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Molecular characterization and genetic diversity analysis of selected maize inbreds using SSR markers

Authors Info

T. Sharma[1,2]*, A. Kumar[3,4], S. C. Dwivedi[1] and R. P. Vyas[2]

[1]School of Biosciences, Suresh Gyan Vihar University, Jaipur-302 017, India

[2]Department of Genetics and Plant Breeding, CSAUAT, Kanpur-208 002, India

[3]UP Council of Agricultural Research (UPCAR), Lucknow- 226 010, India

[4]Department of Botany, Ch. Charan Singh (PG) College, Heonra (Saifai), Etawah- 206 002, India

*Corresponding Author Email : trivenidevsharma@gmail.com

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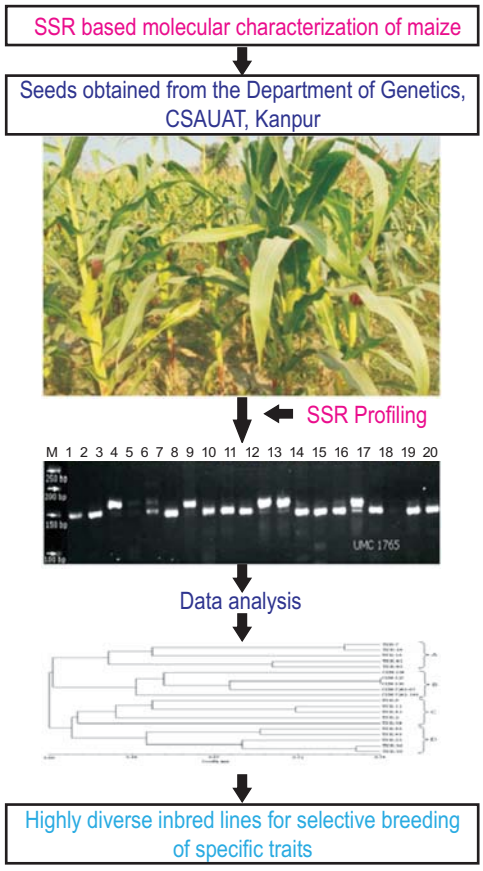
Abstract

Aim : The aim of the current study was to analyze the molecular diversity and genetic relationship among selected maize inbreds using SSR markers.

Methodology : Genomic DNA of individual inbreds was isolated by using the standard CTAB procedure (Doyle and Doyle, 1987) with some modifications. Thermal cycler was used to perform the polymerase chain reactions. 50ng of genomic DNA was used in 10 µl total volume with a final concentration of 10X *Taq* buffer, $MgCl_2$ (50 µM), each primers (10 µM), nucleotides (2.5 mM) and 1 U Red *Taq* DNA polymerase. Amplified PCR products were resolved by electrophoresis on horizontal gel (3.0% Agarose gel) system using 1x TBE Buffer. The gels were photographed with CCD camera attached to a gel documentation system.

Results : In total, 191 alleles were detected which showed a range of 2 to 6 alleles per marker and an average value of 3.82 alleles per locus. Polymorphic information content (PIC) value showed a range of 0.050 (umc1069) to 0.817 (bnlg1823) with an average value of 0.585 indicating the efficiency of markers to study the level of polymorphism available in the maize inbred lines. The value of genetic similarity coefficients for the twenty maize inbreds based on 50 SSR markers ranged from 0.51 to 0.74. The maximum value of similarity coefficient was observed between CIM-127 vs CIM-130, whereas the minimum value of it was between TSK-7 vs TSK-2, TSK-41 vs TSK-2. Cluster analyses, done using matrices of similarity in the NTSYS-pc software, classified studied inbred lines into four distinct clusters, Cluster A, B, C and D comprising of 5, 5, 5 and 5 maize inbred lines, respectively.

Interpretation : In this study, PIC values indicated a good efficiency of markers for studying the polymorphism level available in studied inbred lines. High level of diversity among the inbreds detected with SSR markers indicated their suitability for further breeding programs.



