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RESEARCH ARTICLE

Genetic Divergence Studies and Heterotic Grouping in Maize Inbred Lines using Microsatellite Markers

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Information on genetic diversity and relationship among different maize genotypes is very important in hybrid maize breeding program. The purpose of present study was to elucidate the nature and extent of differentiation and divergence among 18 inbred lines of maize based on the analysis of targeted microsatellite sites. Using 28 primer pairs, altogether 296 allelic variants including 145 shared and 151 unique alleles were detected amongst amplified products and a total of 49 loci were assigned with an average of 6.04 alleles per locus. Polymorphic information content of microsatellite primer pairs ranged from 0.34 (umc1304) to 0.93 (umc1179) with mean of 0.77 per primer. A remarkably higher level of genetic differentiation and divergence was revealed by the use of 28 microsatellite markers, which allowed unique genotyping and unambiguous classification of the maize inbred lines under evaluation. Among the inbred lines under molecular characterization, CML163 and CML467 appeared as the most diverse genotypes. Using the matrix of genetic similarity, the cluster analysis grouped the eighteen inbred lines into four heterotic groups. The markers utilized in the present study were sufficient for discrimination and unambiguous classification of inbred lines.

Key Words: Genetic diversity, inbred lines, microsatellite, maize, heterotic group

Introduction

Globally, maize is the third most important cereal after wheat and rice. It is cultivated in wide range of environments than wheat and rice because of its greater adaptability. Among the cereals, the productivity of maize is recorded to be the highest as compared to rice and wheat. Genetic diversity is an essential element for the genetic improvement and development of new inbred lines, hybrids and synthetic cultivars of maize. Assigning the parental lines into different heterotic groups is fundamental for the maximum exploitation of heterosis through hybrid cultivar development in a cross-pollinated crop like maize. Different methodologies have been used to characterize genetic diversity in the maize germplasm, which are morphological characters (Goodman *et al*., 1977), pedigree analysis (Duvick 1984), heterosis (Smith *et al*., 1989) and detection of variation at DNA level using markers. Morphological differences are usually determined by a small number of genes and may not be representative of genetic divergence in entire genome (Singh *et al.,* 1999; Brown-Guedira *et al.,* 2000).

Recently, molecular markers, which provide reliable and complementary information, have been used by the researchers for the purpose of characterization of inbred lines, assessment of genetic diversity and classification of inbred lines into heterotic groups. Molecular markers developed for the differentiation of genotypes and assessment of genetic diversity are reliable and remain unaffected across different growth stages, seasons, locations and agronomic practices (Efendi *et al*., 2015). Since expression is not influenced by environmental factors, the most important advantage offered by molecular marker is that the actual level of genetic difference can be determined between different genotypes including inbred lines. Amongst the molecular markers, microsatellite markers are regarded as useful tool to assess the genetic diversity among the different maize inbred lines and maize genetic resources. When microsatellites are individually amplified by means of the polymerase chain reaction using a pair of flanking unique oligonucleotides as primers, they almost invariably show extensive polymorphism due to site-specific length

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variation as a consequence of the occurrence of different numbers of repeat units.Microsatellites are widely used in maize, as these markers are genetically co-dominant, hyper-variable, highly polymorphic, abundant, robust, reproducible, distributed throughout the genome and amenable to automation (Dubreuil *et al*., 2006; Prasanna *et al*., 2010). Keeping into consideration that the use of microsatellite markers can help in assessing the nature and extent of genetic diversity among inbred lines, assigning inbred lines efficiently to heterotic groups and making the choice of heterotic parents to develop new hybrids, the present study has been conducted.

Materials and Methods

DNA extraction and Primer based amplification

The total genomic DNA was isolated from leaf samples of eighteen inbred lines (Table 1) of maize which were harvested from 4-5 leaf seedlings using standardized maize genomic DNA extraction protocol (Punya *et al*., 2017). 28 microsatellite primer pairs were chosen from MaizeDB (http://www.maizegdb.org/ssr.php) (Table 2). PCR amplification were performed in 15µl volumes containing 2µl of genomic DNA, 1U *Taq* DNA polymerase, 3µl dNTP (1mM), 1.5µl primer, 2.8µl nuclease free water,1.3µl $MgCl_2$ (10mM). Amplification consisted of initial denaturation for 5 min at 94^oC, 30 cycles of 94^0C (1 min), annealing at 52^0C-60^0C (1 min) and 72^0C (2 min), followed by a final extension at 72^0C (7 min). Amplification products were separated by electrophoresis in horizontal gel system at 110 V for 1 h 30 min on 2% agarose gel stained with ethidium bromide (10mg/ml) using 0.5X TBE buffer. The amplified products were visualized with the help of a gel documentation system (Alpha Innotech, USA) and the size of fragments was estimated with the help of 50bp ladder (Fermentas).

