DNA (50 ng ul^{-1}), 0.5 µl of 10 µM dNTP, 1 µl of 25 mM $MgCl_2$, 2.5 µl of 5X PCR buffer and 0.5 µl of 10 µM of each primers (Table 2) with 0.25 µl of 500 units Taq DNA polymerase (Promega Corp., USA) Reactions incubated at 94 °C for 2 min and following 35 amplification cycles (30 s at 95 °C, 30 s at 50-60 °C, and 30 s at 72 °C) were performed. The final PCR products were visualized under UV light after electrophoresis on ethidium bromide-stained 2% agarose gels. The genetic purity percentage was calculated with the following formula;

$$\text{Seed Genetic Purity (\%)} = \left(1 - \frac{\text{off-type}}{total\ \ samples}\right) \text{x } 100\ \% \quad (1)$$

Polymorphism information content (PIC) values of molecular markers were calculated according to the following formula: $PIC = 1 - \Sigma\ Pi^2$. Where; Pi is the frequency of the i^{th} allele (Anderson et al 1993).

2.3. Genetic diversity analysis

The extracted DNAs of three F_1 hybrids and their six parental inbred lines, obtained from genetic purity test, were used for genetic diversity analysis. Twenty three polymorphic SSR markers were used for analysis (Table 3). After gel electrophoresis of PCR products, each band was considered as a single allele and alleles were scored as present (1) or absent (0). The matrix was analyzed to reconstruct phylogenetic tree using the Unweighted Pair Group Method Arithmetic Mean (UPGMA) on Numerical Taxonomy and Multivariate Analysis System (NTSyS-PC) program (Rohlf 2000).

3. Results and Discussion

Based on parental survey analysis using 50 SSR markers, twenty three markers located on ten different chromosomes of maize genome, were found highly polymorphic for both parents and hybrids of *cv*. Frida, Pasha and PG1661 with an average of 0.69 PIC value (Table 3). These selected markers were used for testing seed genetic purity and diversity analyses (Figure 1).

Table 2- Evaluated SSR Markers' information used for genetic analyses

Çizelge 2- Genetik analizler için değerlendirilen SSR işaretleyicilerinin bilgileri

*Maize SSR markers**	*Located chr. no.*
UMC 1363, UMC1976, UMC1395, UMC1358, UMC1111	1
UMC 1265, UMC1465, UMC1004, UMC1108, UMC1604	2
UMC 1970, UMC1425, UMC2002, UMC1135, UMC1273	3
UMC 1228, UMC1963, UMC1117, UMC1109, UMC1707	4
UMC 2291, UMC1587, UMC1060, UMC1155, UMC1072	5
UMC 1143, UMC1133, UMC1857, UMC1413, UMC1859	6
UMC 1241, UMC1159, UMC1134, UMC1708, UMC1407	7
UMC 1327, UMC1913, UMC1858, UMC1268, UMC1638	8
UMC 1370, UMC1809, UMC1191, UMC1231, UMC1137	9
UMC 1380, UMC1962, UMC2016, UMC1115, UMC1196	10

*, available at maizeGDB website

Table 3- Primer sequences, band size and polymorphism information content (PIC) values of tested microsatellites

Çizelge 3- Testlenen mikrosatellitlerin primer dizisi, bant büyüklüğü ve polimorfizm bilgisi

