Journal of Applied and Natural Science 9 (4): 2427 – 2433 (2017)

JANS

Analysis of genetic diversity among tropical and subtropical maize inbred lines using SSR markers

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Received: March 30, 2017; Revised received: June 20, 2017; Accepted: November 3, 2017

Abstract: Genetic diversity of 24 tropical and subtropical elite maize lines was assessed at molecular level employing 42 Simple Sequence Repeats. A total of 107 alleles with an average of 2.55 alleles per locus were detected. The Polymorphism Information Content (PIC) values of 42 SSR loci ranged from 0.08 (UMC1428) to 0.68 (UMC2189 and UMC2332) with the overall calculated PIC mean value of 0.44, whereas the Discrimination Rate (DR) value for SSR markers ranged from 0.09 (UMC2089) to 0.42 (UMC1311) with the average DR value of 0.26. Pair-wise genetic similarity (GS) values, calculated by Jaccard's coefficients, ranged between 0.25 and 0.78 with a mean genetic similarity of 0.63, indicating the existence of adequate amount of genetic divergence among the genotypes selected for the study. The cluster dendrogram separated all the inbred lines into six main clusters with sub clusters based on genetic similarity. Factorial analysis also confirmed a nearly similar pattern for grouping these inbred lines as presented by cluster dendrogram. In this study, SSR markers were found to be powerful tool for detection of genetic diversity in maize inbred lines. These findings could provide information for effective utilization of these materials for development of maize hybrids as well as for genetic improvement of inbred lines.

Keywords: Genetic diversity, Inbred lines, Maize, SSR markers, Polymorphism information content

INTRODUCTION

Maize (*Zea mays* L, 2n = 20) is one of the most important cereal crop in the world. Globally, it is known as queen of cereals because of its highest genetic yield potential. Maize provides at least 30% of the food calories together with rice and wheat to more than 4.5 billion people in 94 developing countries (Adetonah *et al.,* 2016). Maize contributes maximum among the food cereal crops i.e. 40% annually (>800 mt.) in the global food production (Jozsef and Zoltan 2013). In India, maize contributes nearly 9% in the national food basket and more than 400 billion to the agricultural GDP at current prices apart from generating employment to over 1000 million man-days (Dass *et al.,* 2012). For the effective conservation and utilization of maize genetic resources, a clear understanding of genetic diversity and its relationships with heterosis is essential for any crop improvement programs. The range of heterosis in crops like maize is depending on the genetic diversity present among the genotypes. Based on quantitative genetic theory, the probability of producing unique genotypes possessing desirable gene combination depends on the enrichment of the parents in proportion to the number of genes by which parents diverge.

The genetic diversity among parental lines is necessary to supply an ample quantity of allelic variation that can be used to generate new favorable gene combinations. Several previous reports indicate that, performance of F_1 depends on the genetic variability of parental lines (Devi and Singh, 2011; Prasanna, 2012; Pedram *et al.,* 2012). There are different methodologies exist for the assessment of genetic diversity in maize like morphological traits (Goodman and Bird 1977), isozymes (Revilla *et al.,* 1998) and molecular marker based diversity assessment using Random Amplified polymorphic DNA (RAPDs), Amplified fragment length polymorphism (AFLP) and simple sequence repeats (SSRs) (Smith *et al*., 1997; Ajmone Marsan *et al*., 2001; Moses *et al*., 2015). Since the morphological characters are influenced by the environment, they do not reliably provide genetic diversity information of germplasm. Molecular markers are found free from such limitations and capable to provide more precise information on genetic diversity. The use of molecular markers for diversity analysis serves as a useful tool to discriminate between closely related individuals from different breeding sources (Moses *et al*., 2015).