Introduction

Maize (*Zea mays* L.) is among the most important cereal crops in the world and a highly versatile and high potential crop having diploid chromosome number 20. Maize kernels contain 60 to 68% starch and 7 to 15% protein. It can be consumed as boiled, roasted vegetable by humans as well as a feed for livestock. In India, maize is the third most important cereal after rice and wheat that provides food, feed, fodder and serves as a source of raw material for the number of industrial products, *viz*. starch, oil, protein, food sweeteners, cosmetics, bio-fuel, etc. Genetic diversity in available germplasm plays a key role for future breeding progress. Study of genetic diversity is important in maize breeding program for the selection of suitable diverse parents to obtain heterotic hybrid as well as for germplasm characterization and conservation. Molecular markers being DNA based in nature produce more accurate and repeatable results compared to morphological data.

DNA-based molecular markers are a more precise and environment-independent way to assess the extent of genetic variation of a particular species. Among available DNA-based markers, Simple Sequence Repeat (SSR) markers are highly informative and easy detectable with PCR. They occur frequently in plant genomes, showing an extensive variation in different individuals and accessions (Sharma *et al*., 2010; Comertpay *et al.*, 2012). Since SSR based molecular markers are co-dominant, multi-allelic, highly polymorphic and randomly distributed throughout the genome (Barcaccia *et al*., 2006), they are widely used for assessing maize genetic diversity (Nikolić *et al*., 2015; Salami *et al*., 2016). SSR markers have proved to be useful in various analysis like genetic diversity studies (Hidalgo *et al*., 2013), evolutionary studies (Zheng *et al*., 2013) and genetic map construction (Sa *et al*., 2012). These markers were frequently applied in different molecular genetic studies in maize for assessment of molecular diversity and molecular characterization of inbreds (Shehata *et al*., 2009; Suteum *et al*., 2014) and landraces (Ignjatovic-Micic *et al*., 2013; Molin *et al*., 2013). The objective of this study was genetic diversity and relationship analysis among 20 selected elite maize inbred lines using SSR markers.

Materials and Methods

Plant materials : Seeds of twenty inbred lines of maize were obtained from the Department of Genetics, C. S. Azad University of Agriculture and Technology, Kanpur (India) (Table- 1). The seeds were sown at experimental farm of CSA University of Agriculture and Technology, Kanpur. Healthy seeds of maize inbreds were sown in two replicates using standard agronomical practices. Fresh young leaves of maize were collected to isolate genomic DNA.

Genomic DNA extraction : Genomic DNA of individual genotypes was isolated using the standard CTAB (Doyle and Doyle, 1987) with some modifications. About 5 g leaf materials was collected, frozen and was crushed to a fine powder adding in a pestle and mortar. The powder was transferred to polypropylene centrifuge tubes (50m) containing 20 ml pre-warmed extraction buffer (100 mM Tris, 20 mM EDTA, 1.4 M NaCl and 2% CTAB) which was maintained at 65°C. The content was mixed vigorously. The suspension was incubated (65°C) for one hour with intermittent mixing. Chloroform-isoamyl alcohol (0.6 volume) was added and mixed gently for 5 min by inversion. The content was centrifuged at 15,000 rpm for 10 min at room temperature. Aqueous phase was transferred to fresh centrifuge tubes with a wide bore pipette and 2/3 volume of isopropanol was added to it. The content was mixed by quick gentle inversion. The precipitated DNA was spooled out using a disposable pipette tip and washed two times with 70% ethanol. The pellet was dried under vacuum and dissolved in minimum volume of TE buffer and stored after that. To remove RNA, DNA was treated with 50µg RNAse for one hour at 37°C and stored at 4°C until further use.

Table 1 : List of inbred lines of maize

Inbred line	Inbred line
TSK-7	TSK-41
TSK-9	TSK-43
TSK-11	TSK-45
TSK-14	TSK-48
TSK-16	TSK-49
TSK-22	CIM-7242-67
TSK-33	CIM-7242-149
TSK-34	CIM-130
TSK-36	CIM-127
TSK-39	CIM-104

SSR markers, PCR Amplification and gel electrophoresis : 50 SSR primers were used to analyze genomic DNA of twenty inbreds. The primer sequences were obtained from Integrated DNA Technology, USA. The polymerase chain reactions were performed using thermal cycler (Long Gene). 50 ng of genomic DNA was used in 10 µl total volume with a final concentration of 10X *Taq* buffer, $MgCl_2$ (50 µM), primers (10 µM), 2.5 mM of each nucleotide and 1 U Red *Taq* DNA polymerase (Bangalore GeNei, India).