Marker	*F primer*	*R primer*	*No. of bands*	*PIC values**
UMC1363	AAAGGCATTATGCTCACGTTGATT	TCTCCCTCCCCTGTACATGAATTA	6	0.793
UMC1004	CTGGGCATACAAAGCTCACA	TGCATAAACCGTTTCCACAA	5	0.793
UMC2002	TGACCTCAACTCAGAATGCTGTTG	CACAAAATCCTCGAGTTCTTGATTG	6	0.768
UMC1963	CTCGTTCGAGGGGATGTACAAG	CTTGCACTGGCACAGAGACG	3	0.693
UMC1117	AATTCTAGTCCTGGGTCGGAACTC	CGTGGCCGTGGAGTCTACTACT	6	0.793
UMC2291	CTCGACGAGTTCAAGCGCTAC	AACTTCTCCTGGCGAGCATCT	4	0.521
UMC1587	ATGCGTCTTTCACAAAGCATTACA	AGGTGCAGTTCATAGACTTCCTGG	7	0.818
UMC1060	ACAGGATTTGAGCTTCTGGACATT	GGCCTCTCCTTCATCCTATTCAA	7	0.867
UMC1155	TCTTTTATTGTGCCCGTTGAGATT	CCTGAGGGTGATTTGTCTGTCTCT	7	0.818
UMC1072	GAGGAGACCGCCTCTGGTTC	CTTCGGGTTCCTGGACCTTCT	6	0.818
UMC1133	ATTCGATCTAGGGTTTGGGTTCAG	GATGCAGTAGCATGCTGGATGTAG	6	0.793
UMC1413	CATACACCAAGAGTGCAGCAAGAG	GGAGGTCTGGAATTCTCCTCTGTT	8	0.867
UMC1859	ATATACATGTGAGCTGGTTGCCCT	GCATGCTATTACCAATCTCCAGGT	8	0.867
UMC1407	AGGCTTACCTCCTGAGAAGCAGTT	AGGCTTAGCATCGGTGGAGAG	3	0.644
UMC1241	TGAAGCAAGTCACTGGTAAGAGCA	TGACACACCCATACTTCCAACAAG	5	0.521
UMC1327	AGGGTTTTGCTCTTGGAATCTCTC	GAGGAAGGAGGAGGTCGTATCGT	2	0.232
UMC1858	GTTGTTCTCCTTGCTGACCAGTTT	ATCAGCAAATTAAAGCAAAGGCAG	3	0.496
UMC1638	AGGTGACCTCGACGTCCTACG	GAGGGGAACAAAGACTTGACGTT	2	0.359
UMC1191	AAGTCATTGCCCAAAGTGTTGC	ACTCATCACCCCTCCAGAGTGTC	3	0.570
UMC1370	GGGAGCACACACAGTAGTACTCGAT	AGAGGCTCTCCTCCTTCAAGCTC	7	0.855
UMC1962	ATAAGTGGGGGAGGCGAGCTA	GAGAACCAACCACCAAAGAAGTCC	6	0.793
UMC1196	CGTGCTACTACTGCTACAAAGCGA	AGTCGTTCGTGTCTTCCGAAACT	4	0.644
UMC1380	CTGCTGATGTCTGGAAGAACCCT	AGCATCATGCCAGCAGGTTTT	5	0.644
Average			5.17	0.69

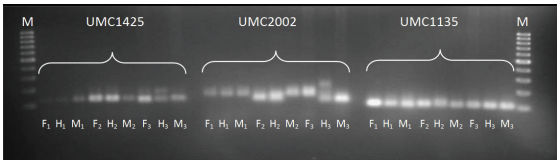

Figure 1- Parental survey analysis results of 3 maize hybrids, *cv.* Pasha, Frida, PG1661 and their parental inbred lines using 3 different SSR Markers (UMC1425, UMC2002, UMC1135). M, 100bp ladder *(Fermentas);* F_1, female parent of *cv.* Pasha; H_1, *cv.* Pasha; M_1, male parent of *cv.* Pasha; F_2, female parent of *cv.* Frida; H_2, *cv.* Frida; M_2, male parent of *cv.* Frida; F_3, female parent of *cv.* PG1661; H_3, *cv.* PG1661; M_3, male parent of *cv.* PG1661

