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Genetic Diversity of Maize (*Zea mays* L.) Inbred Lines Revealed by Simple Sequence Repeat Markers

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A B S T R A C T

A set of 33 maize inbred lines were analyzed using 40 Simple Sequence Repeat (SSR) markers. Out of 40 SSR markers, 25 markers were found reproducible in which 18 were found polymorphic and 7 monomorphic. Total 40 alleles ranges from 2 to 3 with mean of 2.22 alleles per loucs were identified among 33 lines using18 polymorphic SSR markers. The average polymorphism Information Content (PIC) was 0.36 with range of 0.1 to 0.56. Unweighted Paired Group Method using Arithmetic Averages (UPGMA) cluster analysis grouped the genotypes into two major clusters. Cluster I was the biggest one with 25 genotypes which was further sub-divided into two sub-clusters with 21 and 4 genotypes, respectively. The major cluster II contained 9 genotypes in two sub clusters each with 7 and 2 genotypes. The identified clusters may find useful to attempt crossing programme in maize breeding programme. In this study, we found SSR as a good complementing tool along with morphological markers for maize genetic characterization.

Introduction

Maize (Zea *mays* L.), popularly known as the queen of cereals, considered as third most important cereal crop after wheat and rice in the world. India ranks fourth in terms of the maize growing country in the world with 9.4 million hectare area, 24.26 million tonnes of production and 2.57 t/ha average productivity (G.O.I. 2014). Hence, it clearly implies that maize has a unique place in Indian economy. There is wide spread interest towards characterization of genetic diversity among genotypes in various crops species. Maize (*Zea mays* L, 2n = 20) is one of the important food grain crops, in which heterosis has been widely exploited. Knowledge of the genetic diversity among commercially important maize inbred lines helps hybrid maize breeding programme by planned utilization of promising source germplasm (Pushpavalli *et al.*, 2001 and Stich *et al.*, 2005). It is well established that morphological characterization alone does not reliably depict the genetic relationships among the genotypes due to environmental interactions, 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 extensively used 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 (Xu *et al.,* 2004).

Lopes *et al.,* (2015) investigated the genetic diversity of twenty-two sweet corn cultivars viz., seventeen open-pollinated varieties (OPV) and five hybrids (H) by applying 30 polymorphic simple sequence repeat markers. They detected 86 alleles with polymorphism information content ranged from 0.19 (umc2319) to 0.71 (umc2205). Average of 2.67 alleles per locus determined in the study emphasizes broadening of sweet corn germplasm.

Ko *et al.,* (2016) investigated the genetic diversity and genetic relationships of 87 super sweet corn inbred lines using SSR and SSAP markers and analyze their population structure. In their study, SSR markers showed higher average gene diversity and Shannon's information index than SSAP markers. Considering the importance of genetic diversity study in maize and utility of SSRs markers as a good tool for this, the present study has been designed with the aim to identify the diverse groups in available materials which will accelerate the hybrids breeding programme.

Materials and Methods

Plant material

A set of 33 maize inbred lines (Table 1) with different genetic background was selected from the lines available at ICAR-Indian Institute of Maize Research Pusa Campus, New Delhi.

DNA isolation and quantification

DNA was extracted from pooled sample of leaves from 15 seedlings following the method (Saghai-Maroof *et al.,* 1984) with minor modifications. One gram fresh leaf sample was used for DNA isolation using a cetyltrimetyl ammonium Bromide (CTAB) extraction method (Stacey and Issac *et al.,* 1994).The samples were grind in autoclaved mortor and pestle to a fine powder with liquid nitrogen. The fine powder was transferred to label 5 ml centrifuge (Micro tube) tube containing 1 ml CTAB extraction buffer (lysis buffer). Sample tubes (microtubes) were placed in water bath at 65^0 C for 1 hr and gently mixed after every 10-15 minutes. The tubes were removed from water bath and contents were allowed to cool at room temperature. Equal volume of chloroform: isoamylalcohol (24: 1) was added and mixed thoroughly. Upper layer of the centrifuged sample were collected in fresh tubes and 0.6 volume of chilled isopropanol was added and incubated at -20^0C for 1 hour to allow precipitation. Samples were centifuged at 10000 rpm for 10 minutes. Supernatant was discarded and pellet was washed twice with 500 µl of 70 % ethanol.

