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Genetic diversity assessment of inbred lines in maize through SSR markers

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Abstract
Maize (*Zea mays* L.) is an important cereal crop of different countries in the world. Hybrid cultivars have played a vital role in increasing the productivity of maize. The success in identifying heterotic hybrids in maize hybrid breeding depends on the availability of genetically diverse maize inbred lines developed from different heterotic gene pool. In the present study, a set of five inbred lines were analyzed using 50 polymorphic Simple Sequence Repeat (SSR) markers. A total of 111 alleles were generated with a mean of 2.2 alleles per locus. The Polymorphic Information Content (PIC) value ranged from 0.164 to 0.672 with an average of 0.345. Gene diversity (He) value ranged from 0.20 to 0.80 with a mean value of 0.468 while the value of Observed heterozygosity (Ho) ranged from 0 to 1 with a mean value of 0.084. The dendrogram generated with unweighted pair group method with arithmetic mean (UPGMA) cluster analysis revealed three clusters with a degree of genetic distance ranging from 0 to 0.1. The information on diversity of inbred lines generated in this study would be much useful in developing heterotic hybrids.

Keywords: Maize, inbreds, genetic diversity, simple sequence repeats (SSR)

Introduction
Maize is one of the well-known cereals for its adaptability and importance worldwide. It is one of the important food grain crops, in which heterosis has been widely exploited. In any breeding program, knowledge on the genetic diversity and relationships among the commercially important inbred lines will have significant impact on the identification of promising hybrid combinations. The process of identifying promising inbred lines to produce outstanding single crosses is dependent upon procedures such as field evaluation of diallel and topcrosses, the use of pedigree information and dependence on the morphological traits. The methods utilizing the morphological traits are slow, laborious, greatly influenced by environment.

The advancement in use of molecular markers has proven valuable for genetic diversity analysis at the DNA level in plant species (Melchinger and Gumber, 1998) [11]. Unlike the morphological markers, molecular markers are not influenced by environmental factors, thus they reflect the actual level of genetic difference existing among the genotypes (Westman and Kresovich, 1997, Legesse *et al*., 2007) [22, 10]. Assessment of genetic diversity of inbred lines using molecular markers allows the characterization of a greater number of lines, hence potentially increasing the efficiency of maize breeding programs. Genotyping techniques such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs) and simple sequence repeats (SSRs) have allowed assessment of the genetic diversity among maize inbred lines to synthesize most heterozygotic hybrid combinations (Ribeiro *et al*., 2010; Reif *et al*., 2006) [19, 18].

Among the various types of markers, microsatellites or SSRs, which are short sequences containing tandemly repeated copies of one to six nucleotide fragments (Rafalski *et al*., 1996) [16], are currently considered as the molecular markers of choice. They are rapidly being adapted by plant researchers because of their simplicity, high levels of polymorphism (Fufa *et al*., 2005) [4], high reproducibility and co-dominant inheritance patterns. Therefore, this study was conducted to investigate the genetic polymorphism and relationships among five inbred lines of maize.

Material and Methods
The experimental material comprised of a total of five inbred lines procured from Maize Research Centre, Rajendranagar.

The inbred lines selected for the present study were BML-6, BML-7, BML-45, PFSR-3, KML-225.

DNA extraction

Healthy leaves were collected from young plants and the genomic DNA extraction was carried using Cetyl trimethyl ammonium bromide (CTAB) method by Murray and Thompson (1980) [13]. The concentration of genomic DNA was determined by Nanodrop spectrophotometer and agarose gel electrophoresis. The final concentration of the samples was adjusted to 100 ng/µl for polymerase chain reaction (PCR).

PCR and Agarose gel electrophoresis

Polymerase Chain Reaction was carried out in a 10µl reaction mixture consisting of 2 µl of 100 ng/ µl template DNA, 4 µl of TAKARA master mix (PCR buffer, Taq polymerase, $MgCl_2$ and DNTP's), each of 0.5 µl of forward and reverse primers and 3 µl of molecular grade water. The amplification profile was maintained at 94°C for 5 min followed by 35 cycles of 94°C for 45 sec, 55°C for 1 min and 72°C for 1 min with a final extension of 10 min at 72°C. The amplified PCR products were electrophoretically resolved on a 3% agarose gel using 1×TAE buffer. DNA banding patterns were visualized using BIO-RAD Imaging gel documentation system.