Table 1. List of inbred lines used in the present study alongwith their source

Sl. No.	Inbreds	Source
1.	CML 467	CIMMYT, Mexico
2.	CML 468	CIMMYT, Mexico
3.	CML 469	CIMMYT, Mexico
4.	CML 470	CIMMYT, Mexico
5.	CML 471	CIMMYT, Mexico
6.	CML 373	CIMMYT, Mexico
7.	CML 115	CIMMYT, Mexico
8.	CML 196	CIMMYT, Mexico
9.	CML 465	CIMMYT, Mexico
10.	LM 13	SRI, Coimbatore
11.	Dholi 2012	TCA, Dholi
12.	HKI 162	CCS HAU, Hisar
13.	HKI 323-B	CCS HAU, Hisar
14.	HKI 586	CCS HAU, Hisar
15.	HKI 1105	CCSHAU, Hisar
16.	CML 161	CIMMYT, Mexico
17.	CML165	CIMMYT, Mexico
18.	CML 163	CIMMYT, Mexico

Data analysis

Gel photographs were scored manually and bands were binary coded by 1 or 0 for their presence or absence in each genotype. The microsatellite primer scores were used to create a data matrix to analyze genetic relationships using the NTSYS-pc software (Rohlf 2000). The information pertaining to allelic diversity and suitability of SSR based polymorphism for identification of polymorphic and informative markers to characterize and differentiate maize inbred lines was generated on the basis of polymorphism information content (PIC) of primer pairs. Polymorphism information content (PIC) values were calculated manually for each SSR locus according to the formula as described by Smith *et al*., 1997.

$$PIC = 1-\sum f_i^{\,2}$$

Where, f_i is the frequency of ith allele

Genetic similarities among inbred lines were calculated on the basis of presence and absence of common bands. The genetic association among inbred lines were analysed by calculating the similarity coefficients (Dice 1945) for pair wise comparisons based on the proportions of shared bands produced by primers.

$$\text{Similarity coefficient} = 2a/(2a+b+c)$$

Two dimensional plot was constructed using two principle components selected by the NTSYS-pc software 2.1 to infer the level of gene similarity among the inbred lines.

Results and Discussion

Allelic diversity and polymorphism analysis

Altogether 296 allelic variants were detected amongst amplified products generated with 28 primer pairs. Amongst the primer pairs used during amplification reaction, ten primer pairs, namely, umc1266, phi072,

Table 2. Characterization of 28 microsatellite markers used for analysis of 18 maize inbred line