The PCR was programmed for the following steps: initial denaturation at 94°C for 4 min and during subsequent 35 cycles, each with denaturation at 94°C for 1 min, primer annealing (58°C) for 1min and primer extension at 72°C for 2 min. The extension step was performed at 72°C for 7 min. Reactions were stopped with 2.0µl of loading dye (6x loading dye).

Amplified products were resolved by electrophoresis on horizontal gel (3.0% Agarose) system using 1x TBE Buffer. The gel was prepared by melting agarose and 0.1gml^{-1} Ethidium Bromide (5µl EtBr in 100ml of Agarose gel) was added. The gels were run at 50 volt for 3 hrs.

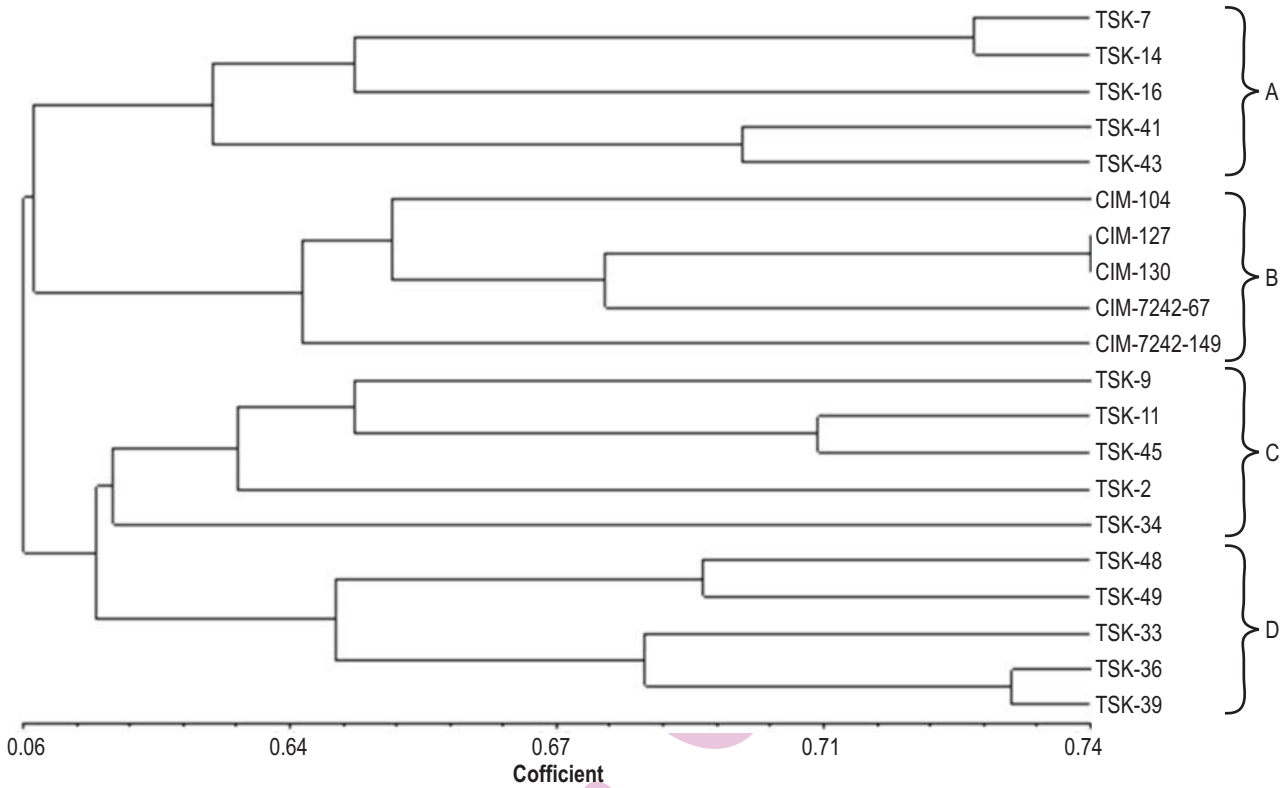


Fig. 1 : UPGMA based cluster analysis of 20 maize inbred lines using SSR primers

Data scoring and analysis : The gel was photographed using CCD camera attached to a gel documentation system with the Quantity One software (Alpha Innotech). Scoring was done manually for each of the gel sections allele was determined base on the positions of the bands. Band pattern for each marker was recorded for each genotype by assigning a letter to each band. Alleles were numbered as 'a_1', 'a_2' etc based on their size. In the data matrix, presence of a band was represented by '1' and absence by '0'. The marker data were entered directly into an excel spreadsheet with the microsatellite allele under rows and genotype under columns.

PIC is synonymous with the term "gene diversity" as described by Weir (1996). The PIC valve ranging from '0' to '1' is an indication of discriminatory power of marker where '0' denotes monomorphic nature of the marker and '1' describe it to be highly discriminative with many alleles in equal frequency. In the present investigation, SSR data was used for cluster analysis. Genetic similarity based on