Modern molecular markers approach facilitate a reliable selection of parents for hybridization and more precise assessment of the levels of genetic variations present in parental lines, especially from different genetic backgrounds (Lu *et al*., 2009). Among molecular marker systems, efficacy of SSR markers in determining genetic variability and relationships among maize germplasm has been effectively demonstrated in several studies (Senior *et al.,* 1998; Kassahun and Prasanna, 2003; Legesse *et al.,* 2007; Kostova *et al.,* 2006). SSR markers are PCR-based, co-dominant, less costly, robust, reliable, highly reproducible, with greater discriminatory ability and are more widely available than other molecular markers like RFLP's and RAPD's etc. (Pushpavalli *et al.,* 2001; Prasanna and Hoisington, 2003). A clear understanding of variability and its relationship with heterosis must be understood in order to undertake any heterosis breeding programme. Therefore, the present study was undertaken to evaluate genetic diversity among tropical and subtropical elite maize lines using SSRs markers.

MATERIALS AND METHODS

Plant material and SSR markers: Plant material comprised of 24 tropical and sub tropical maize inbred lines from different backgrounds (Table 1) was used to study the diversity at molecular level using 42 SSR markers distributed on all chromosomes of maize genome (Table 2). The primer information of these SSR markers is available in public domain (MaizeGDB http://www.maizegdb.org). Seed materials were grown during Kharif season following recommended package of practices (Parihar *et al* ., 2011) at the research farm of IARI, New Delhi and further genotyping work was carried out at Maize Genetics Unit, IARI, New Delhi.

DNA isolation and quantification: A modified CTAB method was used for isolation of genomic DNA from a pooled sample of fresh leaves of 5 plants from each inbred lines (Saghai *et al.,* 1984). The working DNA samples were stored at 4°C and stock solutions at –20°C (Celfrost BFS 345-S). Quantification of DNA samples was done using a Spectrophotometer (Bio-Tek Instruments, USA) and absorbance was recorded at 260nm and 280nm. The quality of DNA samples was checked using 0.8% agarose gel electrophoresis with a known concentration of uncut λ DNA to adjust final concentration for use in PCR reaction.

PCR amplification: DNA amplification was carried out by PCR with a reaction mixture of 15 µl, containing 5 ng of genomic DNA template, 1 µM each of forward and reverse primers, 0.2 mM dNTPs, 0.5 U *Taq* DNA polymerase and 1.5 mM $MgCl_2$ (Fermentas), in a Peltier Thermal cycler-100 or Dyad (MJ Research, USA). The cycling parameters for PCR amplification consisted of initial denaturation at 94°C for 4 min followed by 94°C for 1 min denaturation, primer annealing at 55-65°C (depending on the respective primer annealing temperature) for 1 min and at 72°C for 2 min for primer extension and then 35 cycles of denaturation to extension. The final cycle for primer extension was performed at 72°C for 7 min and then stored at 4°C. The amplified PCR products were resolved on 3.5% metaphor agarose gel (Fermentas) along with 100 bp ladder (Fermentas) using a horizontal submarine gel electrophoresis system (Biorad Submarine Gel Electrophoresis Unit, USA). The gel was run in 1x Tris/Borate/EDTA (TBE) buffer at a constant voltage of 80 V for about 4 hr (until the tracking dye migrated to the end of the gel). The gel images were captured using a Gel Documentation System (Alpha Imager, USA).

Scoring and analysis SSR's data: Marker data was recorded as '1' for the presence and '0' for the absence of a band whereas, the diffused bands or bands depicted ambiguity in scoring were considered as missing data and designated as '9' in the data matrix. Null allele for any specific marker in a genotype was considered as absence of primer binding site, after reruns with specific check and was designated as '0'. The polymorphism information content (PIC) was performed as described as (Bantte and Prasanna, 2003). PIC is a measure of allele diversity at a locus which is equal to $1-\Sigma fi^2$, where f is the frequency of i^{th} allele. Discrimination Rate (DR) was calculated according to Selvi *et al.* (2005). Genetic similarity (GS) based on SSR data was calculated for all possible pairs of genotypes using Jaccard's coefficient (J) (Jaccard, 1908) based on the formula, $GSJ = N_{11} /(N_{11} + N_{10} + N_{01})$, where N_{11} is the number of alleles present in both individuals, N_{10} is the number of alleles present only in one of the pair (individual i) and N_{01} is the number of alleles present only in the individual j. The similarity matrix was implemented by using UPGMA (Unweighted Paired Group Method using Arithmetic Averages) with average linkage. Goodness of fit of clustering was also tested by estimating cophenetic values using COPH and MXCOMP options of the NTSYSpc version 2.2 (Rohlf, 1989).

