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SSR Marker Assay - Based Selection of Parents for the Development of Multiparent Segregating Population in Maize (*Zea mays* L.)

SHRIKRISHNA P. DESAI[1], H. C. LOHITHASWA[2], R. L. RAVIKUMAR[3] AND A. MOHAN RAO[4]
[1&4]Department of Genetics and Plant Breeding, GKVK, [2]GPB, CoA, VC Farm, Mandya, [3]Plant Biotechnology, GKVK, UAS, GKVK, Bengaluru - 560 065
e-Mail : lohithaswa.chandappa@gmail.com

ABSTRACT

Assessment of molecular diversity is fundamental to judge the utility of genotypes in hybridization programme along with the presence of essential characteristic features in them. A panel of 22 inbred lines with useful traits was assembled and was characterised using simple sequence repeats (SSR) in order to assess the diversity. Out of 96 SSRs, 17 amplified PCR products across all the genotypes. The average gene diversity obtained using 17 SSR markers among 22 inbred lines was 0.30 and the SSR loci umc1139 showed the highest gene diversity of 0.47. The highest PIC value of 0.36 was exhibited by the SSR umc1139. Based on molecular diversity and phenotypic response to major biotic and abiotic stresses, eight founder parents were selected namely PDM4341, CML451, CAL1443, VL109545, SKV50, CAL1518, CM202 and CM212. Further, the selected eight founder parents were analysed with 77 SSR markers. A total of 190 alleles were detected wherein the number of alleles scored per SSR locus ranged from two to five (bnlg 1360). This study further led to the identification of six informative SSR loci detecting nine unique alleles to each of the genotypes. The average polymorphic information content obtained was 0.384, with a range of 0.195 (phi097) to 0.746 (bnlg1360). The dissimilarity matrix varied from 0.019 to 0.583 between the genotypes CML 451 and SKV 50 and these two fell in different clusters. The SSR-based diversity detected in this study could be utilized for the exploitation of heterosis and development of genetically diverse multiparental populations in understanding the genetics and dissecting the traits like resistance to many biotic and abiotic stresses.

Keywords : Maize, Founder parents, Molecular diversity, Multiparent segregating population

A major advantage for plant breeder lies in the ability to develop well-structured experimental populations. Such populations are derived with systematic pedigree from the well characterized founder parents. Traditional experimental population uses two founder parents with contrasting phenotypes to dissect the genomic regions of interest. However, such population captures only a part of the variation affecting the trait of interest because of narrow genetic base resulting from two contrasting parents. Hence there is a need for the development of a much-sophisticated design which captures more phenotypic variation and recombination fraction and can efficiently map multiple genomic regions in a single mapping population. In order to overcome the weakness of existing biparental design, development of multi parent population is recommended. In these populations multiple founder parents (up to 12) are intermated for several generations prior to creating an inbred line, resulting in a diverse population whose genomes are fine-scale mosaics from all founder parents.

Success in developing multiparental population to dissect the genetic architecture depends on the careful choice of parents. The parental selection has to be done at two levels, firstly at the phenotypic level, contrasting for multiple traits of interest and at the DNA level which aids in identifying the genomic regions controlling the trait of interest. Hence, the knowledge on the genetic diversity and relationships among the founder parents is helpful in generating multiparental population which serves as a long-term genetic resource to derive superior inbred lines.

Among the morphological, biochemical and molecular methods (Mohammadi & Prasanna, 2003 and Pejic *et al.,* 1998) employed in assessment of genetic