SSR Analysis

Genetic diversity of the inbred lines was estimated through 50 polymorphic markers (Table 1). Gel photographs of 50 polymorphic markers were scored following the conventional binary 0/1 method and the bands were scored by 1 and 0 for their presence and absence in each inbred line. The binary data matrix of 50 SSR markers from five inbred lines was subjected to cluster analysis using the un-weighed pair group method with arithmetic mean (UPGMA) using DARwin software (Perrier *et al*., 2003) [15]. To estimate the discriminatory power of a marker, the Polymorphic Information Content (PIC) for each SSR marker along observed and expected heterozygosity were calculated using CERVUS 3.0.7 software (Kalinowski *et al*., 2007) [7].

Results and Discussion

Genetic diversity is of prime importance for the successful adaptation to certain agro-climatic conditions and improvement of any crop species. In the present study five inbred lines of maize were evaluated through 50 polymorphic markers. Each detected band was considered to be an allele. Variations in alleles were recorded to generate the molecular data *viz*., number of alleles per locus (k), observed heterozygosity (Ho), expected heterozygosity (He) and Polymorphic Information Content (PIC).

A total number of 111 alleles were generated by 50 polymorphic SSR markers among the five inbred lines of maize (Table 2). The number of alleles ranged from 2-4 with an average of 2.2 alleles per polymorphic primer pair. The highest number of alleles were observed in dupssr 27 (four alleles). Warburton *et al*., (2002) [21] reported an average of 4.9 alleles with 85 SSR markers in 57 maize inbreds while Patto *et al*., (2004) [14] reported 5.3 alleles using 80 SSR markers. However, the current results are close to the findings of Gupta and Singh (2010) [5] who recorded an average value of 2.5 alleles using nine polymorphic SSR primers in twenty maize inbreds.

Polymorphic information content demonstrates the informativeness of SSR markers to detect the differences among the inbred lines based on their genetic differences. Usually, markers with PIC value more than 0.5 are considered to be highly informative while markers with PIC value 0.25 to 0.50 are considered to be moderately informative in measuring the polymorphism for a marker locus (DeWoody *et al*., 1995) [3]. Polymorphic Information Content (PIC) value in the present study ranged from 0.164 to 0.672 with an average of 0.345 (Table 2) (Fig 1) indicating moderate level of polymorphism in the SSR markers. The average PIC value determined in the present study was similar to the findings of Legesse *et al*., (2007) [10] who recorded an average PIC of 0.58 in 56 inbred lines of maize while Babu *et al*., (2012) [2] reported average PIC value of 0.49 in 22 maize inbred lines.

Expected heterozygosity (He) or gene diversity gives an idea of SSR loci and their potential to detect differences between the parental inbred lines based on their genetic relation. Expected heterozygosity (He) differed among the markers ranging from 0.20 to 0.80 with a mean value of 0.468. The lesser mean value of expected heterozygosity indicated narrow genetic diversity among the five parental inbred lines. Similar results have been reported by Kondwakwenda *et al*., (2020) [9] where the mean value of expected heterozygosity was 0.363 among 46 genotypes of maize. Morales *et al*., (2010) [12] reported the mean expected heterozygosity as 0.68 among 25 germplasm accessions of maize.

Table 1: List of polymorphic SSR markers used in the genetic diversity assessment of inbred lines in maize