Marker	Bin	Repeat type	Locus	Size range(bp)	No. of alleles	No. of alleles per locus	Unique alleles	Shared alleles	**PP**	**PIC**
phi 227562	1	ACC	2	313-352	07	3.5	2	5	28.5	0.80
bnlg 1429	1	AG(20)	2	197-226	11	5.5	4	7	36.3	0.89
umc 1297	1	(GA)6	2	68-182	13	6.5	5	8	38.4	0.79
nc 133	2	GTGTC	2	62-152	15	7.5	5	10	33.3	0.71
phi 083	2	AGCT	3	66-168	14	4.6	6	8	42.8	0.75
phi029	3	AG/AGCG	2	79-190	11	5.5	6	5	54.5	0.84
phi 053	3	ATAC	2	180-22	16	8	8	8	50.0	0.80
umc1266	3	(CAG)4	1	139-170	11	11	6	5	54.5	0.88
umc1136	3	(GCA)5	2	134-163	06	3	3	3	50.0	0.66
phi072	4	AAAC	1	145-161	06	6	2	4	33.3	0.72
phi093	4	AGCT	1	286-345	11	11	7	4	63.6	0.89
nc 130	5	AGC	1	150-171	09	9	4	5	44.4	0.87
umc1332	5	(CTA)5	2	126-167	13	6.5	6	7	46.1	0.79
umc1152	5	(TCA)4	2	76-187	11	5.5	4	7	36.3	0.58
bnlg118	5	--	2	133-177	13	6.5	8	5	61.5	0.89
bnlg1136	6	AG(14)	2	203-240	11	5.5	5	6	45.4	0.87
umc1083	6	(GA)16	3	61-144	14	4.6	5	9	35.7	0.73
phi034	7	CCT	1	138-172	11	11	7	4	63.6	0.87
phi116	7	ACTG/ACG	1	169-185	08	8	2	6	25.0	0.85
umc 1304	8	(TCGA)4	2	56-155	09	4.5	2	7	22.2	0.34
umc1161	8	(GCTGGG)5	3	63-180	12	4	3	9	25.0	0.55
phi115	8	AT/ATAC	1	290-313	07	7	4	3	57.1	0.81
phi 014	8	GGC	2	64-182	08	4	5	3	62.5	0.70
phi065	9	CACTT	2	141-189	15	7.5	10	5	66.6	0.85
phi 084	10	GAA	1	157-195	10	10	7	3	70.0	0.83
umc1367	10	(CGA)6	2	167-255	11	5.5	7	4	63.6	0.79
umc1196	10	CACACG	1	150-171	07	7	4	3	57.1	0.72
umc1179	10	(AAG)4	1	188-214	06	6	3	3	50.0	0.93
	Total		49	-	296	-	151	145	-	21.7
	Average									0.77

phi093, nc130, phi034, phi116, phi115, phi084, umc1196 and umc1179 generated only one polymorphic amplified product. Interestingly, the primer pairs phi227586, bnlg1429, umc1297, nc133, phi083, phi029, phi053, umc1136, umc1332,umc1152, bnlg118, bnlg1136, umc1083, umc1304, umc1161, phi014, phi065 and umc 1367 generated more than one amplified product due to amplification of more than one locus, which might have resulted from the co-dominant nature of the microsatellite markers . A total of 49 loci were assigned to the 28 primer pairs with an average of 6.04 alleles per locus. The number of alleles ranged from 6 in case of umc1136, phi072, umc1179 to 16 in case of phi053 with a range between 56 to 352 bp (Table 2). A total of 145 shared and 151 unique allelic were detected by using 28 primer pairs. The number of unique alleles ranged from 2 to 10 while number of shared alleles ranged from 3 to 10. Average number of alleles per locus obtained in this study is more or less similar to the results obtained in previous studies conducted by several researchers in maize (Pejic *et al*., 1998; Li *et al*., 2002; Wu *et al*., 2004; Qi-Lun *et al*., 2008; Morales *et al*., 2010). Confirming the earlier reports (Smith *et al*., 1997; Pejic *et al*., 1998; Yuan *et al*., 2000; Senior

et al., 1998), the results of the present study also indicated that the microsatellite loci with di-nucleotide repeat unit were more polymorphic loci than that with tri-nucleotide repeat unit and generated more number of allelic variants per locus. The maximum polymorphism was observed in phi084 (70%) and minimum was observed in umc1304 (22.2%) microsatellite marker.

The Polymorphism Information Content (PIC) demonstrates the informativeness of SSR loci and their potential to detect differences among the inbred lines based on their genetic differences. In the present study, PIC values of microsatellite loci ranged from 0.34(umc1304) to 0.93(umc1179) with mean of 0.77 (Table 2), reflecting the presence of high allelic variation in the marker loci. Average value calculated for this parameter in the present study is very close to the value obtained by several earlier research workers (Hoxa *et al.*, 2004; Reid *et al*., 2011). The highest PIC value was observed in umc 1179 (0.93) whereas, lowest was observed in case of umc1161 (0.55). Considering the number of alleles generated by different primer pairs in conjunction with the level of polymorphism detected in the present study, the primers umc1297, phi053, umc1266, phi093, bnlg118, phi034, phi115, phi065 and phi084 appeared to be more informative primers (Fig. 1).