diversity, the morphological method offers a cost-effective approach for selection of diverse inbred lines based on observable phenotype. At present, the genetic diversity of plants has been more accurately assessed following the implementation of methods that reveal polymorphism directly from the DNA levels. Among the various marker systems, RFLPs (Lee *et al*., 1989 and Bernardo, 1994) were the first molecular markers used in maize breeding programs. Among the different molecular markers such as AFLP, ISSR, RAPD and SSR, Simple sequence repeats (SSRs) have been successfully employed in maize genetic diversity studies because of their high reproducibility, multi-allelic nature, co-dominant inheritance, relative abundance and good genome coverage (Senior *et al.,* 1998; Bantte & Prasanna, 2003 and Ranatunga *et al.,* 2009). Hence, SSRs have been extensively used for diversity analysis in maize because they provide a high level of polymorphism (Smith *et al.,* 1997) and detects the polymorphism at the DNA level. This will make it easier to differentiate inbred lines into well-defined groups based on genetic distance. With this background an investigation was carried out to select the eight best diverse founder parents among the set of 22 maize elite inbred lines based on molecular diversity and also considering their response to biotic and abiotic stress resistance. Further, SSR marker-based assessment of diversity among eight selected founder parents was performed.

Material and Methods

Plant Material

A panel of 22 maize inbred lines (Table 1), as an initial material for development of multiparent segregating population, was evaluated over two seasons during summer 2016 and *kharif* 2016 for their responses to major biotic stress such as Turcicum leaf blight (TLB), Sorghum downy mildew (SDM) and Fusarium stalk rot (FSR) and to abiotic stress such as drought with combining ability. These 22 inbred lines differing in their response to different biotic and abiotic stresses were used for the assessment of SSR based molecular diversity.

Table 1

Panel of 22 maize elite inbred lines conferring resistance to major biotic and abiotic stress

Maize inbred line	Summer 2016	*kharif* 2016
Resistance to Turcicum leaf blight (Per cent disease severity)		
VL109545	27.53	28.5
CAL1443	22.22	33.33
SKV50	31.09	30.00
CML39	29.56	30.25
NAI125	22.22	33.33
NAI162 (Moderate resistant)	44.44	44.44
Resistance to Sorghum downy mildew (Per cent disease incidence)		
VL109138	8.46	7.23
SKV50	1.66	1.53
Moderate resistance to Fusarium stalk rot (Per cent disease severity)		
VL1012903	3.6	3.4
CM202	3.5	3.0
CM212	3.15	3.06
VL1218	5.6	5.1

Drought tolerant maize inbred lines

Inbred lines	Proline content	
	Control (µ moles/g)	Stress (µ moles/g)
CML451	0.039	0.0108
CML444	0.078	0.0108
CML505	0.0051	0.0180
CML151	0.042	0.0102
CML538	0.0015	0.0160
SKV50 (Susceptible)	0.0005	0.0021

Best inbred lines with significant GCA effects for yield and its attributing traits in desirable direction

Inbred lines	GCA effect	Trait
NAI137	0.112 ***	Plot yield
V351	0.12 **	Plot yield
CAL1518	1.27 **	Test weight
MAI105	0.115 ***	Plot yield
LM13	3.38 **	Kernels per row
AH7005	0.20 **	Plot yield

DNA Isolation

Total genomic DNA from each of the genotypes was extracted from the leaves of three-week old maize seedlings using modified CTAB protocol (Hoisington *et al.,* 1994). The DNA concentration was estimated using Nanodrop, the final concentration of genomic DNA was adjusted to 50 ng / microliter by diluting the DNA in TE buffer.

SSR Markers and PCR Amplification

In the present study, 96 SSR markers chosen from the maize genome database (Woodhouse *et al.,* 2021) were used for diversity analysis among 22 inbred lines. Total volume of the PCR reaction was 10 µl, consisting of 1µl DNA template, 1µl of each forward and reverse primer, 4 µl of Ampliqon Taq 2x Master mix and 3 µl of double distilled water. Cyclic amplification of the DNA was carried out using Eppendorf Master cycler gradient by adhering to the following amplification conditions : initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C 1min, 58 °C 45s, 72°C 1min and final extension at 72 °C for 7 min. The SSR-PCR products were resolved using 3 per cent agarose, in 1X TBE buffer contained in horizontal gel electrophoresis system and visualization of DNA polymorphisms was done through a gel documentation system.