Şekil 1- Pasha, Frida ve PG1661 mısır hibritleri ile ebeveyn hatlarının 3 farklı SSR işaretleyicisi (UMC1425, UMC2002, UMC1135) kullanılarak yapılan ebeveyn tarama analizi sonucu. M, 100bp ladder; F_1, Pasha'nın anne ebeveyni; H_1, cv. Pasha; M_1, Pasha'nın baba ebeveyni; F_2, Frida'nın anne ebeveyni; H_2, cv. Frida; M_2, Frida'nın baba ebeveyni; F_3, PG1661'in anne ebeveyni; H_3, cv. PG1661; M_3, PG1661'in baba ebeveyni

Out the 94 samples of *cv.* Pasha, two samples (sample 5 similar to parent A, sample 14 similar to parent B) were detected as off-type using UMC1858 and UMC1413 markers which detected most off-types among the selected ten markers (UMC1004, UMC1587, UMC1060, UMC1155, UMC1858, UMC1191, UMC1962, UMC1371, UMC1413, UMC1380) (Figure 2). Two seeds of *cv*. Frida and one seed of *cv.* PG1661 out of 94 seeds were detected as off-types using UMC1191 marker (among UMC2002, UMC1963, UMC1117, UMC1363, UMC1859, UMC1638, UMC1858, UMC1191,

UMC1196, UMC2291 markers) and UMC1155 markers (among UMC1004, UMC1963, UMC1060, UMC1155, UMC1133, UMC1241, UMC1638, UMC1371, UMC1196 markers), respectively. The confirmation of off-types detected by one marker with analysis by another marker indicated reliability of the test. According to seed genetic purity analysis of *cv*. Frida, Pasha and PG1661, it was detected that the tested hybrids have 97.8%, 97.8% and 98.9% seed homogeneity, respectively (Table 4). It is suspected that mixing occurred during pollination, harvesting or processing. Self-pollination of female parent is one of the main reasons for contamination in hybrid production. This is as result of incomplete removal of its tassel (Salgado et al 2006). Also, the purity level of parental inbred lines could affect the purity of their hybrids.

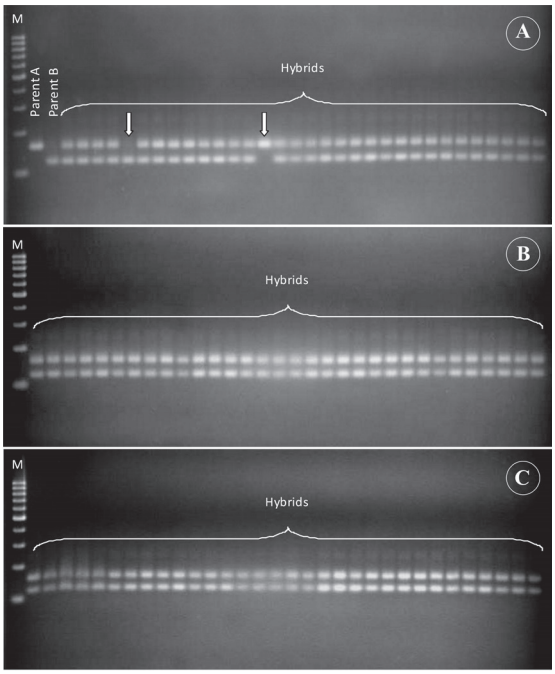

Figure 2- Agarose gel electrophoresis results of seed genetic purity testing of "Pasha" maize hybrids with their parents using UMC1858 primers. M, 100 bp ladder *(Fermentas)*; arrows indicate off-types

Şekil 2- UMC1858 primerleri kullanılarak yapılan "Pasha" mısır hibriti ve ebeveynlerinin tohum genetik saflık testinin agaroz jel elektroforezi sonucu; M, 100 bp ladder (Fermentas); oklar, tip-dışlarını göstermektedir

Table 4- The genetic purity analysis results of hybrid seeds *cv.* Frida, Pasha and PG1661 based on SSR analysis

Çizelge 4- SSR analizlerine göre; Frida, Pasha ve PG1661 hibrit çeşit tohumlarının genetik saflık analizi sonuçları

