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Molecular characterization of inbreds in maize (*Zea mays* L.)

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Abstract

A set of 8 inbred lines *viz.*, HKI-163, HKI-1105, HKI-1128, DMRPE6, CML-285, CML-292, CML-132, LM-16 were analyzed using 36 Simple Sequence Repeats (SSR) markers 22 markers out of 36 were found to be polymorphic. In total 50 SSR alleles were identified with a mean of 2.27 alleles per locus. The average polymorphic information content (PIC) was 0.41. These markers showed high level of polymorphism and therefore can be use for fingerprinting and mapping studies. Genetic similarity expressed as Jaccard's coefficient varied from 0.57-0.15. Three clusters were obtained by using Unweighted Paired Group Method using Arithmetic Averages (UPGMA). Coefficient of similarity matrix showed inbred lines CML 292 and CML285 tobe more similar while CML132 and DMRPE6 were found to be most diverse. Inbred lines used in present study revealed heterozygosity ranges from 4.3-17.4%. In this study we found SSR as a good tool for characterization of maize genotypes.

Key words: *Zea mays, Simple sequence repeat, Diversity, Dendogram.*

Introduction

The maize (*Zea mays* L, 2n = 20) is suitable to diverse environments due to its heterozygous and well adapted nature, which makes it matchless. Breeding for high yielding hybrids is most pertinent to any breeder. For this, identification and utilization of diverse germplasm is the central issue in any plant breeding programme. Characterization of germplasm lines especially in any hybrid maize breeding programme, where the recognition and exploitation of heterotic patterns are vital for maximizing heterosis. During the past decade numerous studies have been conducted in maize to estimate genetic diversity using morphological characters. But alone they have remained insufficient due to number of constraints, like stage and tissue specific expression of the traits, influence of environment, existence of pleiotropy and largely unknown genetic control of these traits and inadequate sampling of the genome (Reif, *et al.,* 2005) Biochemical markers such as isozymes, and chromatographic data of zein, have been used extensively to examine the genetic diversity in commercial maize genotypes (Bar-Hen, *et al.,* 1995) However, limited number of available marker loci and low level of polymorphism are some of the major limitations of the biochemical markers (Liu and Liu, 2004) Hence, molecular markers characterize lines at DNA level. Several studies have successfully

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used SSRs (Simple sequence repeats or microsatellites) to study maize genetic diversity and define heterotic groups particularly in maize germplasm (Dubreuil and Charcosset, 1998; Lu and Bernardo, 2001 and Vigourous, *et al,* 2005). SSR markers are PCR-based, codominant, robust, reliable and are highly reproducible, with greater discriminatory ability than RFLP and RAPD markers (Pushpavalli, *et al,* 2001; Smith, *et al,* 1997 and Legesse, *et al,* 2007). Microsatellites or SSR markers are stretches of tandemly arranged short motifs (ranging from two to six nucleotides) which are abundant and highly polymorphic in several eukaryotic genomes, including maize. Nearly 2000 SSR loci have been placed on all 10 chromosomes of maize (Maize GDB http://www. Agron.missouri.edu/ssr.html). This database is very useful for selecting SSR markers. The objective of this study was to characterize a set of important maize inbred lines by using SSR markers**.**

Material and Methods

Plant Materials - Molecular characterization was done for assessment of diversity of 8 inbred lines of maize (*Zea mays* L.) (Received and assessed at Indian Institute of Maize Research, New Delhi) using Simple Sequence Repeat markers. Their sources have been presented in Table-1.

Table 1: **Source of 8 inbred lines**.

S.N.	Parents	Source	Source of material
1.	HKI-163	CML 163	IIMR New Delhi
2.	HKI-1105	Kargil 633	IIMR New Delhi
3.	HKI-1128	Hybrid (FF)	IIMR New Delhi
4.	DMRPE-6	CA 14524-1	IIMR New Delhi
5.	CML-285	CIMMYT line	IIMR New Delhi
6.	CML-292	CIMMYT line	IIMR New Delhi
7.	CML-132	CIMMYT line	IIMR New Delhi
8.	LM16	SE 539	IIMR New Delhi

DNA isolation– DNA was extracted from a pool sample of leaves following the method of Saghai-Maroof *et al,* (1984), with minor modifications. Fresh leaves (1000 mg) ground to fine powder in liquid nitrogen, were transferred to 50 ml centrifuge tubes containing 10 ml prewarmed CTAB extraction buffer (1M Tris-Cl, 0.5M EDTA, 2% CTAB, pH 8.0).