SSR Data Analysis

The SSR allele sizes were determined using Image lab software (Bio Rad). Major allele frequency, number of alleles detected, gene diversity, heterozygosity and polymorphic information content (PIC) were computed using Power Marker 3.25 (Liu and Muse, 2005) and finally Dendrogram was constructed with the help of NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System (Rohlf, 1998) for 22 inbred lines and DARWIN (Dissimilarity Analysis and Representation for WINdows) software for eight parent diversity analysis using UPGMA (Unweighted Paired Group Method with Arithmetic Mean) algorithm method.

Criteria for Selection of Eight Founder Parents

Among the different strategies for selection of a number of founder parents like 2-way, 4-way, 8-way and 16-way, the optimum number of founder parents to initiate the development of multiparent segregating population is 8-way, which is a good compromise rather than going for 16-way, because increasing the number of founder parents from four to eight drastically increases the number of unique haplotypes that could be generated but after that trend of increasing the unique haplotypes decreases. Hence, the optimum number of founder parents to initiate the development of multiparent population is to start with eight founder parents to capture the greater recombination and diversity (Ladejobi *et al.,* 2016).

The main criteria for the selection of eight parents from a panel of 22 inbred lines was based on dendrogram constructed from SSR marker data using UPGMA algorithm method and also considering the difference in reaction to major biotic and abiotic stress resistance.

Assessment of SSR Based Diversity among Selected Eight Founder Parents and Identification of Unique Alleles

Further diversity analysis with more markers for increased accuracy was investigated to ensure the diversity among selected eight founder parents and to understand the relationship between the founder parents contrasting for biotic and abiotic resistance based on molecular diversity using 77 SSR markers. In addition, an attempt was made to identify the unique alleles among selected eight inbred lines which further aids in SSR based finger printing of elite inbred lines.

Results and Discussion

Prior to initiating population development, the selection of founder parents plays a significant role in the success of selection of desirable segregants. In addition to molecular diversity, the phenotypically diverse parents must be carefully chosen in order to complement and capture the diversity for multiple traits.

Out of 96 SSR markers studied, 17 markers produced PCR products in all the 22 genotypes and hence were considered for further analysis. Gene diversity is defined as the probability that two randomly chosen alleles from the population are different (Liu and Muse, 2005). The average gene diversity obtained using 17 SSR markers among 22 inbred lines was 0.30. The SSR loci namely phi126, bnlg1371, umc1085, bnlg1327, umc1152 and umc1139 exhibited the highest gene diversity (≥ 0.4). Among them umc1139 showed the highest gene diversity of 0.47 whereas, umc1319 was the lowest in gene diversity (0.09). Polymorphism information content (PIC) value reveals the informativeness of the SSR loci among 22 inbred lines and their ability to detect differences among the genotypes based on their molecular diversity. The PIC value for umc1139 was found to be 0.36 which is highest among the markers used. This marker showed the highest discriminating power among 22 inbred lines. The marker umc1319 recorded the lowest PIC value of 0.08 (Table 2).

TABLE 2
Gene diversity and PIC values of 17 SSR markers

Marker	Gene diversity	PIC
umc1139	0.47	0.36
umc1152	0.46	0.35
phi126	0.40	0.32
bnlg1371	0.40	0.32
umc1293	0.13	0.11
bnlg1422	0.35	0.29
phi382202	0.26	0.22
umc1319	0.09	0.08
mmc0111	0.20	0.17
bnlg1327	0.44	0.34
umc1928	0.21	0.18
bnlg1909	0.23	0.20
umc1884	0.38	0.30
umc1085	0.42	0.33
umc2181	0.23	0.20
umc1945	0.20	0.17
umc1719	0.28	0.23

Cluster Analysis among 22 Maize Inbred Lines

The dendrogram of 22 maize inbred lines was constructed using 17 polymorphic SSR markers using UPGMA clustering. When we cut the dendrogram at 0.43 similarity coefficient, it grouped the 22 inbreds into three major clusters namely A, B and C and further six sub clusters were observed (Fig. 1). The major cluster A consisted of two sub clusters namely A1 and A2. Eight genotypes namely, PDM4341, VL101293, VL10938, MAI105, NAI125, VL109545, LM13 and CML444 grouped into one cluster. In cluster B1, SKV50, NAI162, CM202, CM212, CML451 and CML505 were grouped together whereas, CAL1443, CML39, CML538 and NAI137 fell in cluster B2. The cluster C consisted of four genotypes namely, CAL1518, CML151, V351 and VL1218.