RESULTS AND DISCUSSION

A total of 42 SSR markers were used to understand the genetic relationship among 24 tropical and subtropical elite maize inbred lines collected from different maize research centers in India. The SSR markers failed to amplify any band and showed monomorphic pattern was not included in any calculation. The majority of SSR fragments size ranged from 50 - 200 bp. Analysis of molecular polymorphisms led to detection of a total of 107 alleles, ranged from 2 to 5 with an average of 2.55 alleles per locus (Table 2). A total of 23 SSR loci reported with two alleles, 16 with three alleles, 2 with four alleles and remaining one locus revealed five alleles. The repeat motifs information of SSR loci analyzed in present study were as follows: tri-repeat motif

Table 1. Seed source/pedigree of 24 maize inbreds investigated under present study.

S. No.	Name of inbred lines	Sources/Pedigree	Types
1	DM-RIL-47	GENETICS IARI	Subtropical
2	NAI-147	NAGANHALLI/EV 25-CD (Y) AIM-C7	Subtropical
3	CM-142-393-1	GENETICS IARI /1PA 3-20-1-1-1	Subtropical
4	MGU-138-3053	GENETICS IARI	Tropical
5	CM-212-3142	GENETICS IARI /CI 21 E	Subtropical
6	CML-152-3058	CIMMYT/ S8862Q-1-4-4-5-B-#/Pop62	Tropical
7	SE-547-3037	GENETICS IARI	Tropical
8	BLSB-RIL-92	GENETICS IARI	Subtropical
9	VQL-1	GENETICS IARI /(CM-212XCML-180) BCJ P1@b@b@b@b-#	Subtropical
10	HKI-193-1	KARNAL/CML193	Subtropical
11	CM-152	GENETICS IARI /POP 31 C4 HS bulk (Alm) ###@b-###-@#,U3-1	Subtropical
12	HKI-209	KARNAL/Pop10	Subtropical
13	HKI-287	KARNAL/CML 287	Subtropical
14	SC-7-2-1-2-6-1	DMR	Subtropical
15	CML-119	CIMMYT/B807-2-3-3-3-1-4-b1-b1#-b1/ R 109	Tropical
16	HKI-1025	KARNAL/BC175	Subtropical
17	HKI-1105	KARNAL/Cargil 633	Subtropical
18	HKI-335	KARNAL/POOL10	Subtropical
19	HKI-323-8	KARNAL/POOL28	Subtropical
20	CML-147	CIMMYT/Pob63c2HC53-1-1-B-B-B-9-B-B-#/POp63	Tropical
21	CML-150	CIMMYT/P 24 QPM	Tropical
22	CML-395	CIMMYT/11TA	Tropical
23	HKI-162	KARNAL/CML162	Subtropical
24	CML-421-CML-170	CIMMYT	Tropical

(39), di-repeat and tetra-repeat (one loci each), while the information about one repeat motifs was not available at Maize GDB. The tri and tetra repeats are having high polymorphism as compared to di-repeats.