Sample tubes were incubated in water bath at 65°C for 1 h with occasional swirling. Equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added to the tubes and mixed thoroughly followed by centrifugation at 15000 rpm for 15 min at 20°C. Aqueous phase was pipette out and 5 µl RNase (10 mg ml-1) was added. The mixture was incubated at 37°C for 1h and then 10 ml phenolchloroform- isoamly alcohol (25:24:1) was added and centrifuged at 15000 rpm for 15 min at 20°C. The aqueous phase was pipetted out carefully in a tube and 0.6 volume of

isopropanol was added and incubated at -20°C for 1 h to allow precipitation. Tubes were centrifuged at 15000 rpm for 15 min. Supernatant was discarded and pellets were washed twice with 70% ethanol. Finally dried pellet was dissolved in 1 ml TE (100 mM Tris-Cl, 10 mM EDTA, pH 8.0).

Primers and PCR amplification – A set of 22 SSR primers, distributed uniformly on both arms of all the 10 chromosomes was used in the study (Table. 3). The PCR amplification cycle consisted of initial denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 30 sec and primer extension at 72°C for 1 min. The final extension step was performed at 72°C for 7 min. PCR was carried out in a volume of 15 µl, containing 50 ng of template DNA, 1 µM of each forward and reverse primers, 0.2 mM dNTPs, 0.5 U *Taq* polymerase and 1.5 mM $MgCl_2$ (Fermentas). The amplified products were resolved on 3.5% metaphore agarose gel along with 50 bp ladder (Fermentas). The gel was run in 1x TBE buffer at a constant voltage of 70 V for about 4 h (until the tracking dye migrated to the end of the gel). The gel image was documented using a gel documentation system (AlphaImager® HP).

SSR data analysis – Scoring of the SSR alleles was performed manually in terms of positions of the bands relative to the ladder sequentially from the smallest to the largest-sized bands. Diffused bands or bands revealing ambiguity in scoring were considered as missing data and designated as '9' in comparison with '1' for the presence of a band and '0' for the absence of a band in the data matrix.Genotypes showing two allelic bands with equal intensity were considered as heterozygous for the locus. Null allele for any specific marker in a genotype was considered as absence of primer binding site, after re-runs with specific check and was designated as '0'. The polymorphism information content (PIC) was determined by Senior and Henn (1993), which is equal to 1- ΣP_{ij}^2, where $P_{ij}$is the frequency of j^{th} allele at i^{th} locus summed across allalleles in the locus. Alleles with frequency of less than 0.20 were considered as rare alleles and such allele representing a particular genotype was considered as unique allele for that genotype. Jaccard's (1908) coefficient was used to calculate the genetic similarities among pair wise comparison of genotypes based on SSR data, as follows: $J = N_{11} / (N_{11} + N_{10} + N_{01})$ where N_{11} is the numberof bands present in both genotypes; N_{10} is the number ofbands present in one genotype (lane) and N_{01} the numberof bands present in the other genotype. The similarity matrix was analyzed using NTSYS pc 2.02 to produce an agglomerative hierarchical classification by employing UPGMA (Unweighted Paired GroupMethod using Arithmetic Averages) with average linkage.

Results

Characterization of 8 inbred lines was attempted using 36 SSR markers ensuring at least two markers (one each from long and short arm) for the first six chromosomes and three for the last four chromosomes. 22 markers out of 36 were found to be polymorphic. The maximum numbers of alleles detected at particular locus were three. Table.3 provided details about the allelic frequency and polymorphic information

content (PIC). Out of 22 polymorphic SSR markers, 6 markers revealed 3 alleles and 16 markers revealed 2 alleles. A total of 50 alleles could be detected from 22 polymorphic SSR markers, giving an average of 2.27 alleles per locus.