Genetic similarity and cluster analysis

Genetic similarity between pair-wise combinations of entries was calculated on the basis of allelic data obtained with regard to 296 allelic variants amongst 18 maize inbred lines. The magnitude of similarity coefficient ranged from zero to 0.52 (Table 3), indicating thereby the existence of ample genetic differences. Thus, the estimates of similarity coefficients indicated a considerably greater extent of variation among the inbred lines under evaluation in the present study and provided greater confidence for the classification and assessment of genetic relationships. Similar inference has been derived in the studies conducted on the molecular markers including microsatellite marker-based divergence analysis in maize by earlier researchers (Efendi *et al*., 2015; Li *et al*., 2002; Hoxa *et al*., 2004).

By drawing the phenon line at 25 similarity units in the dendrogram (Fig.2), the eighteen entries were divided into six clusters for the purpose of deriving inference about the pattern of divergence amongst the entries at the molecular level. Out of six clusters, three clusters, namely, cluster A, cluster C and cluster F were

Table 3. Estimates of microsatellite markers based Dice similarity coefficients among eighteen maize inbred lines used in the present study

Genotypes	CML467	CML468	CML469	CML470	CML471	CML373	CML115	CML196	CML465	LM13	DH2012	HKI162	HKI323B	HKI586	HKI1105	CML161	CML165
CML468	0.30																
CML469	0.14	0.38															
CML470	0.22	0.32	0.33														
CML471	0.18	0.19	0.22	0.45													
CML373	0.08	0.17	0.32	0.26	0.50												
CML115	0.11	0.11	0.03	0.27	0.36	0.29											
CML196	0.13	0.17	0.11	0.23	0.41	0.23	0.32										
CML465	0.13	0.08	0.14	0.14	0.19	0.11	0.14	0.40									
LM13	0.10	0.08	0.05	0.08	0.18	0.14	0.20	0.27	0.30								
DH2012	0.08	0.08	0.05	0.02	0.10	0.05	0.14	0.08	0.21	0.29							
HKI162	0.08	0.05	0.08	0.08	0.05	0.05	0.05	0.14	0.14	0.19	0.22						
HKI323B	0.00	0.02	0.05	0.05	0.02	0.02	0.05	0.02	0.08	0.16	0.19	0.20					
HKI586	0.02	0.06	0.09	0.15	0.08	0.00	0.00	0.08	0.02	0.05	0.11	0.15	0.26				
HKI1105	0.00	0.06	0.03	0.12	0.05	0.03	0.09	0.09	0.09	0.08	0.08	0.18	0.03	0.19			
CML161	0.02	0.14	0.02	0.08	0.08	0.08	0.08	0.11	0.05	0.05	0.08	0.11	0.08	0.08	0.23		
CML165	0.02	0.09	0.00	0.09	0.05	0.03	0.09	0.09	0.06	0.17	0.11	0.06	0.09	0.15	0.12	0.52	
CML163	0.05	0.02	0.02	0.05	0.02	0.00	0.00	0.02	0.02	0.00	0.00	0.05	0.11	0.20	0.15	0.25	0.27