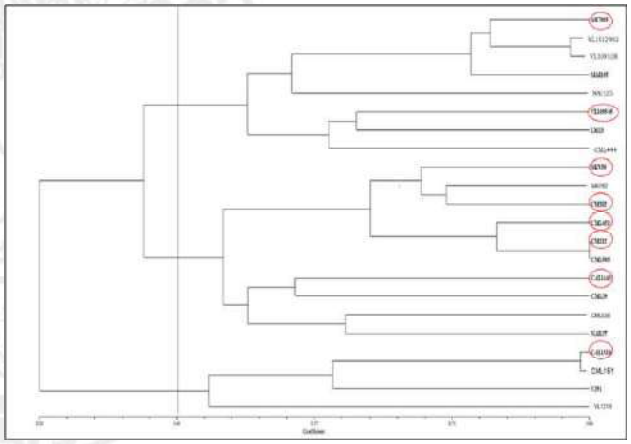

Fig. 1: SSR marker-based construction of dendrogram for 22 elite maize inbred lines

Selection of Eight Diverse Parents for the Development of Multi Parental Segregating Population

In order to select the eight diverse genotypes, we employed the two criteria, firstly based on marker diversity at the DNA level and secondly based on their phenotypic response to major biotic and abiotic stress resistance. Dendrogram depicts the hierarchical relationship between the individuals. All the genotypes that fall under one cluster is considered to be more similar than the genotypes that fall in different clusters. The 22 genotypes

formed the three major clusters and each major cluster consisted of two sub clusters. Genotypes were carefully chosen so that they fall in different sub clusters in order to capture greater diversity since our aim was to develop the population segregating for major diseases and drought tolerance.

Among the five genotypes in the first sub cluster A1, PDM4341 was chosen because the *gca* effect for plot yield was highly significant in the desirable direction. The inbred VL109545 was chosen from the second sub cluster A2 because of resistance to TLB (Table 1). B cluster comprised of B1 and B2 sub clusters and among the genotypes present in B cluster SKV50, CM202, CML451 and CM212 were chosen even though they were present in the same cluster because these genotypes have complementarity among them for resistance and susceptibility to multiple diseases and drought (Fig. 1). For instance, SKV50 is resistant to multiple diseases such as TLB, SDM and Polysora rust but susceptible to drought (Table 1). Whereas, CM202 and CM212 are moderately resistant to FSR but susceptible to TLB and SDM. To account for abiotic stress, CML451 was selected which is drought tolerant. The genotype CAL1443 which falls in B2 cluster was selected because of its resistance to Turcicum leaf blight. Finally, CAL1518 from Cluster C was selected which is having highly significant *gca* effect for test weight in desirable direction. Apart from selecting parents for biotic and abiotic stress resistance, weightage was also given for yield and its attributes such as plot yield and test weight to derive resistant inbred lines with good *gca* effect for economically important traits, especially yield.

Assessment of Diversity among Eight Selected Founder Parents

Eight founder parents (Table 3) selected were further analyzed using 77 SSR markers. A total of 190 alleles were detected across eight genotypes used in the study. The representative gel picture depicting SSR polymorphism among the eight maize inbreds is depicted in Fig. 2. The number of alleles scored per SSR locus ranged from two to five (bnlg1360), with a mean of 2.46. The PIC of the SSR loci was in the range of 0.195 (phi097) to 0.746 (bnlg1360) with a mean of 0.384 (Table 4). Four SSR loci were found to have high PIC value (>0.60) namely, bnlg1360, umc1040, bnlg1200 and bnlg1432 which indicates the high discriminating power of these SSR loci.

