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ORIGINAL ARTICLE

Application of simple sequence repeat (SSR) markers for molecular diversity and heterozygosity analysis in maize inbred lines

Afaf I. Shehata, Haila A. Al-Ghethar, Ali A. Al-Homaidan *

Department of Botany and Microbiology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia

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Abstract There is an important role of understanding the genetic diversity among and within inbred lines at the molecular level for maize improvement in different breeding programs. The present study was devoted to estimate the level of genetic diversity among the inbred lines of maize using the simple sequence repeat analysis (SSR). The application of six different SSR markers successfully provided the information on similarity or diversity as well as the heterozygosity of the allelic loci for all the eight inbred line of maize.

1. Introduction

Genetic fingerprinting of maize is an efficient method for large scale application to aid breeders in the placement of breeding lines and populations into the correct heterotic group, to aid in the curation of gene bank collections by refining the core subsets formed from field evaluation and to have a better understanding of the evolution of major tropical maize races (Dubreuil and Charcosset, 1998; Franco et al., 2001; Warburton et al., 2002). Previous studies have used restriction fragment length polymorphism (RFLP) markers to place temperate line into known heterotic groups with considerable success (Anthony et al., 2001; Ajmone-Marsan et al., 1998; Dillman et al., 1997; Dubreuil et al., 1996; Jones et al., 1997). In a study involving 148 US maize inbred lines, Mumm and Dudlley (1994) used 46 RFLP markers to cluster all the inbred lines into the two major heterotic groups. They were also able to identify subgroups within the major heterotic groups. Dillman et al. (1997) used RFLPs and morphological distances to study 145 maize inbreds released in France. They concluded that RFLP markers could serve as tools to discriminate between closely related individuals from different breeding sources. Other investigators have used random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR) and amplified fragment length polymorphism (AFLP) for maize diversity analysis (Ajmone-Marsan et al., 1998; Castigloione et al., 1993; Jones et al., 1997; Hernandez et al., 2001; Gomez et al., 2001). In a study of 33 inbred lines, SSR produced twice as more information as AFLPs and RAPDs, and 40% more than RFLP's in terms of numbers of alleles per locus (Powell et al., 1996).

* Corresponding author.
E-mail address: homidan@ksu.edu.sa (A.A. Al-Homaidan).

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Many studies have reported genetic diversity or relatedness of maize inbred lines at the molecular level (Bornet and Branchard, 2001; Dangle et al., 2001; Kenis and Keulemans, 2000; Selvi et al., 2003; Senior et al., 1998). However, studies on measuring genetic variation at this level within and among identically named inbred lines maintained by different programs are lacking. The approach of molecular fingerprinting is complementary to phenotypic measures in quantifying genetic changes because it shows variations in DNA that may not be phenotypically expressed. Historically important public inbred lines continue to play an important role in maize improvement in many different breeding programs. Owing to their continued use, they have undergone numerous seed generations in diverse programs since their original release (Warburton et al., 2002). The objective of this study was to estimate the level of genetic diversity both among and within inbred lines of maize by means of SSR markers.

2. Materials and methods

2.1. Plant materials

Plant tissue samples were obtained from each plant per inbred of eight maize lines. The plants were grown in the greenhouse at 25 °C under four weeks of light and darkness. After that time, approximately 100 mg of plant tissue was harvested and stored at −80 °C until DNA was extracted.

2.2. Molecular analysis

Plant genomic DNA was extracted by a modified cetyltrimethylammonium bromide (CTAB) method (Mitchess et al., 1997). Leaf tissues (100 mg) were ground in 100 μm of CTAB extraction buffer (100 mM Tris, 1.4 M NaCl, 20 mM EDTA, 0.2% β-mercaptoethanol and 2% CTAB, pH 8.0) and heated at 60 °C for 30 min. DNA was extracted with one volume of chloroform: isoamyl/alcohol mix (24:1) and precipitated in presence of isopropanol (40% [v/v] final concentration). The DNA pellet was washed with 5 mM ammonium acetate and 70% ethanol, dried, and dissolved in 100 μL of TE (10 mM Tris–HCl, 1 mM EDTA pH 8.0). After addition of 1 μl of RNase (10 mg/mL), DNA concentrations were determined with fluorometer (Hoeffer TKO 100) using bisbenzimide as a fluorescent dye. DNA was quantified with the picogreen ds DNA quantification kit (Molecular probes, Eugene, OR) Table 1.