The Polymorphism Information Content (PIC) value of a SSR locus is influenced by the number of alleles (allele richness) as well as distribution of these alleles across the genotypes. PIC values of the SSR loci varied from 0.03 (umc1178) to 0.68 (umc1353) with overall mean value of 0.44 (Table 2). This overall PIC value may be influenced by various factors, *viz.,* the nature of germplasm used for the study; number of SSR loci as well as inbred lines analyzed; nature and type of repeats of SSR loci and the methodology employed for allele detection (agarose versus PAGE). Out of 42 SSR markers, only 28 gave rise to high (≥ 0.40) PIC values; such loci could be highly useful in genotype differentiation and genetic diversity analysis among the inbred lines (Gurung *et al*., 2010). Rest of the fourteen SSRs gave low (≤ 0.40) PIC values indicating their inability in discriminating the genotypes. The markers amplifying high number of monomorphic bands were having low PIC value whereas the markers showing more unique and null alleles were having high PIC value (Nepolean *et al.,* 2013). Discrimination rate (DR) ranged from 0.09 (umc1428) to 0.42 (umc1452) with an average of 0.26. Out of 42 SSR markers, 14 gave rise to high DR values (≥ 0.30), while remaining 28 primers depicted low DR values (< 0.30). PIC values and DR gave a positive significant correlation of 0.58. Except few of markers with high PIC value did show high DR, however, *vice versa* was always not true. Some markers like umc1596, umc1428, umc1913, umc2089, umc2017 and umc2021 gave low DR (<0.29), which also showed very low PIC values. The pair wise genetic similarity was calculated by Jaccard's coefficients, this ranged from 0.25 between two pairs (VQL-1 with HKI-193-1) and 0.78 between (CM -142-393-1 with HKI 1025; HKI 1105 with DMRIL-47) with an average value of 0.63. Cluster analysis revealed a good fit to the data matrix as evidenced from high cophenetic correlation coefficient value of 0.58.

The factorial analysis showed a scattered distribution of 24 maize inbred lines in the two dimensional plot diagram (Figure 2), confirmed a nearly similar pattern for grouping as presented in cluster dendrogram (Figure 1), and separated all the inbreds into four major groups. Group II was the largest consisted of eight inbred lines namely CML-395, SE-547-3037, BLSB-RIL-92, SC-7-2-1-2-6-1, CML-421-CML-170, CML-119, NAI-147 and HKI-287. Similarly group I composed of six HKI323-8, CML1-147, CM-212-3142, CM-142-393-1, DM- RIL-47 and HKI-162, Group III with four HKI-209, MGU-138-3053, CM-152 and HKI -1105, group IV with HKI-1025, HKI-193-1, VQL-1, CML-150 and HKI-335 inbred lines. One inbred line CML-152-3058 did not fall into any group. From the result of this study, it was found that some pairs of inbred lines were consistently close falling in one cluster as per the pedigree details, but other inbreds did not follow this pattern. Clustering of inbred lines indicate the presence of enough genetic diversity among maize inbred lines collected from different maize research centers. In sub-clusters presence of inbred lines of different group indicate the minor genetic difference be-

Table 2. Information of 42 SSR loci with bin location, repeat type, product size, No. of amplified alleles, polymorphism information content (PIC) and discrimination rate (DR).