TABLE 3

List of eight maize inbred lines selected based on SSR marker diversity for development of MAGIC population

Inbred	Characteristic feature
PDM4341	Significant GCA effects for plot yield
VL109545	Resistant to TLB
CML451	Drought tolerant
CAL1443	Resistant to TLB
CM212	Moderate resistant to FSR
SKV50	Resistant to TLB, SDM and Polysora rust
CAL1518	Significant GCA effects for test weight
CM202	Susceptible to TLB, SDM but moderate resistant to FSR

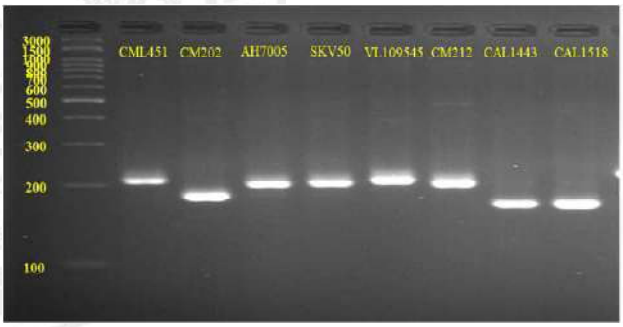

Fig. 2: Gel Picture depicting the tri-allelic SSR loci of bnlg1600 among eight inbred lines

The molecular marker-based assessment of heterozygosity is also useful for determining the purity of the seed lot (Nepolean *et al.,* 2013). The average heterozygosity revealed across 77 SSR loci was 0.015 (Table 4), which confirms the achievement of a high degree of homozygosity in the inbred lines. Heterozygosity observed in a few loci may be due to mutation in a particular SSR locus (Semagn *et al.,* 2006).

Average major allele frequency was 0.649, with a range of 0.250 (bnlg1360) to 0.875 (umc2393, phi097, bnlg1118, bnlg2228, umc1914, phi083, umc2257 and