SSR data can be calculated for all possible pairs of genotype using various coefficients. Jaccard´s coefficients (J), calculated as $J = N_{ij} / (N_{ij} + N_i + N_j)$ was used to estimate genetic similarities, where N_{ij} represents the number of bands present in the both individual, N_i depicts the total number of bands in the individual i and N_j is the total number of bands in the individual j. The similarity matrix was worked out using NTSYS-pc version 2.2 to produce an agglomerative hierarchical classification (Rohlf, 2000), by employing UPGMA (Unweighted Paired Group method using Arithmetic Mean) method with average linkage (Sneath and Sokal, 1973).

Results and Discussion

Simple sequence repeat markers (SSR) were used to assess the genetic divergence among 20 inbreds of maize (*Zea mays* L.). Based on the specificity of genotype of a plant, a particular DNA profile can be ascribed to a particular plant. Analysis of the distribution of the SSR alleles across the maize lines revealed that almost all the alleles were shared by *Zea mays*.. Analysis of PIC values revealed that all of the SSR markers studied were found to be highly polymorphic. The PIC value ranged from 0.050 to 0.817 with an average of 0.585 among the studied primers. All these primers were found to be polymorphic (Table 2). A total of 191 alleles were recorded at 50 SSR loci with an average of 3.82 alleles per locus with their distribution as 2 alleles in 12 primers, 3 alleles in 13 primers, 4 alleles in 6 primers, 5 alleles in 10 primers and 6 alleles in 9 primers. Maximum number of alleles (6 alleles) were observed in primers *viz*; bnlg1823, umc1136, umc2227, umc1015, bnlg2235, umc2383, bnlg1194, umc1705, umc1353 and minimum number of alleles (2 alleles) were observed in primers *viz*; umc2069,