S. No.	SSR locus	Bin	Repeat type	Repeat	Product size	Amplified alleles	PIC	DR
1	UMC1452	1.04	(GCC)4	tri	96	2	0.23	0.28
2	UMC1122	1.06	(CGT)7	tri	156	3	0.66	0.37
3	UMC2151	1.06	(CAG)4	tri	127	3	0.49	0.30
4	UMC1446	1.08	(TAA)7	tri	160	2	0.47	0.24
5	UMC2189	1.10	(CAG)4	tri	160	2	0.68	0.34
6	UMC1534	1.10	(AAG)5	tri	92	3	0.44	0.24
7	UMC1353	1.10	(AAC)4	tri	159	3	0.66	0.35
8	UMC1262	2.02	(GTC)4	tri	145	2	0.38	0.20
9	UMC2125	2.05	(GAG)7	tri	151	2	0.33	0.17
10	UMC2129	2.07	(CGC)5	tri	80	3	0.45	0.28
11	UMC1256	2.09	(CAT)5	tri	143	2	0.48	0.25
12	UMC2103	3.00	(GCG)5	tri	158	3	0.54	0.28
13	UMC2050	3.07	(CGC)4	tri	85	2	0.36	0.30
14	UMC1813	3.09	(CAG)8	tri	121	2	0.54	0.28
15	UMC1010	3.09	(GA)10	di	94	3	0.50	0.28
16	UMC1228	4.01	(CAG)10	tri	50	3	0.66	0.35
17	UMC1313	4.09	(CTT)5	tri	86	2	0.52	0.27
18	UMC2011	4.10	NA	NA	155	3	0.37	0.30
19	UMC1597	5.03	(CCT)4	tri	107	2	0.21	0.11
20	UMC2298	5.04	(GCG)4	tri	NA	3	0.53	0.27
21	UMC1171	5.04	(GTT)4	tri	90-100	3	0.54	0.28
22	UMC1153	5.09	(TCA)4	tri	111	2	0.50	0.26
23	UMC1818	6.01	(CAG)6	tri	148	2	0.47	0.24
24	UMC1178	6.02	(GGC)6	tri	160	2	0.45	0.28
25	UMC1857	6.04	(TAA)6	tri	146	2	0.41	0.22
26	UMC2364	7.01	(GGA)7	tri	NA	4	0.45	0.36
27	UMC2325	7.01	(TGG)7	tri	NA	3	0.29	0.36
28	UMC1428	7.02	(CCG)5	tri	105	2	0.08	0.14
29	UMC1708	7.04	(GGA)4	tri	84	4	0.66	0.35
30	UMC2332	7.04	(CTC)5	tri	NA	2	0.68	0.34
31	UMC1913	8.02	(TTG)6	tri	217	3	0.29	0.19
32	UMC1872	8.02	(GCA)6	tri	141	3	0.50	0.30
33	UMC1735	8.03	(AGC)5	tri	97	2	0.34	0.18
34	UMC1596	9.01	(GGC)6	tri	157	2	0.50	0.25
35	UMC2336	9.02	(TGT)4	tri	102	3	0.39	0.28
36	UMC2133	9.05	(AGC)4	tri	160	3	0.42	0.30
37	UMC2089	9.07	(CGC)4	tri	178	2	0.15	0.09
38	UMC2017	10.03	(CAA)4	tri	52	2	0.29	0.15
39	UMC1280	10.04	(AAT)7	tri	111	2	0.40	0.21
40	UMC2043	10.05	(TCC)4	tri	NA	2	0.53	0.28
41	UMC1311	10.60	(TCTT)4	tetra	136	5	0.55	0.42
42	UMC2021	10.07	(TGG) 4	tri	NA	2	0.28	0.14
Total						107/42	18.62/42	11.07/42
Mean						2.55	0.44	0.26

tween these lines of a main cluster. Such grouping may expose a slight gene flow among inbred lines of different origin. According to Sun *et al.,* (2001), the discrepancy between pedigree and molecular markers-based genetic diversity estimates may be caused by selection pressure for different breeding objectives. The results also revealed that pattern of grouping did not match well with available pedigree information which is in accordance with the results reported in oats (O'Donoughue *et al*., 1994), bread wheat (Barrett *et al*., 1998; Almanza-Pinzon *et al*., 2003, Soleimani *et al*., 2002).