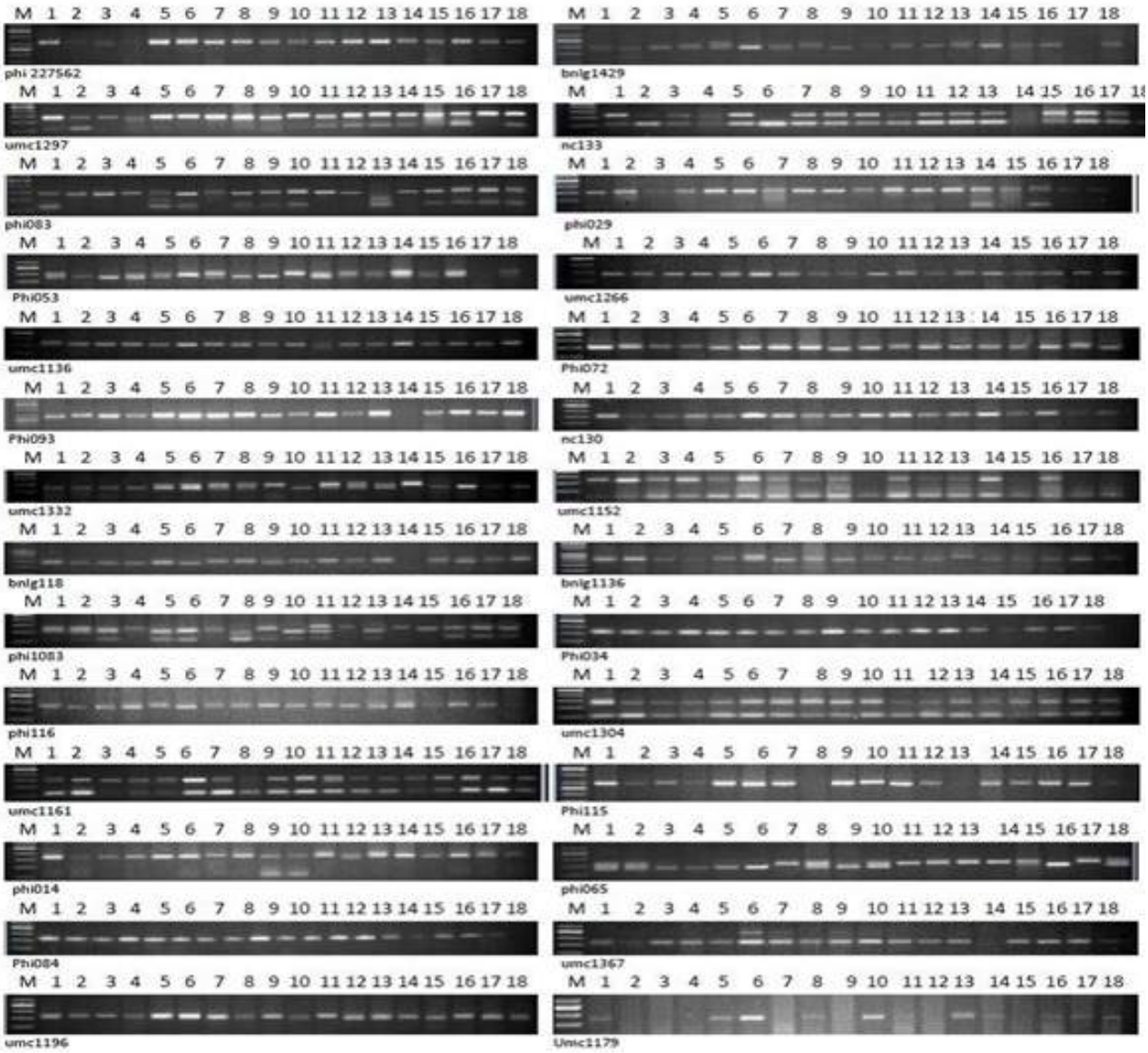

Fig. 1. Primer pairs dependent amplification of region of genomic in eighteen maize inbred lines used in the study

1. CML 467	4. CML470	7. CML115	10. LM13	13. HKI323B	16. CML161
2. CML468	5. CML471	8. CML 196	11. DH2012	14. HKI586	17. CML165
3. CML469	6. CML373	9. CML465	12. HKI162	15. HKI1105	18. CML163

tri-genotypic. Cluster B was mono-genotypic and cluster D was di-genotypic, whereas cluster E was multi-genotypic. At 50 and 75 similarity units, the cluster A was further sub-divided into cluster AI comprising of two entries, namely, CML 161and CML165, whereas cluster AII consisting of CML 163. Cluster B was mono-genotypic consisting of HKI1105. Cluster C was sub-divided into CI, CII and CIII accommodating LM13, DH2012 and HKI162, respectively. Cluster D was sub-divided into DI and DII comprising of HKI586 and HKI323B, respectively.

Keeping phenon line at 50 similarity units, the cluster E was divided into EI and EII. At 75 similarity units, sub-cluster EI was further sub-divided into sub-sub cluster EIa accommodating CML471 and CML373, sub-sub-cluster EIb containing CML470 and sub-sub-cluster EIc accommodating CML115. Similarly, EII was sub-divided into two mono-genotypic sub-sub-clusters EIIa and EIIb containing CML465 and CML196, respectively. At 50 similarity units, cluster F was divided into FI and FII. At 75 similarity units, sub-cluster FI was further sub-divided into FIa and FIb incorporating CML469

and CML468, respectively. Mono-genotypic sub-cluster FII contained CML467 (Table 4). Amongst pair-wise combinations of entries under evaluation in the present study, the magnitude of similarity coefficient between CML 165 and CML 161 was found to be the maximum, reflecting close similarity of these two inbred lines with respect to the regions of the genome targeted by the primer pairs used for amplification in the present study while CML 163 and CML467 appeared as the most diverse genotypes.