Hybrids	*Number of tested seeds*	*Number of off-types*	*Genetic purity (%)*
PG1661	94	1	98.9
Pasha	94	2	97.8
Frida	94	2	97.8

The cluster analysis based on genetic distance matrix obtained with UPGMA displayed 2 main groups with 2 subgroups (Figure 3). The hybrid *cv*. Pasha was clustered together with its parents in one group as expected. *cv*. PG1661 was clustered together with its male parent and parents of *cv*. Frida were clustered in the same group. The hybrid *cv.* Frida was detected genetically distant from their parents. The broad genetic diversity detected within the samples demonstrates the genetic purity and potentials of SSR markers for seed genetic purity analysis in maize.

Seed contamination is always a problem in hybrid seed production of maize. The SSR marker technology is currently used for purity identification in many crops. Microsatellite markers (phi96100, phi328175 and phi072) were reported highly polymorphic for genetic purity analysis of maize hybrids (*cv.* Bima-3 and Bima-4) by Hipi et al (2013). Six SSR markers tested on maize hybrids and inbred lines were analyzed for genetic purity and diversity by Daniel et al (2012) and they were reported that these markers were powerful biotechnological tools capable of detecting genetic purity status of maize hybrids. Shehata et al (2009), showed the application of six SSR markers for molecular diversity and heterozygosity analysis in 8 different maize inbred lines. Also, Mingsheng et al (2006) were reported that SSR markers are useful for assessing genetic purity of maize hybrid, even if the hybrid derived from two related parental lines. All these studies confirm the efficiency of SSR markers in maize hybrid for seed genetic purity

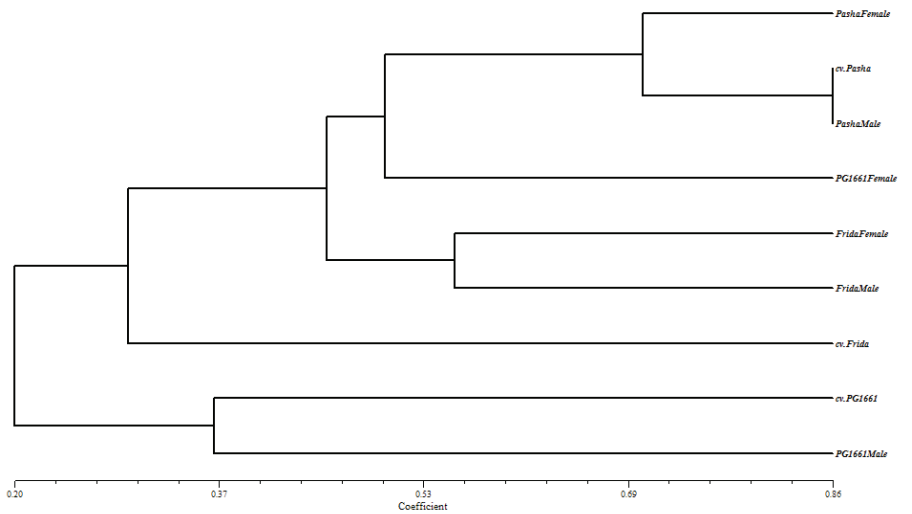

Figure 3- Dendrogram of the hybrids along with their inbred parents developed from SSR data

Şekil 3- Bu çalışmada kullanılan çeşitlerle ebeveynlerinin SSR verileri kullanılarak oluşturulan dendrogram

as indicated in our study. In this study, an efficient and precise method was established for rapid and reliable genetic purity testing of commercial maize hybrid seeds and genetic diversity of hybrids were determined.

4. Conclusions

It is concluded from this study that seed genetic purity analysis and differentiation of the maize hybrids, can be performed more accurately and efficiently using molecular markers. These molecular markers would be more efficient, fast and cheap than GOT. The SSR marker information developed through this study will be helpful for hybrid maize seed industry to select appropriate marker combinations and assess genetic purity of the crop.

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