Finally pellet was kept at room temperature for 37^0C. After that dried pellet was dissolved in 1ml TE (100Mm) and stored in 4^0C for further use. Agarose gel (1%) electrophoresis was utilized to estimate quantity and quality of DNA. DNA samples for loading were prepared by taking 2µl quantity of DNA. Good quality, uncut lambda DNA of known quality 50ng, 100ng, 200ng, 400ng lambda DNA having concentration of pure DNA as 50ng/µl, was also prepared. The electrophoresis DNA samples were visualized using a UV trans-illuminator gel documentation system.

Primers and PCR amplification

A set of 40 SSR markers, distributed uniformly across 10 chromosomes were used in the study (Table 1). Out of 40 SSR marker only 25 markers were amplified and found

reproducible. The PCR amplification cycle consisted of initial denaturation at 94^0C for 10 min followed by 35 cycles of denaturation at 94^0C for 1 min, primer annealing at 55^0C for 30 sec and primer extension at 72^0C for 1 min. The final extension step was performed at 72^0C for 1 min. PCR was carried out in a volume of 20µl, containing 50ng of template DNA, 1.5µl of each forward and reverse primers, 0.5 U *Taq* Polymerase, 0.4mm $MgCl_2$. The amplified products were resolved on 3% agarose gel with 50bp ladder.

SSR data analysis and clustering

Scoring of the SSR alleles was performed manually in terms of position of bands. Summary for each marker was estimated. The diversity parameters like PIC value and major allele frequency was also estimated.

The SSR data set was subjected estimation of allele frequency which was used to calculate the pairwise distances between individuals. Grouping of genotypes was done using GGT 2.0 software (Berloo, 2008) to produce an agglomerative hierarchical classification by employing Unweighted Paired Group Method using Arithmetic Averages (UPGMA).

The polymorphism information content (PIC) was determined as described by Senior and Henn (1993), which is equal to 1- ΣPij^2, where Pij is the frequency of jth allele at ith locus summed across all alleles 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 coefficient (J) (Jaccard *et al.,* 1908) was used to calculate the genetic similarities among pair wise comparison of genotypes based on SSR data, as follows: J = N11 / (N11+N10+ N01) where N11 is the number of bands present in both genotypes; N10 is the number of bands present in one genotype (lane) and N01 the number of bands present in the other genotype. Genetic distances between the genotypes were calculated using 1- coefficient of genetic similarity.

Results and Discussion

Total 40 alleles among the 33 lines were identified by 18 polymorphic SSR markers which range from 2 to 3 with an average value of 2.22 (Table 2). Four loci (bnlg108, bnlg1523, phi112 and bnlg128) gave 3 alleles and 14 loci (phi056, bnlg2086, phi038, phi96100, bnlg1940, nc133, umc2104, umc1908, phi085, umc1378, umc1889, umc1161, umc1061 and umc1249) provide 2 alleles. The size of allele ranges from 50-400bp (Fig. 1 and 2; Table 2). The polymorphism information content (PIC) ranges from 0.1 to 0.56 with an average of 0.36 (Table 2). Only three markers *viz*. umc1249, umc1061, and umc1908 provide the highly polymorphic values (0.56, 0.5 and 0.5).

These values are equal to or more than 0.5, indicating their potential utility to detect differences among the inbred lines. However, three markers *viz*. provide/give very low PIC value of less than 0.2 and they may not be useful in discriminating inbreds. Those primers revealing more number of monomorphic bands were showing less PIC value and vice versa for primers, which were showing more unique and null alleles.

The resulting dendrogram indicated that all the genotypes could be differentiated and clustered into two major clusters (Fig. 3). Cluster I was the biggest one with 25 genotypes which was further divided into two sub-clusters with 21 and 4 genotypes, respectively. The cluster II contained 9 genotypes in two sub clusters with 7 and 2 genotypes, respectively.