Table2 : PIC values of SSR loci across 20 inbred lines of maize

Primers	Alleles	No. of accessions sharing alleles	Frequency of SSR alleles	No. of accessions showing amplification	No. of alleles	PIC
umc1078	a_1	8	0.228	19	5	0.771
	a_2	10	0.298			
	a_3	8	0.210			
	a_4	2	0.052			
	a_5	9	0.210			
bnlg1823	a_1	4	0.205	17	6	0.817
	a_2	5	0.205			
	a_3	3	0.088			
	a_4	5	0.176			
	a_5	5	0.235			
	a_6	2	0.088			
umc2120	a_1	1	0.017	20	4	0.504
	a_2	5	0.092			
	a_3	18	0.658			
	a_4	9	0.233			
umc1136	a_1	13	0.403	19	6	0.751
	a_2	6	0.149			
	a_3	2	0.044			
	a_4	3	0.105			
	a_5	9	0.219			
	a_6	3	0.078			
umc1746	a_1	12	0.333	20	4	0.738
	a_2	7	0.233			
	a_3	6	0.167			
	a_4	8	0.267			
umc2227	a_1	3	0.037	20	6	0.774
	a_2	7	0.096			
	a_3	9	0.133			
	a_4	9	0.125			
	a_5	20	0.304			
	a_6	20	0.304			
umc1015	a_1	5	0.117	20	6	0.795
	a_2	9	0.187			
	a_3	6	0.133			
	a_4	8	0.154			
	a_5	4	0.071			
	a_6	14	0.337			
bnlg2235	a_1	5	0.101	19	6	0.804
	a_2	11	0.228			
	a_3	8	0.232			
	a_4	4	0.078			
	a_5	5	0.101			
	a_6	10	0.258			
bnlg1847	a_1	6	0.202	19	4	0.649
	a_2	8	0.307			
	a_3	11	0.465			
	a_4	1	0.026			
phi109275	a_1	5	0.092	20	5	0.745
	a_2	4	0.083			
	a_3	10	0.192			
	a_4	8	0.267			
	a_5	11	0.367			

umc2204	a_1	6	0.158	20	5	0.744
	a_2	4	0.108			
	a_3	6	0.158			
	a_4	10	0.417			
	a_5	5	0.158			
umc2383	a_1	3	0.050	20	6	0.760
	a_2	8	0.167			
	a_3	7	0.125			
	a_4	5	0.125			
	a_5	15	0.408			
	a_6	7	0.125			
bnlg1831	a_1	3	0.050	20	5	0.684
	a_2	5	0.092			
	a_3	13	0.308			
	a_4	14	0.450			
	a_5	3	0.100			
bnlg1367	a_1	3	0.075	20	4	0.628
	a_2	2	0.075			
	a_3	9	0.400			
	a_4	11	0.450			
bnlg1194	a_1	2	0.031	19	6	0.783
	a_2	10	0.337			
	a_3	5	0.110			
	a_4	5	0.140			
	a_5	6	0.188			
	a_6	6	0.193			
phi059	a_1	4	0.096	19	3	0.506
	a_2	17	0.649			
	a_3	8	0.254			
umc1705	a_1	4	0.092	20	6	0.607
	a_2	7	0.146			
	a_3	4	0.079			
	a_4	20	0.596			
	a_5	2	0.050			
	a_6	2	0.037			
umc1907	a_1	4	0.139	18	3	0.440
	a_2	3	0.139			
	a_3	15	0.723			
bnlg1043	a_1	10	0.315	19	5	0.742
	a_2	4	0.114			
	a_3	7	0.315			
	a_4	2	0.035			
	a_5	6	0.219			
umc2069	a_1	4	0.117	17	2	0.209
	a_2	17	0.882			
umc1141	a_1	14	0.360	19	4	0.693
	a_2	2	0.043			
	a_3	14	0.394			
	a_4	10	0.149			
umc1353	a_1	2	0.039	19	6	0.679
	a_2	3	0.065			
	a_3	14	0.360			
	a_4	13	0.425			
	a_5	4	0.083			
	a_6	2	0.026			