Six SSR markers were used for genotyping as reported earlier (Warburton et al., 2002). The sequences and length of primers as well as the fluorescent dye labeling strategy are given in Table 2. Primer pairs were chosen on the basis of their properties of detecting single loci, their broad coverage of the genome and their high levels of polymorphism when applied to a broad range of maize germplasm. The sequence of the six primer pairs were chosen from the maize database project, Mias DB at the University of Missouri (http://www.agron.Missouri.edu).

Table 1 Types of inbred maize lines.

Inbred maize lines	Variety
A	Coral
A1	Merit
A2	Iochief
A3	Ne + 7007
A4	Ne + 7007
A5	Panama
A6	Giubileo
A7	Bonanza F1

2.3. Polymerase chain reaction (PCR)

Each 20 μl PCR reaction consisted of 1 × PCR buffer, 0.4 mM dNTPs, 1.2 mM $MgCl_2$, 0.2 unit of Taq polymerase, 1 μl (4 pmul/μl) of each primer and 5 μl (25–50 ng) of DNA. The amplification conditions were 95 °C for 2 min, 55 °C for 1 min, 72 °C for 5 min and a terminal extension step at 72 °C for 10 min. To prepare the PCR products for detection 0.5 μl of the amplified DNA was mixed with 0.1 μl Genescan 500 XL RoX standard (Applied Biosystems, Foster City, CA) and 1 μl of 50% formamide loading buffer and the DNA was denatured by heating at 95 °C for 2 min and then promptly cooled on ice.

The samples were loaded and electrophoresed on 2% (w/v) denaturing long ranger (FMC) 36-cm well-to-read gels. DNA samples were electrophoresed in 1 × TBE buffer (pH 8.3) at constant voltage (3.00 kV) for 3 h. Microsalellite loci repeats were assayed on the basis of their observed hetorozygosity and number of alleles detected with the PCR amplification profile. All samples were replicated to verify the results.

2.4. Statistical analysis

Hetrerozygosity (H) was calculated using Neis (1973) formula $H = n\,(n-1) * (1-\sum P_i^2)$, where P_i is the frequency of alleles i in the analyzed trees and n is the number of alleles. The power of discrimination (PD) for each locus was calculated using the formula $PD = 1 - \sum P_i^2$, where P_i is the frequency of genotype i (Kloosterman et al., 1993).

3. Results

The determined concentration of DNA ranged between 125 ng/μl for line A7 and 560.5 ng/μl for line A1.The genetic relationship of eight inbred lines were analyzed by simple sequence repeats (SSRs) markers and the six SSR primers gave stable amplified band pattern detected over 50 alleles among the tested lines (Table 3). The average number of alleles per SSR locus was 4.35 with a range from 2 to 10. The value of polymorphism information content (PIC) for each SSR locus varied between 0.42 1 and 0.88 with an average of 0.58. Clustering analysis with UPGMA showed that Merit and Ne + 7007 could not be compartmentalized as other genes, but the genetic distance between BonanzaF1and one of the other was quite big. The difference in mean genetic distance between Merit and Ne + 7007 compared to the mean genetic distance between Merit and Ne + 7007 was significant at 0.01 levels. Among the eight germ plasmas, Ne + 7007 is more closed to Merit.

Tables 4 and 5 showed that the alleles length for sir locus UMC 1061 were equal in lines A, A2, A3 and A6 (105 bp) and for line A1, A5 and A7 was 102 BP.

For SSR locus UMC 1122, the lines A, A1 and A4 have similar allele length (164 BP), but the A2 and A5 have 156

Table 2 Details of SSR primers used.

Marker	Position	Dye label	Sequence (5–3)	Size (bp)
UMC 1061	Forward	Fam	AGCAGGAGTACCCATGAAAGTCC	23
	Reverse		TATCACAGCACGAAGCGATAGATG	24
UMC 1122	Forward	Hex	CACAACTCCATCAGAGGACAGAGA	24
	Reverse		CTGCTACGACATACGCCA GGC	21
UMC 1136	Forward	Tet	CTCTCGTCTCATCACCTTTCCCT	23
	Reverse		CTGCATACAGACATCCAACCAAAG	24
UMC 1152	Forward	Fam	CCGAAGATAACCAAACAATAATAGTAGG	28
	Reverse		ACTGTACGCCTCCCCTTCTC	20
UMC 1399	Forward	Hex	GCTCTATGTTATTCTTCAATCGGGC	25
	Reverse		GGTCGGTCGGTACTCTGCTCTA	22
UMC 1555	Forward	Tet	ATAAAACGAACGACTCTCTCACCG	24
	Reverse		ATATGTCTGACGAGCTTCGACACC	24

Table 3 Statistical analysis of SSR markers according to Neis (1973).