Table.1 List of Genotypes used for genetic diversity study using simple sequence repeat markers

S.NO.	Genotype	Source Germplasm
1	DML-127	P4855
2	DML-19	EC44612
3	DML-242	P5133
4	DML-310	Seed Tech 2324
5	DQL-1005	HQPM1
6	HK1-42050	NA
7	UMI-1200	NA
8	DQL-630-(ORANGE)3-6	NA
9	DQL-574-2	NA
10	DQL-593-3	NA
11	DQL-633-1-1	NA
12	CM-125	NA
13	CML-114	P45
14	CML-172	G25Q
15	CML-220	P30
16	CML-312	P500
17	CML-334	P590
18	CML-40BBB	P36
19	CML-451XE62	NPH-28
20	CML494	P43
21	CML-207	EV7992
22	CML-208BBB	EV7992
23	CML-163	G26Q
24	LM-16	NA
25	IML13-17	DML177 X BML6 (17)
26	IML13-22	DML177 X BML6 (22)
27	IML-13-84	DML177 X BML6 (84)
28	IML-15-7	CML269 X HKI4C4B (7)
29	IML-15-60	CML269 X HKI4C4B (60)
30	IML-15-288	CML269 X HKI4C4B (288)
31	IML-15-299	CML269 X HKI4C4B
32	IML-16-210	P72clXbrasil1177XESM13 (210)
33	IML-16-279	P72clXbrasil1177XESM13 (279)

Table.2 Details of SSR markers used for study of genetic diversity in maize inbred lines

S. No.	Marker	Forward	Reverse	No. Alleles	An.Tm	PIC	Bin
1	phi056	ACTTGCTTGCCTGCCGTTAC	CGCACACCACTTCCCAGAA	2	55	0.37	1.00
2	bnlg2086	CGGAACCTGCTGCAGTTAAT	GAGATGCAGGAATGGGAAAA	2	55	0.26	1.04
3	phi038	TCAGACTCCGCCCAGCAATCA CTG	AGCCTAGTGCTTATCTTGAAGGCTT	2	55	0.17	1.08
4	phi96100	AGGAGGACCCCAACTCCTG	TTGCACGAGCCATCGTAT	2	55	0.49	2.01
5	bnlg108	GCAC TCACGCGCACAGGTCA	CGCCTGCCAAGGTACATCAC	3	55	0.35	2.04
6	bnlg1940	CCTTTTGTTTCAGGCCGTTA	CAGCAGCCTGATGATGAACA	2	55	0.42	2.08
7	nC133	AATCAAACACACACCTTGCG	GCAAGGGAATAAGGTGACGA	2	55	0.17	2.05
8	umc2104	CTGCTGGCAGTGGCAGTATTC	TACTGCTACACCTTTGTCGTCACC	2	55	0.40	3.00
9	bnlg1523	GAGCACAGCTAGGCAAAAGG	CTCGCACGCTCTCTCTTCTT	3	55	0.43	3.03
10	umc1908	CGTACACTCAATCACGATCCAAAC	AACTTTGGGTACAAGTCAAGA	2	55	0.50	3.04
11	phi085	AGCAGAACGGCAAGGGCTA	TTTGGCACACCACGACGA	2	55	0.48	5.06
12	umc 1378	GAAGTCGCTGATGAGAACGTAACC	GCTAGCTAGTGTGAGTTCTTCCGC	2	55	0.11	7.00
13	phi112	TGCCCTGCAGGTTCACATTGAGT	AGGAGTACGCTTGGATGCTCTTC	3	55	0.11	7.01
14	umC1889	CCAATGTGCTGACGAACCATT	TCACCCTCCTTCTATGTTGTCCAT	2	55	0.26	8.05
15	umc1161	GCTCGCTGTTGGTAGCAAGTTTTA	GGTACCGCTACTGCTTGTTACTGC	2	55	0.37	8.06
16	bnlg128	CACCTGGAGGGACCCATTCC	AGGACCACAGGATCCATCCATCATCCT	3	55	0.49	9.07
17	umc1061	AGCAGGAGTACCCATGAAAGTCC	TATCACAGCACGAAGCGATAGATG	2	55	0.50	10.06
18	umc1249	GACCAGCAGCACTAGAGGACATTT	CTTCTGTTACTTTGGCAGCGGTT	2	55	0.56	10.10
			Average	2.22		0.36	