bnlg1346	a_1	3	0.095	14	5	0.772
	a_2	4	0.155			
	a_3	8	0.333			
	a_4	4	0.202			
	a_5	5	0.214			
umc1844	a_1	12	0.272	19	3	0.661
	a_2	14	0.324			
	a_3	15	0.403			
bnlg1070	a_1	6	0.214	14	4	0.735
	a_2	9	0.357			
	a_3	6	0.178			
	a_4	6	0.250			
bnlg1017	a_1	2	0.037	18	3	0.535
	a_2	14	0.509			
	a_3	13	0.453			
bnlg1117	a_1	8	0.267	15	3	0.605
	a_2	3	0.200			
	a_3	12	0.533			
phi114	a_1	15	0.394	19	3	0.584
	a_2	17	0.500			
	a_3	5	0.105			
umc1002	a_1	3	0.138	18	3	0.449
	a_2	4	0.194			
	a_3	13	0.667			
umc1055	a_1	3	0.156	16	2	0.266
	a_2	14	0.843			
umc1254	a_1	7	0.368	19	2	0.467
	a_2	12	0.631			
umc1276	a_1	7	0.225	20	3	0.648
	a_2	11	0.425			
	a_3	12	0.350			
umc1297	a_1	2	0.069	18	5	0.730
	a_2	9	0.194			
	a_3	9	0.194			
	a_4	5	0.125			
	a_5	9	0.421			
umc1350	a_1	6	0.357	14	3	0.645
	a_2	5	0.214			
	a_3	9	0.428			
umc1765	a_1	7	0.263	19	2	0.390
	a_2	16	0.736			
umc1655	a_1	15	0.442	20	3	0.612
	a_2	4	0.142			
	a_3	14	0.417			
umc1766	a_1	7	0.340	18	3	0.655
	a_2	7	0.340			
	a_3	7	0.340			
umc1858	a_1	5	0.187	16	2	0.307
	a_2	15	0.812			
umc1871	a_1	12	0.445	18	2	0.493
	a_2	14	0.556			
bnlg1189	a_1	10	0.468	16	2	0.500
	a_2	11	0.531			
umc2071	a_1	9	0.464	14	2	0.499
	a_2	10	0.535			

umc2175	a_1	6	0.184	19	3	0.618
	a_2	12	0.500			
	a_3	11	0.315			
bnlg1006	a_1	5	0.131	19	5	0.785
	a_2	7	0.210			
	a_3	8	0.210			
	a_4	4	0.149			
	a_5	9	0.298			
umc1415	a_1	19	0.973	19	2	0.054
	a_2	1	0.026			
umc1065	a_1	3	0.105	19	5	0.660
	a_2	7	0.184			
	a_3	5	0.210			
	a_4	16	0.500			
	a_5	2	0.052			
bnlg1037	a_1	19	0.815	19	2	0.303
	a_2	7	0.184			
umc1035	a_1	8	0.245	19	5	0.741
	a_2	7	0.140			
	a_3	13	0.394			
	a_4	5	0.140			
	a_5	2	0.078			
umc1069	a_1	20	0.975	20	2	0.050
	a_2	1	0.025			
umc1569	a_1	20	0.700	20	2	0.150
	a_2	12	0.600			
umc2115	a_1	3	0.095	14	3	0.518
	a_2	12	0.631			
	a_3	6	0.274			
Total					191	29.255
Average (Mean)					3.82	0.585

umc1055, umc1254, umc1765, umc1858, umc1871, bnlg1189, umc2071, umc1415, bnlg1037, umc1069, umc1569. Summarized data regarding the allele number, allele frequencies and allele distribution for various primers studied on 20 maize inbred lines are provided in Table2.

PIC values were estimated for different SSR loci based on the allele frequencies. Range of PIC value was found to be from 0.050 to 0.817 with an average of 0.585. On the basis of alleles, number of sharing alleles and their frequencies, the highest value of PIC (0.817) was found for the primer bnlg1823 because of well distributed presence of six alleles across the inbred lines followed by 0.804 for primer bnlg2235, 0.795 in primer umc1015, 0.785 with primer bnlg1006, 0.783 for primer bnlg1194 and 0.774 with primer umc2227. The lowest value of PIC (0.050) was shown by primer umc1069. The reason for low PIC value may be the poor distribution of alleles in the genome. Various workers have already reported similar results in maize (Jambrovic *et al*., 2008; Shehata *et al*., 2009; Suteu *et al*., 2013; Nikolić *et al*., 2015). Lu and Bernardo (2001) reported an average of 4.9 alleles for 83 SSR loci in 40 U.S. maize inbred lines. Similarly, Senior *et al*., (1998) reported an average of 5.0 alleles per locus. Warburton *et al*., (2002) amplified 416 bands in CIMMYT maize inbred lines, with an average number of 4.9 and a range of 2 to 14 alleles per locus among the 85 SSR loci studied. On the other hand, Liu *et al*., (2003) reported average 21.7 alleles per locus at 94 SSR loci studied among 260 US inbreds.