SSR markers	Repeat	P_i	Number of alleles
UMC 1061	Tri	0.62	8
UMC 1122	Tri	0.34	8
UMC 1136	Tri	0.73	8
UMC 1152	Tetra	0.74	7
UMC 1399	Tetra	0.62	6
UMC 1555	Tetra	0.76	6

Table 5 Genetic Index of the six SSR markers used to generate DNA profiles of eight maize inbred lines.

SSR markers (locus)	Heterozygosity (*H*)	Power of differentiation (PD)
UMC 1061	0.709	0.62
UMC 1122	1.005	0.88
UMC 1136	0.537	0.47
UMC 1152	0.525	0.45
UMC 1399	0.744	0.62
UMC 1555	0.504	0.42
Mean	0.670	0.57
Total	–	3.46

BP and A3 and A6 have 165 BP. For UMC 1136, the lines A and A2 were similar, while A1, A3, A4, A6 and A7 have an allele length of 167 BP. For UMC 1152, the lines A1 and A4 and A2 and A5 has 160 BP, and for UMC 1399 locus, the lines A, A2, A3 and A6 have similar allele length. The allele length for lines A1 and A3 in UMC 15bb was 330 bp and for A2 and A6 it was 261 bp.

The similarity frequency for marker UMC 1061 among the lines A, A2, A3 and A6 was 100% (Table 6). The similarity between lines A1, A5 and A7 was 100%, whereas the inbred line A4 was close to all lines with 66.7% similarity using the same marker (Table 6). The allele similarity between lines A, A1 and A4 was 10%. A very close similarity was between lines A2, A5 and A3 and line A6 in UMC 1122. There was a decrease in the allele similarity (33.3%) between A and A5 as well as A2 and A5 for UMC 1136. A 100% similarity was observed between lines A and A2, A6 and A7, and A1, A3 and A4. A percentage similarity of 66.7% was found between the lines A1, A6 and A4, A3, A6 and A7, and A4, A6 and A7 (Table 6).With the marker UMC 1152, the genetic similarity was found to be 100% among lines A, A3 and A7 as well as lines A1 and A4, and A2 and A5. However a lower degree of similarity (66.7%) was noticed among lines A, A6; A3, A6 and A6, A7. For UMC 1399, the lines A, A2, A3 and A6 were similar (100%) and also the lines A1, A4, A5 and A7. In UMC 1555 primer, a similarity (100%) was found between A, A4 and A4; A1 and A3; A2 and A6; and A1 and A3 (Table 6).

Table 4 Alleles length by six SSR markers in eight maize inbred lines. Sizes are given in bp.

Line	Alleles length						DNA/Conc. (ng/μl)
	UMC 1061	UMC 1122	UMC 1136	UMC 1152	UMC 1399	UMC 1555	
A	105	164	144/155	167	70/113	260/333	349.4
A1	102	164	146/157	220/200	72/118	330	560.5
A2	105	156	144/155	160	70/113	261	320.6
A3	105	165	146/157	167	70/113	330	295.4
A4	102/105	164	146/157	220/200	72/118	260/333	432.9
A5	102	156	143/155 134/122	160	72/118	260/333	507.6
A6	105	165	138/146 157/127	167/216	70/113	261	187.6
A7	102	–	138/146 157/127	167	72/118	200	~125

Table 6 Similarity percentage of alleles length showing eight maize inbred lines using six different SSR markers.

Primers	Line	A	A1	A2	A3	A4	A5	A6	A7
UMC 1061	A		0	100	100	66.7	0	100	0
	A1	0		0	0	66.7	100	0	100
	A2	100	0		100	66.7	0	100	0
	A3	100	0	100		66.7	0	100	0
	A4	66.7	66.7	66.7	66.7		66.7	66.7	66.7
	A5	0	100	0	0	66.7		0	100
	A6	100	0	100	100	66.7	0		0
	A7	0	100	0	0	66.7	100	0	
UMC 1122	A		100	0	0	100	0	0	0
	A1	100		0	0	100	0	0	0
	A2	0	0		0	0	100	0	0
	A3	0	0	0		0	0	100	0
	A4	100	100	0	0		0	0	0
	A5	0	0	100	0	0		0	0
	A6	0	0	0	100	0	0		0
	A7	0	0	0	0	0	0	0	
UMC 1136	A		0	100	0	0	33.3	0	0
	A1	0		0	100	100	0	66.7	66.7
	A2	100	0		0	0	33.3	0	0
	A3	0	100	0		100	0	66.7	66.7
	A4	0	100	0	100		0	66.7	66.7
	A5	33.3	0	33.3	0	0		0	0
	A6	0	66.7	0	66.7	66.7	0		100
	A7	0	667	0	66.7	66.7	0	100	
UMC 1152	A		0	0	100	0	0	66.7	100
	A1	0		0	0	100	0	0	0
	A2	0	0		0	0	100	0	0
	A3	100	100	0		0	0	66.7	100
	A4	0	0	0	0		0	0	0
	A5	0	0	100	0	0		0	0
	A6	66.7	0	0	66.7	0	0		66.7
	A7	100	0	0	100	0	0	66.7	
UMC 1399	A		0	100	100	0	0	100	0
	A1	0		0	0	100	100	0	100
	A2	100	0		100	0	0	100	0
	A3	100	0	100		0	0	100	0
	A4	0	100	0	0		100	0	100
	A5	0	100	0	0	100		0	100
	A6	100	0	100	100	0	0		0
	A7	0	100	0	0	100	100	0	
UMC 1555	A		0	0	0	100	100	0	0
	A1	0		0	100	0	0	0	0
	A2	0	0		0	0	0	100	0
	A3	0	100	0		0	0	0	0
	A4	100	0	0	0		100	0	0
	A5	100	0	0	0	100		0	0
	A6	0	0	100	0	0	0		0
	A7	0	0	0	0	0	0	0	