Polymorphic information content (PIC), which is a measure of allelic diversity at a locus, was estimated for various SSR loci used during study (Table 3). The values ranged from 0.05 (umc1402) to 0.78 (umc1962). The mean PIC values estimated across all SSR loci was 0.41. The PIC values of 8 primers were found to be greater than this mean value. The number of alleles detected per locus was relatively high in case of 'umc' markers even though some of the 'umc' markers showed only two alleles in comparison with the other primers. Among the primers used for analysis, some of the primers showed 3 alleles per locus, while other showed 2 alleles per locus.

Inbred parents, in true sense, are expected to be totally homozygous for all the loci, except residual heterozygosity, which could be acceptable at the maximum level of 10-12%. However, in some instance a higher level of residual heterozygosity for some alleles was found. This can be very clearly revealed by the SSR markers as they are co- dominant and are able to show both the alleles in case of a heterozygote which is seen as double/triple bands in the gel. This deviation from the expected single locus profiles has been show in Table 2. Out of 8 lines namely HKI1128, HKI1105, HKI163, LM16 showed permissible level of residual heterozygosity, However, there is no residual heterozygosity was found in inbred line CML132, remaining all the lines analyses showed moderate to high level of residual heterozygosity and highest residual heterozygosity was found in CML292.

Table 2:Percentage of heterozygote found in eight inbred lines using twenty two SSR markers.

S.N.	Genotype	Source	Total polymorphism	Heterozygote loci (Nos.)	Frequency of heterozygote	%Heterozygote
1.	CML292	CIMMYTline	22	4	0.174	17.4
2.	CML285	CIMMYTline	22	3	0.130	13.0
3.	CML132	CIMMYTline	22	0	0.000	0.0
4.	HKI1128	Hybrid(FF)	22	1	0.043	4.3
5.	HKI1105	Kargil 633	22	1	0.043	4.3
6.	HKI163	CML 163	22	2	0.087	8.7
7.	LM16	SE 539	22	2	0.087	8.7
8.	DMRPE6	CA 14524-1	22	3	0.130	13.0

Table3: List of twenty two SSR markers, their bin location; annealing temperature, number of alleles detected and Polymorphic Information Content (PIC) value which were identified as polymorphic in eight inbred lines while evaluation.

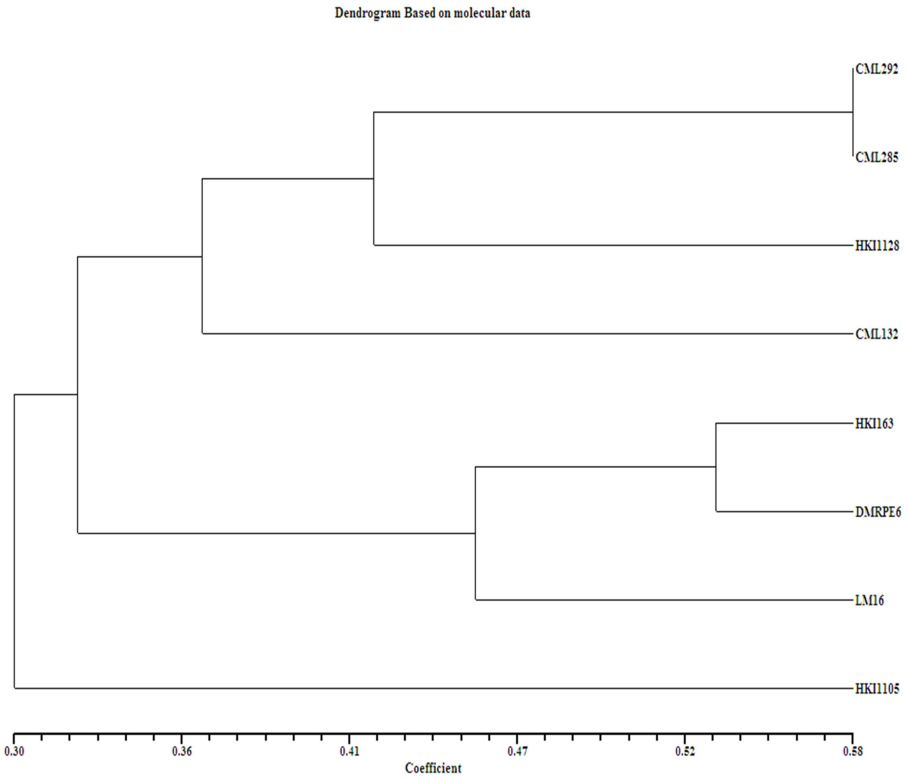
S.N.	Marker	No of Repeats	Bin location	Annealing temp.	No. of alleles found	PIC value
1	bnlg1126	(AG)20	4.02	60^0 C	2	0.17
2	bnlg1108	(AG)21	4.01	60^0 C	2	0.22
3	umc2039	(CAG)5	4.09	60^0 C	2	0.55
4	umc2053	(CAG)4	10	60^0 C	2	0.17
5	umc2307	(CAG)4	5.08	60^0 C	3	0.55
6	bnlg1523	(AG)17	3.03	60^0 C	2	0.36
7	phi 109188	AAAG	3.05	60^0 C	3	0.72
8	umc1845	(AG)8	2.03	60^0 C	3	0.23
9	umc1265	(TCAC)4	2.02	60^0 C	2	0.3
10	umc1962	(GAGA)5	8.02	60^0 C	3	0.78
11	umc1072	(GGA)10	5.01	60^0 C	3	0.53
12	umc1674	(AC)6	3.06	60^0 C	2	0.37
13	umc1008	(GT)4 (GA)6	4.01	60^0 C	2	0.37
14	umc1296	(GGT)7	6.06	60^0 C	2	0.3
15	umc1415	(GAC)10	8.04	60^0 C	2	0.37
16	umc1021	(GT)4	1.03	60^0 C	3	0.67
17	umc1402	(GCCC)4	10.01	60^0 C	2	0.05
18	dupssr6	(CA)6(A)5(CA)9	9.02	60^0 C	2	0.69
19	umc1673	(TCC)5	8.08	60^0 C	2	0.37
20	bnlg1268	AG(20)	1.09	60^0 C	2	0.47
21	umc1378	(CGC)6	7	60^0 C	2	0.5
22	umc1267	(CGG)4	9.03	60^0 C	2	0.37
Average						0.41