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TABLE 4

Summary statistics of the SSR genotyping assay for eight inbred lines

Marker	Major Allele Frequency	Allele detected	Gene Diversity	Heterozygosity	PIC
bnlg109	0.500	3	0.594	0.000	0.511
bnlg1556	0.750	3	0.406	0.000	0.371
bnlg1502	0.833	2	0.278	0.000	0.239
umc1082	0.375	3	0.656	0.000	0.582
phi097	0.875	2	0.219	0.000	0.195
bnlg1018	0.750	3	0.406	0.000	0.371
bnlg198	0.857	2	0.245	0.000	0.215
phi127	0.625	2	0.469	0.000	0.359
bnlg1413	0.563	2	0.492	0.375	0.371
bnlg1396	0.625	3	0.531	0.000	0.468
umc1594	0.750	2	0.375	0.000	0.305
phi053	0.563	3	0.586	0.125	0.520
bnlg1185	0.563	3	0.570	0.125	0.496
umc1288	0.500	3	0.594	0.000	0.511
bnlg490	0.625	2	0.469	0.000	0.359
umc1532	0.500	2	0.500	0.000	0.375
bnlg589	0.625	3	0.531	0.000	0.468
bnlg2323	0.750	3	0.406	0.000	0.371
umc1155	0.625	2	0.469	0.000	0.359
bnlg1118	0.875	2	0.219	0.000	0.195
bnlg1792	0.571	2	0.490	0.000	0.370
umc1141	0.625	2	0.469	0.000	0.359
bnlg1065	0.625	2	0.469	0.000	0.359
phi022	0.625	2	0.469	0.000	0.359
bnlg127	0.625	2	0.469	0.000	0.359
bnlg1360	0.250	5	0.781	0.000	0.746
phi078	0.813	2	0.305	0.125	0.258
bnlg125	0.625	2	0.469	0.000	0.359
bnlg2328	0.625	2	0.469	0.000	0.359
umc1065	0.750	2	0.375	0.000	0.305
bnlg1144	0.375	3	0.664	0.125	0.590
bnlg1350	0.500	3	0.594	0.000	0.511
bnlg1724	0.625	2	0.469	0.000	0.359
bnlg1014	0.750	3	0.406	0.000	0.371
umc2226	0.500	3	0.625	0.000	0.555
umc2228	0.875	2	0.219	0.000	0.195
umc1323	0.500	2	0.500	0.000	0.375
mmc0041	0.500	3	0.594	0.000	0.511
umc1914	0.875	2	0.219	0.000	0.195
bnlg2123	0.750	3	0.406	0.000	0.371
phi083	0.875	2	0.219	0.000	0.195
umc1331	0.625	2	0.469	0.000	0.359
umc2363	0.500	2	0.500	0.000	0.375
bnlg1017	0.625	3	0.531	0.000	0.468
umc1265	0.750	2	0.375	0.000	0.305
umc1448	0.625	3	0.531	0.000	0.468
umc2007	0.375	3	0.656	0.000	0.582
umc2372	0.750	3	0.406	0.000	0.371
mmc0381	0.571	3	0.571	0.000	0.501
umc1736	0.750	2	0.375	0.000	0.305
phi101049	0.625	2	0.469	0.000	0.359
bnlg149	0.625	2	0.469	0.000	0.359
umc2186	0.688	2	0.430	0.125	0.337
umc1165	0.750	2	0.375	0.000	0.305
umc2246	0.750	2	0.375	0.000	0.305
umc2255	0.500	3	0.594	0.000	0.511
umc1931	0.750	2	0.375	0.000	0.305
umc1746	0.750	3	0.406	0.000	0.371
umc2071	0.750	2	0.375	0.000	0.305
umc2257	0.875	2	0.219	0.000	0.195
umc2376	0.625	3	0.531	0.000	0.468
phi126	0.500	3	0.594	0.000	0.511
umc1996	0.875	2	0.219	0.000	0.195
umc1143	0.625	2	0.469	0.000	0.359
bnlg1043	0.750	2	0.375	0.000	0.305
bnlg1600	0.625	3	0.531	0.000	0.468
bnlg1139	0.750	2	0.375	0.000	0.305
bnlg1165	0.625	3	0.531	0.000	0.468
bnlg1432	0.500	4	0.656	0.000	0.605
umc1409	0.500	2	0.500	0.000	0.375
bnlg1200	0.500	4	0.656	0.000	0.605
umc2393	0.875	2	0.219	0.000	0.195
umc1279	0.750	2	0.375	0.000	0.305
umc1040	0.500	4	0.656	0.000	0.605
umc2084	0.625	2	0.469	0.000	0.359
umc2018	0.563	2	0.492	0.125	0.371
mmc0111	0.750	2	0.375	0.000	0.305
Mean	0.649	2.465	0.457	0.015	0.384

umc1996). In the present investigation bnlg1360 was found to be highly diverse. Average gene diversity obtained was 0.457, the SSR loci namely, phi097, bnlg1118, umc2228, umc1914, phi083, umc2257, umc1996 and umc2393 recorded the lowest average gene diversity whereas, bnlg1360 exhibited the highest average gene diversity of 0.781 (Table 4).

Identification of Unique Alleles among Eight Founder Parents

Out of 77 SSR loci tested, six SSR loci (bnlg1018, umc1288, bnlg589, bnlg1360, umc2007 and bnlg2123)

TABLE 5

UPGMA dissimilarity coefficient matrix for eight maize inbreds based on SSR marker analysis