However, in the present study low and high alleles showed polymorphism level on the basis of highest and lowest amplification. On the basis of PIC values, the genotypes sharing alleles and distribution of alleles in the genome can be identified. PIC values ranged from 0.050 to 0.817 with an average of 0.585 indicates a considerable efficiency of markers for studying the polymorphism level in maize inbred lines. SSRs undoubtedly represent a useful molecular tool that, either alone or in combination with additional approaches defining the internal genetic relationships among genotypes of large germplasm collections. The robustness of the systems of identification based on SSRs has been progressively implemented by augmenting the number of SSRs that are concomitantly used rather than increasing the length of their core, repeated unit (Gavazzi *et al.,* 2016).

Range of genetic similarity coefficients for twenty maize inbred lines based on 50 SSR markers was from 0.51 to 0.74. The

highest similarity coefficient value (74%) was observed between CIM-127 vs CIM-130 and the lowest similarity coefficient value (51%) was observed between TSK-7 vs TSK-2. For TSK-41 vs TSK-2, it was 51%. The resulting dendrogram classified studied inbred lines into 4 distinct clusters *i.e*. Cluster A, B, C and D comprising of 5 inbred lines each (Fig.1).

Inbreds in cluster A were further grouped into two subclusters *viz*; A-1 and A-2. Subcluster A-1 comprised of three maize inbred lines namely TSK-7, TSK-14 and TSK-16. In subcluster A-1, the maximum similarity coefficient occurred (0.72) between TSK-7 vs TSK-14 and the minimum similarity coefficient (0.61) occurred between TSK-7 vs TSK-16. Subcluster A-2 consisted of only 2 maize inbred lines namely TSK-41 and TSK-43, which showed the similarity coefficient value of 0.69. Cluster B consisted of five maize inbred lines namely CIM-104, CIM-127, CIM-130, CIM-7242-67 and CIM-7242-149. In this cluster, the maximum (0.74) similarity coefficient was observed between CIM-127 vs CIM-130 and the minimum similarity coefficient (0.61) was found between CIM-104 vs CIM-7242-149 and CIM-130 vs CIM-7242-149. Inbreds in cluster C were grouped into two subclusters *viz*; C-1 and C-2. Subcluster C-1 consisted of three maize inbred lines namely TSK-9, TSK-11 and TSK-45. In Subcluster C-1, the maximum similarity coefficient (0.70) was observed between TSK-11 vs TSK-45 and the minimum similarity coefficient (0.62) occurred between TSK-9 vs TSK-11. Subcluster C-2 consisted of only two inbreds namely TSK-2 and TSK-34, which showed the similarity coefficient value of 0.60. Cluster D's inbreds were grouped into two subclusters *viz*., D-1 and D-2. Subcluster D-1 consisted of only two maize inbred lines namely TSK-48 and TSK-49, which showed the similarity coefficient value of 0.69. Subcluster D-2 consisted of three maize inbred lines namely TSK-33, TSK-36 and TSK-39. In subcluster D-2, the maximum similarity coefficient (0.73) occurred between TSK-36 vs TSK-39 and the minimum similarity coefficient (0.66) occurred between TSK-33 vs TSK-39. SSR markers have been used to investigate genetic diversity and relationships several important crops like maize (Senior *et al*., 1998), rice (Saini *et al*., 2004), wheat (Plaschke *et al*., 1995), soybean (Rongwen *et al*., 1995) and rice bean (Muthusamy *et al.,* 2008). Identifying genetic subpopulations provides key insight into a sample's ecology and evolution (Bryc *et al*., 2010; Glover *et al*., 2012; Moore *et al*., 2014), reveals ethnic variation in disease phenotypes (Moreno-Estrada *et al*., 2014), and reduces spurious correlations in genome-wide association studies (Galanter *et al.,* 2012; Behr *et al.* 2016).

SSR markers have been increasingly used as efficient tools to determine genetic diversity and relationship among maize inbred lines. In addition, SSR markers used in this study were highly polymorphic and revealed differences in maize inbred lines. The presence of high level of diversity among the inbred lines as deciphered by SSR markers indicated their suitability for breeding purposes.

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