S. No	Name of the primer	Forward sequence	Reverse sequence	Chromosome location
1	bnlg 1347	GTGGTCACGACGAAATCCTT	TTGCAATCACACAGGTGGTT	1
2	umc 1245	TGGTTATGTGCATGATTTTTCCTG	CATGCGTCTGATCTTCAGAATGTT	1
3	bnlg 1025	TGGTGAAGGGGAAGATGAAG	CCGAGACGTGACTCCTAAGC	1
4	umc 1590	CAGAGTCTGATAGTCCGAACCCAG	GTAAAGCTCACAGCTTCCGACAG	1
5	umc 1244	AACCTCAGCTAAAACTGCTCGTGT	TTGTGCGTGTTTGGAAGTGATAGT	1
6	umc 2236	GCCACGCCTTCCATTATTAGAGTA	CGGTACTGTTCTGGGATTCGTTT	1
7	bnlg 2042	TGTCGCGTACTCGCATTTAG	TTTGATTGGTGATCTCGCAG	2
8	bnlg1297	TCTCGATCGCTCCGATCTAT	GACTCAACTCCAAAAGGCGA	2
9	bnlg1233	GAACACCAGAGGAGAGTGGG	TTCACTTGTCCACCACTGGA	2
10	umc 2245	GCCCTGTTATTGGAACAGTTTACG	CGTCGTCTTCGACATGTACTTCAC	2
11	umc 1798	TATAACAACGTAGCAAAGCACGGG	GATCGACCCTAATCGTCCTCCTAC	2
12	bnlg 1138	TGCTCTAGCCGACCTCAATT	ATGCCTGAACCGTGATTAGG	2
13	bnlg 1175	ACTTGCACGGTCTCGCTTAT	GCACTCCATCGCTATCTTCC	2
14	bnlg 1914	ATGCAACATTTCGTGATCCA	GATTTTTCTAGCACTCGCGC	2
15	umc 2088	ACGACAAGAAGGAGGCCAAAG	CAAGTAGATCGATCGAGCAGCAG	2
16	umc 2079	CGGCCTCGCTGTCTTCTAGC	ATGATCACGTCGTGCTGGTAGTG	2

17	bnlg 381	TCCCTCTTGAGTGTTTATCACAAA	GTTTCCATGGGCAGGTGTAT	2
18	mmc 0231	GAGCGACTGCGAGACGG	AGATCGCGCCACCGCTC	2
19	umc 2246	AGGCTCCAGCTCTAGGGGAGT	GTGAACTGTGTAGCGTGGAGTTGT	2
20	bnlg 2277	TTACGGTACCAATTCGCTCC	GACGACGCCATTTTCTGATT	2
21	bnlg 1092	TATTCTGGTCAAGTTGGGGC	GCTTGATCTCCAATCCTTGC	2
22	bnlg 1338	GTGCAGAATGCAGGCAATAG	GCAAATGTTTTCACACACACG	2
23	dupssr 27	CTATAGTTGCCACCACATCC	ACCCTTTGTGTAACTTTTCA	2
24	mmc 0191	GGTGTTCAGTGTGAAAGGTTA	AAGATTTCCGCAAGGTTAAAC	2
25	bnlg 1754	CCATCGCTGTACACATGAGG	TACCCGAAGGATCTGTTTGC	3
26	bmc 2136	TGCTCCTTCTCGAGCACC	ATGGACGTACGGCAGACTCT	3
27	umc 1641	CTCCCTTCGTCTCCCGACTC	CAGATCGGCTCAGCCACAAC	3
28	bnlg 2241	GTGCACACTCTCTTGCATCG	TAGTCAGCATCTGCCGTGTC	3
29	mmc 0132	ATATTCATCGGTTCAACTTCC	AGCGCCAGCCTCCCGTAGTC	3
30	umc 1012	TTCTTGCGGACCTCAAACTTGT	CTCCATCACCACTCAGAATGTCA	3
31	bnlg 1325	CTAAATGCGCAGCAGTAGCA	TGCTCTGCAACAACTTGAGG	3
32	bnlg 1137	ATGAGCTCAGTCACACTGTAGTG	ACTGATGACTGGTCCATGCA	4
33	bnlg 1159	GTGTGCCTATCCTTCCGAGA	AAGGACGTCAACAACGAACC	4
34	bnlg 1006	GACCAGCGTGTTGATCCC	GGAGACCCCGACTCTCTCTC	5
35	umc 1822	GGTATAATTTTGCAAGCAGAAAGGG	GGTTTGCTCAGGAAGAGCATGT	5
36	umc 1308	GCAGATGGACACAAACAAATGAAG	GCTACTGATGCTGGCAATCTTACA	5
37	phi 048	GCAAACCTTGCATGAACCCGATTGT	CAAGCGTCCAGCTCGATGATTTC	5
38	Bnlg 1136	TAACCGGATGAGCATCTTCC	CATCAGCTTCAACGAGTTCG	6
39	bnlg 1043	TTTGCTCTAAGGTCCCCATG	CATACCCACATCCCGGATAA	6
40	umc 1014	GAAAGTCGATCGAGAGACCCTG	CCCTCTCTTCACCCCTTCCTT	6
41	umc 1653	GAGACATGGCAGACTCACTGACA	GCCGCCCACGTACATCTATC	6
42	umc 2208	ACCAAATCAAACGAAGAAAAGTGG	CTTGATCGACATTTCGTTATGCTG	6
43	umc 1753	AAGATCTTGCTCCGTTTCCTCTCT	TTCAGATGCAAATCTCTTTTCGCT	6
44	bnlg 1884	TTCGGATGCATGTGTAACGT	CGGAAGTCCCATCTGTTTGT	9
45	bnlg 1724	CTGACCCAGAGCATTGTGAA	GATGAAGAGCTTGCAGTCCC	9
46	umc 2163	AAGCGGGAATCTGAATCTTTGTTC	GAAATTGCTGGGGTTCTCATTTCT	10
47	bnlg 1185	CGGTCCAGGCAGGTTAATTA	GACTCGAGGACACCGATTTC	10
48	bnlg 210	GCCTCGCACCAAGACATAATA	TGCCCCATTTGAGTAGACTTC	10
49	umc 1038	CGTCACACTCCTCTGCCACTT	GAGGATTCAGAACTCGACTCGG	10
50	umc 1077	CAGCCACAGTGAGGCACATC	CAGAGACTCTCCATTATCCCTCCA	10