	CML 451	CM 202	PDM4341	SKV 50	VL 109545	CM 212	CAL1443
CM 202	0.575						
PDM4341	0.583	0.583					
SKV 50	0.583	0.583	0.460				
VL 109545	0.575	0.378	0.583	0.583			
CM 212	0.583	0.583	0.460	0.414	0.583		
CAL1443	0.575	0.480	0.583	0.583	0.480	0.583	
CAL 1518	0.575	0.480	0.583	0.583	0.480	0.583	0.019

produced nine unique alleles specific to particular inbred line. The SSR bnlg1018 produced a unique SSR allele for PDM4341 and CM 212 whereas, umc1288 generated a specific allele for SKV 50. The line VL 109545 could be differentiated from rest of the seven inbreds at bnlg589. Similarly, CML 451 and SKV 50 could be easily identified using the unique alleles produced by bnlg1360. The SSR loci umc2007 differentiated the CM 202 among eight genotypes. High level of genetic similarity was observed between CAL 1443 and CAL 1518. Out of 77 SSR loci tested, 76 SSR loci showed mono morphism between these genotypes. The marker bnlg2123 was polymorphic, which exhibited its ability to differentiate between these two genotypes.

Cluster Analysis among Eight Founder Parents

The dendrogram of eight maize genotypes was constructed using 77 polymorphic SSR markers. The genetic dissimilarity coefficient ranged from 0.019 to 0.583 (Table 5). The highest genetic dissimilarity coefficient was 0.583 between genotypes CML 451 and SKV 50 (Table 5). The lowest genetic dis similarity coefficient (0.019) was observed between CAL 1443 and CAL 1518 indicating the highest similarity between these genotypes. The dissimilarity matrix generated using simple matching coefficient was used to construct a tree using the UPGMA algorithm in hierarchical clustering method. It grouped the eight genotypes into two major clusters (Fig. 3). Cluster A comprised of five inbreds and could be further grouped into three sub clusters. Clustering pattern indicated that sub cluster A1 comprised of CM 202 and VL 109545, while CAL 1443 and CAL 1518 fell in the sub cluster A2 whereas, CML451 fell in the cluster A3. In Cluster B1, CM 212 and SKV 50 were grouped together and PDM4341 was found in cluster B2.

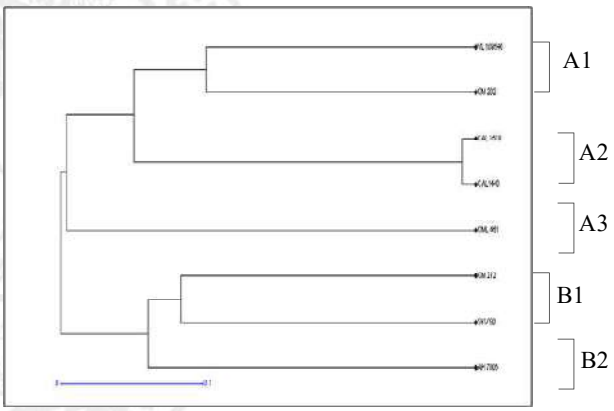

Fig. 3: Dendrogram showing clustering of eight maize inbred lines based on SSR marker analysis

In the criteria for the selection of parents, four parents namely SKV50, CM202, CM212 and CML451 were chosen although they fell in the single sub cluster initially. However, when we analyzed the eight parents with more markers CML451 and CM202 fell in A cluster (Fig. 3) and SKV50 and CM212 in cluster B (Fig. 3). Since SKV50 is resistant to TLB, SDM and PR and its contrasting counterpart CM202 was susceptible to TLB and SDM, they were grouped in separate clusters and said to be diverse as per SSR based diversity and have contrasting phenotype as well. Similar results were obtained for CML451 (drought tolerant) and SKV50 (drought susceptible) as well. Hence, the utility of large number

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of SSR markers in revealing the true diversity among eight founder parents was observed and served the objective of identification of unique alleles for all eight founder parents.

The study indicated the existence of molecular diversity and interrelationship among eight maize genotypes. Since above studied eight inbred lines are superior with respect to biotic and abiotic resistance and also diverse at molecular level, systematic crosses among these diverse inbred lines could be used in the development of multiparent segregating population for isolating inbreds with multiple disease resistance and in understanding the genetic architecture of resistance to biotic and abiotic stress.

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