Cluster analysis was done using scores of molecular markers for 8 inbred lines. The analysis revealed three major groups which further has been classified into sub-group.

Table.3 Coefficient of similarity matrix between eight inbred lines generated using twenty two polymorphic SSR markers

Similarity Coeffi.	CML292	CML285	CML132	HKI1128	HKI1105	HKI163	LM16 DMRPE6
CML292	1.0000000						
CML285	0.5757576	1.0000000					
CML132	0.3428571	0.3636364	1.0000000				
HKI1128	0.4285714	0.4117647	0.3870968	1.0000000			
HKI1105	0.3888889	0.2631579	0.1944444	0.3142857	1.0000000		
HKI163	0.4571429	0.4848485	0.2941176	0.4242424	0.3823529	1.0000000	
LM16	0.3243243	0.3428571	0.2727273	0.2162162	0.2857143	0.4375000	1.0000000
DMRPE6	0.3684211	0.3513514	0.1538462	0.2000000	0.2972973	0.5312500	0.46875001.0000000

Average Linkage (Between Groups)



Dendogram Based on Molecular Markers

Group I CML292, CML285, CML132 and HKI1128

Sub-group (a) CML292, CML285, and HKI1128

Sub-group (b) CML132

Group II HKI163, DMRPE6 and LM16

Group III HKI1105

Coefficient of similarity matrix between eight inbred lines generated using 22 polymorphic SSR markers presented in Table.3 showed highest coefficient 0.57 between CML292and CML285. However lowest coefficient 0.15 was found between CML132 and DMRPE6.

Conclusion

The study demonstrates the utility of SSR markers in determining genetic relationship among maize germplasm, which also have been demonstrated by various authors in recent past (Liu *et al*, 2003,; George *et al,* 2004.; Kumar *et al,* 2012,; and Wegary *et al,* 2013). We could establish reliable fingerprint for the inbred lines. It has been demonstrated that PIC will give true representation of the in formativeness of a SSR markers. The study clearly indicates importance of maintenance breeding by detecting considerable amount of heterozygosity among the genotypes. Similar study taking more number of genotypes and more number of markers may give rise to better understanding of the situation.

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