This analysis led to detection of 107 alleles (Table 2), with a mean of 2.55 alleles per locus. Earlier studies reported 4.9 alleles using 85 SSR markers (Warburton *et al.,* 2002), 3.25 alleles with 36 SSR loci (Bantte and Prasanna, 2003), and 5.3 alleles using 80 SSR loci (Vaz Patto *et al.,* 2004). In this study, mean of alleles per locus was considerably lower than reported earlier in the maize. Karanja *et al.,* (2009) reported 2.0 averages of alleles using 14 loci. The SSRs used in this study were tri-repeat motifs (thirty nine loci), di and tetra-repeat (one locus each) whereas; information of one repeat motif was not available at MaizeGDB. All these loci were found to be significantly associated with the genome as depicted by the average number

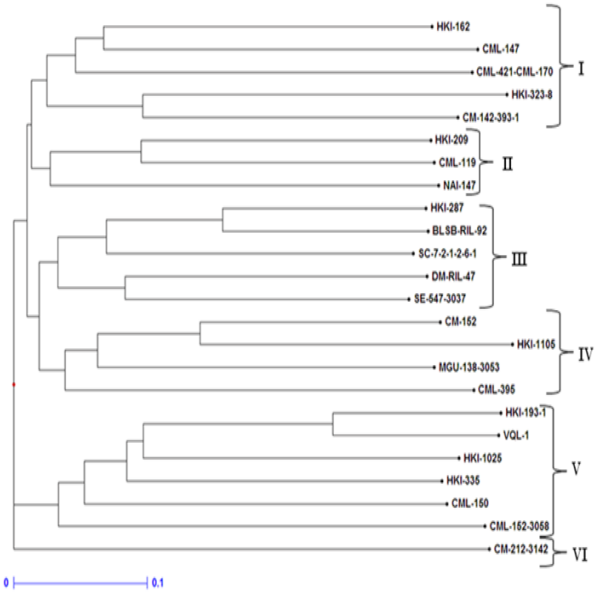

Fig.1. *Dendrogram generated based on 42 SSR loci to expose the genetic relationships among 24 maize inbred lines.*

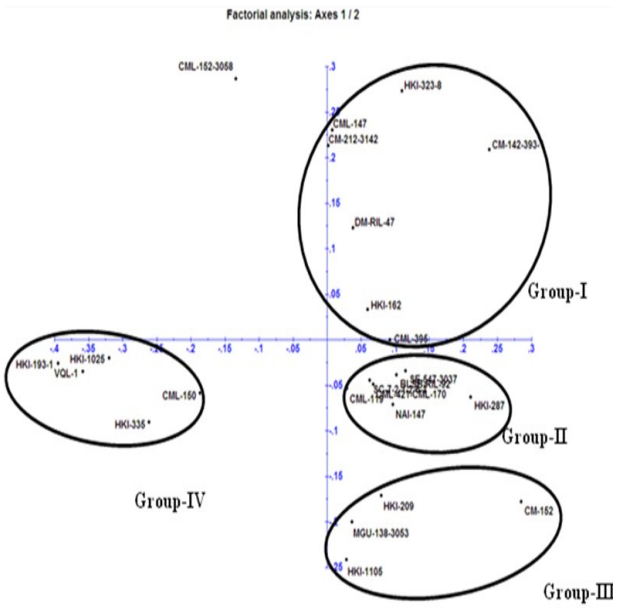

Fig.2. *Factorial analysis revealed the genetic relationships among the maize inbreds.*

alleles (2.55) amplified per primer (Table 2). The mean PIC and DR values were 0.44 and of 0.26 respectively in our study, which support the observations made by other workers (Legesse *et al.,* 2007; Enoki *et al.,* 2002). In the present study, the less PIC value might be due to the use of metaphor agarose gel electrophoresis, whereas polyacrylamide gel or automated analysis approaches used by (Yap *et al.,* 1996; Tobias *et al*., (2008) reported high PIC values.