The values of observed heterozygosity (Ho) ranged from 0 to 1 with a mean value of 0.084 indicating that the parental inbred lines attained an appreciable level of homozygosity. Similar results were also reported by Josia *et al*., (2021) [6] where mean value of observed heterozygosity was 0.09 among the 26 inbred lines of maize.

Clustering of inbred lines

Cluster analysis is useful in revealing the complex relationships among genotypes of diverse origins in a more simplified manner. The dendrogram constructed using the UPGMA clustering algorithm grouped the inbred lines into three clusters (Fig 2). The degree of genetic distance ranged from 0 to 0.1 indicating narrow genetic diversity. Two inbred lines KML-225 and BML-7 were grouped into cluster I. PFSR-3 alone was grouped into cluster II and the other two inbred lines BML-45 and BML-6 were grouped into cluster III. The results revealed that inbred lines in the same cluster were genetically similar to each other than the inbred lines in the other cluster. High yielding hybrids could be developed by combining the inbred lines from different clusters. The results obtained in the present study were close to the findings of Kanagarasu *et al*., (2013) [8] where twentyseven inbred lines were grouped into five clusters. Azam *et al*., (2018) [1] also reported the association between fifteen inbred lines of maize by 6 SSR markers. This is in agreement with the other investigators Senior *et al*., (1998) [20] and Reif *et al*., (2003) [17] who demonstrated the correspondence of SSR markers with cluster analysis in maize.