Fig.1 Diversity among the maize genotypes amplified with primer set bnlg1523 along with a 50bp ladder (M). The lane sequence in the gel corresponds to the identification of the genotypes as indicated in Table 1

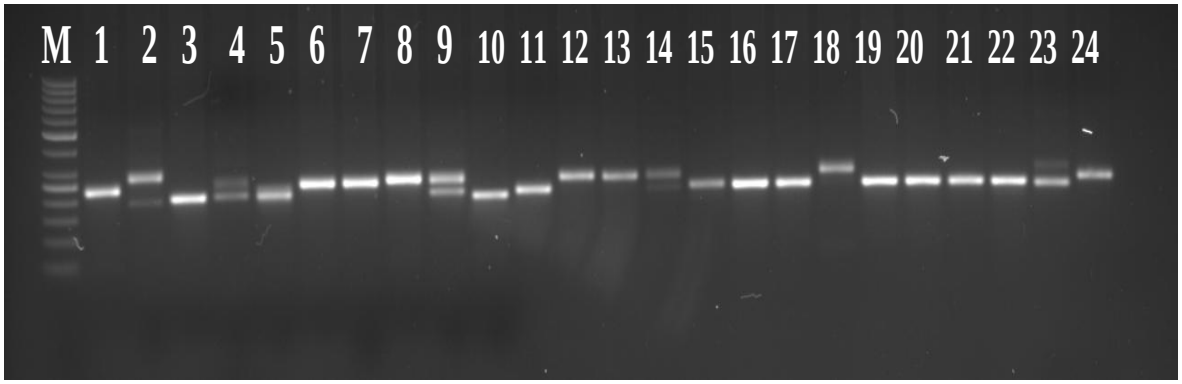


Fig.2 Diversity among the maize genotypes amplified with primer set phi033 along with a 50bp ladder (M). The lane sequence in the gel corresponds to the identification of the genotypes as indicated in Table 1