4. Discussion

Since the mid 1980s, genome identification and selection has progressed rapidly with the help of PCR technology. A large number of marker protocols that are rapid and require only small quantities of DNA have been developed. Three widely-used PCR-based markers are random amplified polymorphic DNA (RAPD) (Williams et al., 1990), SSRs or microsatellites (Tautz, 1989), and AFLP (Vos et al., 1995). Each marker technique has its own advantages and disadvantages. RAPD markers are very quick and easy to develop (because of the arbitrary sequence of the primers) but lack reproducibility (Karp et al., 1997; Hansen et al., 1998; Virk et al., 2000). AFLP has medium reproducibility but is labor intensive and has high operational and development costs (Karp et al., 1997). Microsatellites are specific and highly polymorphous (Karp et al., 1997; Jones et al., 1997), but they require knowledge of the genomic sequence to design specific primers and, thus, are limited primarily to economically important species.

SSRs are co dominant molecular markers that distinguish homozygotic and hetrozygotic individuals and also possess a large number of alleles. In fact, the use of single SSR marker

may not provide authentic information hence we used six different SSR markers for reliable and accurate differentiation of maize plants, as recommended earlier (Warburton et al., 2002). The value of PD between the genetic loci for SSR makers provides quantitative information to use these markers to measure the genetic diversity of different lines of any species. Also SSR marker can be used to differentiate homozygotic and heterozygotic alleles between the lines from the same origin. The line A4 in UMC 1061 marker contained two alleles of different sizes (102/105 bp) indicating that the line A4 might have originated by out breaking of one or both genetically different parents (Table 4). Similar explanation applies to A1, A4 and A6 in UMC 1152 and A, A4 and A5 in UMC 1555 suggesting the usefulness of SSR markers for molecular differentiation of plant species at the taxonomic level (Kenis and Keulemans, 2000). All six SSR markers provided unique patterns of similarity indices (Table 6) indicating the multiplexing of these markers for reliable interpretation of results. Thus, the analysis of eight maize lines by six SSR markers resulted in the definition of six different genotypes, providing the possibility of distinguishing between the cultivars. However, it was not possible to differentiate between some lines of maize most probably because both cultivars have been obtained as UMC 1152, UMC 1399 and UMC 1555 from the same controlled cross between the same parents.

The expected heterozygosity proved to be significantly lower than the results obtained previously by Guilford et al. (1997) and those detected by specific loci analyzed. This study showed that different seed sources of the same inbred contribute a potentially important source of genetic variation. Establishing the level of heterozygosity in seed is critical because it improves the usefulness of data to other breeder using identically named materials. It also ensures uniformity and stability of any materials developed from them. The similarity level in maize inbred lines based on ANOVA and Neis similarity coefficient, although sufficient for general identification and in testing programs, it may not be sufficient in studies that require higher resolution, such as fine mapping of quantization trait loci and development of marker such as single nucleotide polymorphisms. On the basis of our results, studies done with the same inbred line should be comparable regardless of the seed source. Also this study was done with public inbred lines which are no longer used in hybrid seed production. Breeders needs to be aware at the variation arising from seed sources as they continue to use these inbreeds for genetic studies and as testers. The detection of low variation within an inbred from one source should be taken into account, especially, when sampling the earlier released materials. No evidence was found to support high mutation rates at the loci examined because no unique alleles were detected and the number of alleles did not vary greatly among inbred-seed source.

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