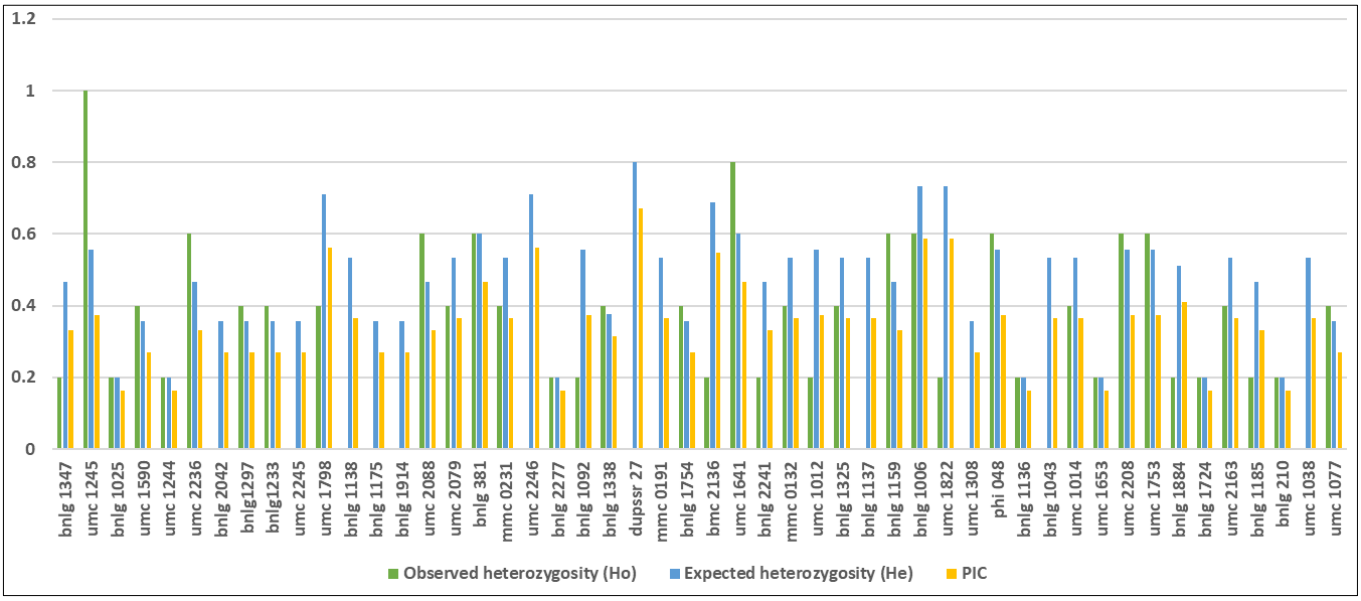

Fig 1: Histogram representing the Observed heterozygosity, expected heterozygosity and PIC values of 50 polymorphic markers among the inbred lines of maize

Table 2: Number of alleles, heterozygosity and PIC values of polymorphic SSR markers among the inbred lines of maize

S. No	Name of the primer	Chromosome No	Number of alleles per locus (k)	Observed heterozygosity (Ho)	Expected heterozygosity (He)	PIC
1	bnlg 1347	1	2	0.200	0.467	0.332
2	umc 1245	1	2	1.000	0.556	0.375
3	bnlg 1025	1	2	0.200	0.200	0.164
4	umc 1590	1	2	0.400	0.356	0.269
5	umc 1244	1	2	0.200	0.200	0.164
6	umc 2236	1	2	0.600	0.467	0.332
7	bnlg 2042	2	2	0.000	0.356	0.269
8	bnlg1297	2	2	0.400	0.356	0.269
9	bnlg1233	2	2	0.400	0.356	0.269
10	umc 2245	2	2	0.000	0.356	0.269
11	umc 1798	2	3	0.400	0.711	0.563
12	bnlg 1138	2	2	0.000	0.533	0.365
13	bnlg 1175	2	2	0.000	0.356	0.269
14	bnlg 1914	2	2	0.000	0.356	0.269
15	umc 2088	2	2	0.600	0.467	0.332
16	umc 2079	2	2	0.400	0.533	0.365
17	bnlg 381	2	3	0.600	0.600	0.466
18	mmc 0231	2	2	0.400	0.533	0.365
19	umc 2246	2	3	0.000	0.711	0.563
20	bnlg 2277	2	2	0.200	0.200	0.164
21	bnlg 1092	2	2	0.200	0.556	0.375
22	bnlg 1338	2	3	0.400	0.378	0.314
23	dupssr 27	2	4	0.000	0.800	0.672
24	mmc 0191	2	2	0.000	0.533	0.365
25	bnlg 1754	3	2	0.400	0.356	0.269
26	bmc 2136	3	3	0.200	0.689	0.548
27	umc 1641	3	3	0.800	0.600	0.466
28	bnlg 2241	3	2	0.200	0.467	0.332
29	mmc 0132	3	2	0.400	0.533	0.365
30	umc 1012	3	2	0.200	0.556	0.375
31	bnlg 1325	3	2	0.400	0.533	0.365
32	bnlg 1137	4	2	0.000	0.533	0.365
33	bnlg 1159	4	2	0.600	0.467	0.332
34	bnlg 1006	5	3	0.600	0.733	0.586
35	umc 1822	5	3	0.200	0.733	0.586
36	umc 1308	5	2	0.000	0.356	0.269
37	phi 048	5	2	0.600	0.556	0.375
38	bnlg 1136	6	2	0.200	0.200	0.164
39	bnlg 1043	6	2	0.000	0.533	0.365
40	umc 1014	6	2	0.400	0.533	0.365
41	umc 1653	6	2	0.200	0.200	0.164