Principal component analysis

Principal component analysis was performed to examine the relationship among 18 inbred lines. Five principal components (Vectors) explained 18.64, 10.81, 8.43, 7.30 and 6.23 percentage of variability, respectively (Table 5). Principal component analysis represented the relative position and clustering pattern of inbred lines (Fig. 3), confirming the results obtained by cluster analysis. Several research workers have studied (Pebendon *et al*., 2008; Kashiani *et al*., 2013) divergence analysis on the basis of principal component analysis.

Obviously, the results of the present study clearly indicated that utilization of 28 microsatellite primer pairs in the analysis of maize inbred lines revealed a remarkably higher level of genetic polymorphism, which allowed unique genotyping of eighteen entries included in the analysis. The markers utilized in the present study were sufficient for discrimination and unambiguous classification of inbred lines.

Heterotic grouping on the basis of molecular markers

Parental line selection and breeding strategies for the successful and efficient hybrid development program are greatly facilitated by heterotic grouping of parental lines. Assigning lines to heterotic groups assists in avoiding the development and evaluation of crosses that should be discarded, allowing maximum heterosis to be exploited by crossing inbred lines belonging to different heterotic groups (Terron *et al*., 1997). Several approaches have been suggested and adopted for the classification of inbred lines into heterotic groups. Recently, microsatellite markers have been developed and used as a tool to assess the genetic diversity among inbred lines of maize and to assign them to different heterotic groups (Rajendran *et al*., 2014). The advantage of using molecular markers is the possibility of evaluating only the more promising crosses between the most divergent lines.

Table 4. Composition of clusters based on similarity coefficients for 28 primer pairs used for amplification in eighteen inbred lines of maize

Clusters identified at different phenon levels*			Entries included in each clusters
25	**50**	**75**	
A (3)	AI (2)	AI (2)	CML161,CML165
	AII (1)	AII (1)	CML163
B (1)	B (1)	B (1)	HKI1105
C (3)	CI (1)	CI (1)	LM13
	CII (1)	CII (1)	DH2012
	CIII (1)	CIII (1)	HKI162
D (2)	DI (1)	DI (1)	HKI586
	DII (1)	DII (1)	HKI323B
E (6)	EI (4)	EIa (2)	CML471,CML373
		EIb (1)	CML470
		EIc (1)	CML115
	EII (2)	EIIa (1)	CML465
		EIIb (1)	CML196
F (3)	FI (2)	FIa (1)	CML469
		FIb (1)	CML468
	FII (1)	FII (1)	CML467

Figures in parenthesis indicate number of entries in different clusters
*: Phenon levels indicate 25, 50 and 75 units of similarity coefficient

Table 5. Different characteristics features of principle components having Eigen value > 1

Component	Eigen value	Variance(%)	Cumulative Variance (%)
PC1	3.35695096	18.6497	18.6497
PC2	1.94598945	10.8111	29.4608
PC3	1.51797503	8.4332	37.8940
PC4	1.31469729	7.3039	45.1978
PC5	1.12140056	6.2300	51.4279