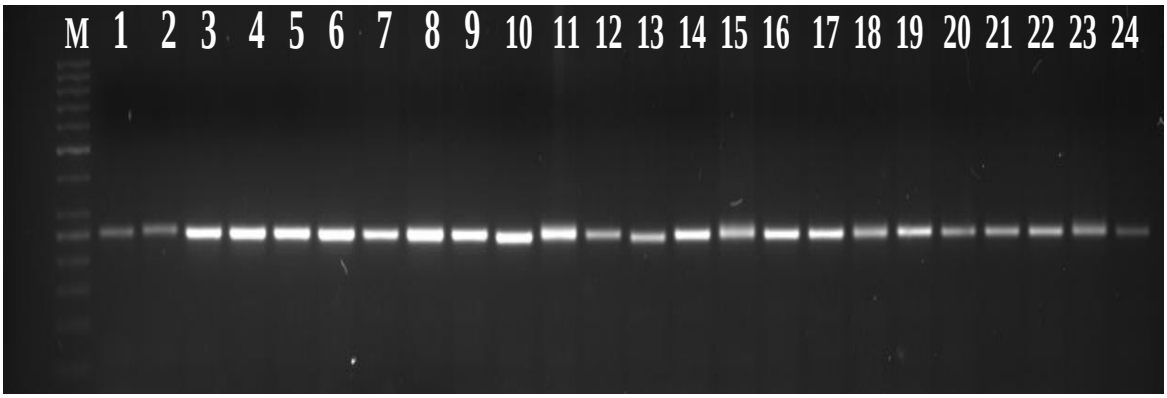
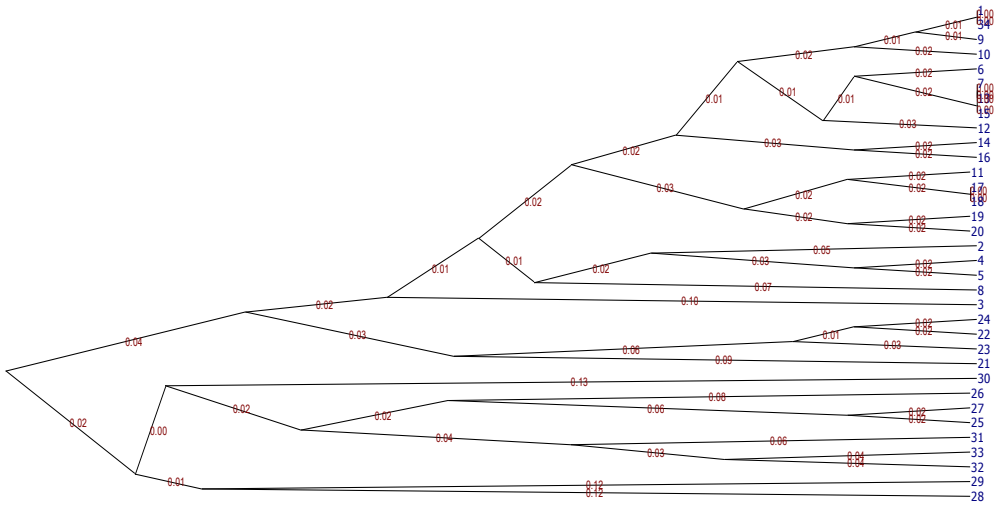


Fig.3 UPGMA cluster analysis grouped the genotypes into two major clusters. Cluster I was the biggest one with 25 genotypes which was further divided into two sub-clusters. The sequence in the cluster is corresponds to the identification of the genotypes as indicated in Table1



Realization of heterosis in any crop is dependent on the understanding of genetic diversity among the materials. The number of loci identified per microsatellite is a major limiting factor to detect genetic diversity among the lines studied. Other factors, like the repeat type and the methodologies employed for detection of polymorphic markers also influence allelic difference (Legesse *et al.*, 2007). The average number of alleles (2.22), we observed in this study was considerably lower than those reported in maize by different researchers. Kumar *et al.*, (2008) observed 2.96 in 16 popular inbred lines using 24 SSR markers. Warburton *et al.*, *(2002*) found 4.9 alleles with 85 SSR markers. However, However, Bantte and Prasanna (2003) reported 3.25 alleles using 36 SSR loci. All these investigators used agarose gel system to screen the microsatellite loci, which reveals low allele number as compared to Poly acryl amide gel electrophoresis (PAGE) based screening. Agarose gel electrophoresis analysis being less cumbersome as compared to Poly acryl amide gel electrophoresis (PAGE) and less costly as compared to automated system, is most appropriate technology for routine analysis. However, resolving power of automated detection system and polyacrylamide gel is higher and thus number of alleles obtained would be higher than agarose based system. This becomes more relevant in case of SSR loci containing dinucleotide repeats. Laborda *et al.*, (2005) detected 5.2 alleles per locus with 50 SSR markers using polyacrylamide gel. Amplicons differing in terms of two base pairs cannot be resolved using agarose gel (Legesse *et al.*, 2007; Sibov *et al.*, 2003). The lines used in the present investigation are less diverse in term of pedigree information as against other reports. Many of the lines have been derived from same base population. This may be the other reason for obtaining less average number of alleles (2.22) as compared to earlier reports. Genetic similarity as calculated by Jaccard's coefficient indicated considerable variability among the lines under study, therefore may be utilized in hybrids breeding programme. Further, the 18 polymorphic markers found out of total 25 (72% polymorphism), has also given an

indication of unexploited variation in the selected lines. If the numbers of markers get increases, the variation can be exploited further. The genotypes belonging to two different clusters can be tried for development of high yielding maize hybrids. 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. 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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