42	umc 2208	6	2	0.600	0.556	0.375
43	umc 1753	6	2	0.600	0.556	0.375
44	bnlg 1884	9	3	0.200	0.511	0.410
45	bnlg 1724	9	2	0.200	0.200	0.164
46	umc 2163	10	2	0.400	0.533	0.365
47	bnlg 1185	10	2	0.200	0.467	0.332
48	bnlg 210	10	2	0.200	0.200	0.164
49	umc 1038	10	2	0.000	0.533	0.365
50	umc 1077	10	2	0.400	0.356	0.269
	Total		111			-
	Average		2.2	0.084	0.468	0.345

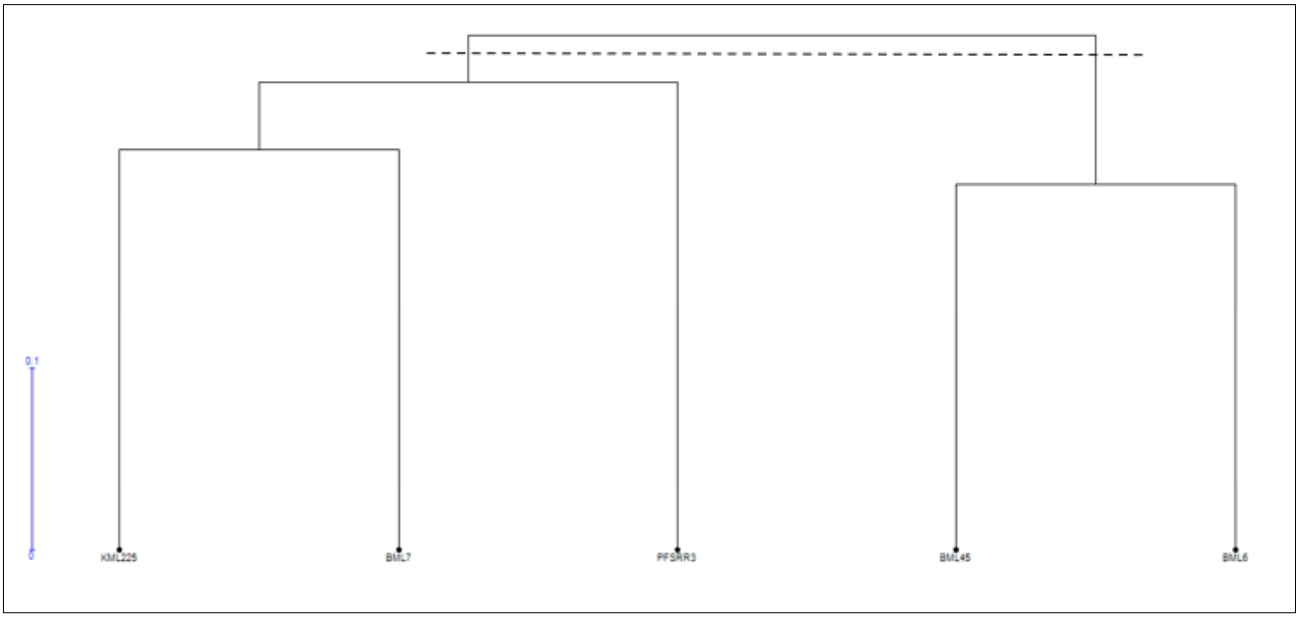

Fig 2: Dendrogram exhibiting relationship among the five inbred lines of maize generated by cluster analysis

Conclusion

In the present study five inbred lines were characterized using 50 polymorphic SSR markers for diversity analysis and inbred grouping in which 111 alleles were generated. The mean PIC value (0.345) and the mean gene diversity value (0.468) indicated the moderate level of polymorphism in SSR markers. UPGMA clustering algorithm grouped the lines into three clusters where the genetic distance ranged between 0 to 0.1 indicating the narrow genetic diversity among the inbred lines. However heterotic hybrids can be developed by utilizing the inbreds in different clusters.

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