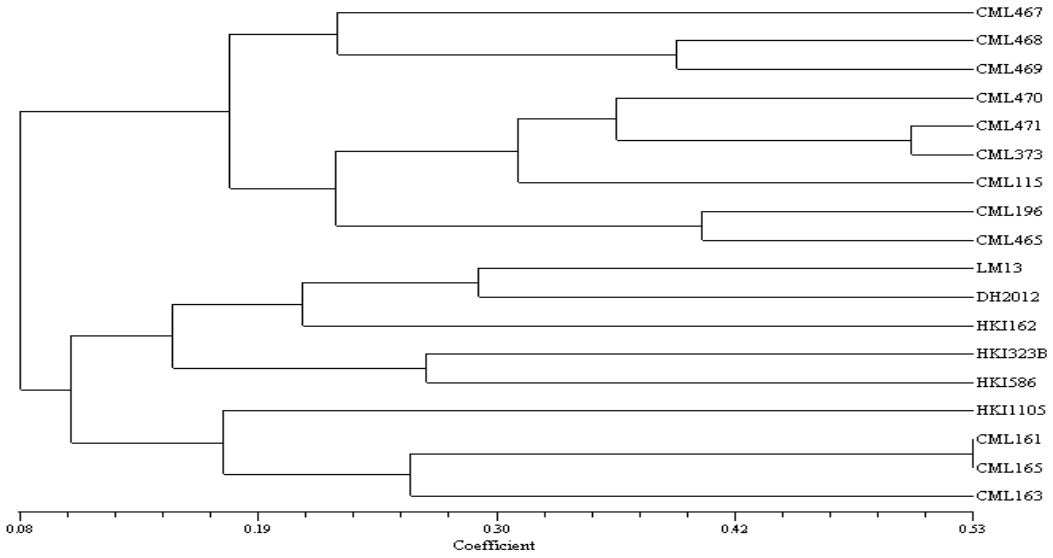

Fig. 2. Dendrogram based on Dice similarity coefficients for 28 microsatellite primer pairs among 18 maize inbred lines

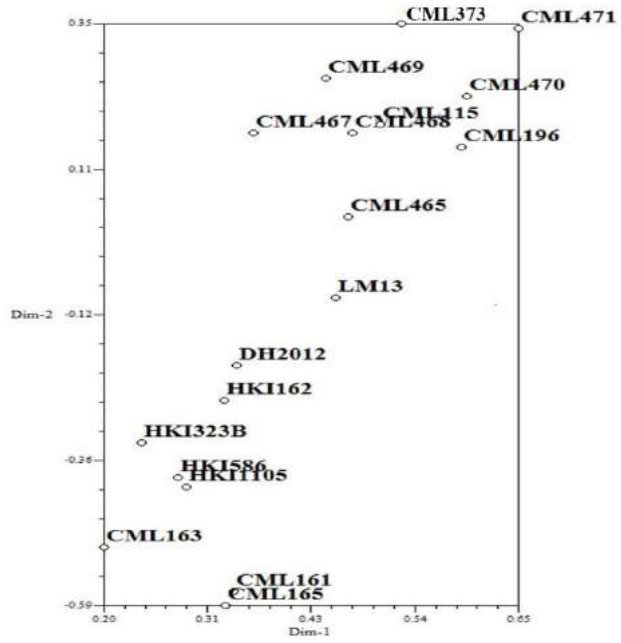

Fig. 3. Plot of the first two axes of a principal component analysis among 18 inbred lines studied

Using the matrix of genetic similarity, the cluster analysis grouped the eighteen inbred lines into four broad groups (Table 6). All the CML lines except CML161, CML165 and CML163 were included in group 1 in which there were nine inbred lines, namely, CML470, CML471, CML373, CML115, CML196 and CML465, CML467, CML468 and CML469. Heterotic group 2 consisted of inbred lines HKI323B and HKI586. Similarly, heterotic group 3 comprised of inbred lines LM13, DH2012 and HKI162. Heterotic group 4 accommodated the inbred lines HKI1105, CML161, CML165 and CML163. Microsatellite markers based discrimination and classification of inbred lines was found highly effective in heterotic grouping simply because remarkably greater number of inbred lines procured from the same source were placed in the same heterotic group.

Table 6. Heterotic grouping of inbred lines on the basis of molecular markers

Heterotic groups	Inbreds in groups
Group 1	CML470,CML471,CML373,CML115,CML196,CML465
	CML467, CML468, CML469
Group 2	HKI323B,HKI586
Group 3	LM13,DH2012,HKI162
Group 4	HKI1105,CML161,CML165,CML163

Conclusion

Understanding the genetic divergence pattern and relationship among inbred lines at genotypic level is of great importance for formulation and implementation of a systematic breeding plan for the purpose of further genetic improvement of maize. Altogether 296 allelic variants were detected amongst amplified products generated with 28 primer pairs. A total of 49 loci were assigned to 28 primer pairs with an average of 6.04 alleles per locus Considering the number of alleles generated by different primer pairs in conjunction with the level of polymorphism, the primers umc1297, phi053, umc1266, phi093, bnlg118, phi034, phi115, phi065 and phi084 appeared to be more informative primers. Among the inbred lines under molecular characterization, CML163

and CML467 appeared as the most diverse genotypes. A remarkably higher level of genetic polymorphism was revealed by the use of 28 microsatellite markers. Cluster analyses revealed that inbred lines CML 468 and CML469 are closely related to each other. Remarkably greater extent of similarity was also noticed between inbred lines HKI323B and HKI586.

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