The inbred lines used in the present investigation are less dissimilar in term of pedigree information as compared to previous reports (Prasanna and Hoisington,

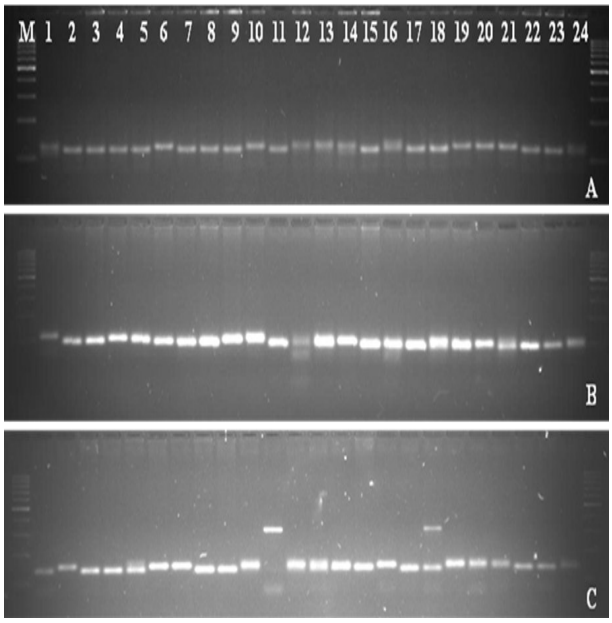

Fig.3. *SSR polymorphisms in 24 selected maize inbred lines, illustrated by umc1415 (A); umc2151 (B) and umc2129 (C). The lane order (1 to 24) is same as the list of genotypes presented in table.1.*

2003; Warburton *et al.,* 2002; Vaz Patto *et al.,* 2004). This may be due to many of the lines used for present investigation are from same groups *viz*, HKI, CML and CM (Table 1). Genetic similarity as analyzed by Jaccard's coefficient indicated considerable variability among the inbred lines used in present study. The average genetic similarity among the lines (0.63%) was relatively low, indicating high level of polymorphism between the inbred lines. This has also been suggested by various authors (Bantte and Prasanna, 2003; Beyene *et al*., 2005; Sharma *et al*., 2010). The dendrogram created using UPGMA clustering algorithm fit well with the similarity matrix with high cophenetic correlation (r = 0.55). The dendrogram placed these inbred lines into six main clusters with minor sub grouping within the major clusters. However, the grouping by UPGMA analysis did not follow the genetic relatedness as indicated by their pedigree data.

Present investigation revealed that, PIC alone will not give true representation of the informativeness of a SSR marker, but DR may also be taken into consideration. Similarity matrices obtained using the markers of high DR and the whole set of data fits wells to the goodness of fit test. Selvi *et al.,* (2005) proposed the use of DR in identifying primers combination for AFLP analysis. The result indicated that, DR may be a more reliable indicator for selecting markers for their ability to differentiate lines as against widely accepted PIC values. The factorial analysis revealed a nearly similar pattern for grouping of inbred lines as cluster dendrogram. All the genotypes showed a clear grouping except in group II. Group II depicted a partial

overlapping between inbred lines indicating that these lines were previously derived from the same group. Finally it could be concluded that, for genetic diversity analysis and grouping the genotypes, molecular distances is the most effective method. Similar study taking more number of genotypes and more number of markers may give rise to a better understanding of the situation. In present study, estimation of accurate genetic relations among parental lines may be useful for determining the material should be combined in crosses for obtaining superior genotypes in future breeding programs. Selection of parents from each cluster and crossing them in a series of breeding fashion could be highly fruitful.

Conclusion

Marker based studies are repeatable and give insight in the variability present at DNA level. In the present study diversity assessed using molecular markers visualized the genetic differences in the maize genotypes. The average PIC value of 0.44 indicated that the SSR markers are a powerful tool for detection of genetic diversity. Molecular markers separated all the inbred lines into six main clusters and the genotypes from the diverse clusters may be a potential candidates for inbred and hybrid development.

ACKNOWLEDGEMENTS

Senior author gratefully acknowledge the help and guidance for conducting the research work provided by Dr. Firoz Hossain, (Maize Genetics Unit, Division of Genetics, I.A.R.I., New Delhi-110012).

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