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SSR MARKER BASED PARENT SELECTION OF MAIZE (*Zea mays* L.) INBRED LINES

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ABSTRACT

An experiment was designed to study the molecular diversity present in eighteen maize inbred lines during *Kharif* – 2016-17. A panel of thirteen highly polymorphic SSR primers generated allelic variants ranging from seven in the cases of nc133, umc1161, umc1367 and umc1196 to ten in the cases of, phi029, umc1332, phi034 and umc1266. A total of 110 alleles were detected among the eighteen maize inbred lines with an average of 8.46 alleles per locus. The PIC value was found to vary from 0.805 in the case of umc1367 to 0.886 in the case of phi065 with an average of 0.849 per primer. Analysis of divergence pattern based on SSR markers allowed differentiation of maize inbred lines into basically three clusters. The large range of similarity coefficient revealed by SSR markers provided greater confidence for the assessment of genetic divergence and interrelationship among the maize inbred lines. A perusal of similarity coefficients clearly reflected that a very high degree of similarity exists between maize genotypes Pool 33- 193 and Pool 34- 193, whereas CML-165 and HKI-586 found more diverse, may be used in breeding programme to generate more recombinants. Use of SSR markers appeared more efficient in achieving unique and unambiguous characterization and differentiation of maize inbred lines used in the present study. The SSR analysis also revealed unique or variety specific allele, which could be useful as DNA fingerprints in the identification and preservation of maize genotypes.

INTRODUCTION

Maize (Zea mays L.) is most important cereal crop belonging to the tribe Maydeae, of the grass family, Poaceae is a diploid (2n = 20) and cross pollinated (monoecious) crop. It is an important food, feed and industrial crop in India and other countries of the world. It is believed to have originated in Southern Mexico or Northern Guatemala (Weatherwax, 1955). Maize is the third most widely distributed crop of the world after rice and wheat (Poehlman, 2006), being grown in diverse seasons and ecologies with highest production and productivity among food cereals. Maize consumption in India has grown up to 19 million tonnes (Anonymous, 2014-15). Maize in India is used as a source of poultry food (43%), human feed (23%), cattle feed (17%), starch industry (14%), brewery (2%) and seed (1%) (IMA, KPMG Analysis 2012-13). There is no other cereal crop which has such immense productivity potential as maize and therefore maize occupies the unique place as "Queen of Cereals" and "Miracle crop". Inbred lines once released are maintained for decades through periodic seed increases in breeding programs and at germplasm repositories. Molecular markers developed for the differentiation of genotypes and assessment of genetic diversity are reliable and remain unaffected across different growth stages, seasons, location and agronomic practices Kumari *et al.,* 2005; Nikolic *et al.,* 2015. Microsatellites or simple sequence repeats (SSR) are tandomly repeated sequence motifs ubiquitously distributed throughout the maize genome. They can be easily am plified by polymerase chain reaction using primers specific to the unique flanking sequences of the SSR and polymorphic amplified fragments can be produced due to difference in the number of the repeat units (Kumar *et al*., 2012; Kanagarasu *et al*., 2013; Nidhal *et al*., 2014 and Sivaranjani *et al*., 2014). Keeping the above findings and facts under consideration, the present investigation was done to characterize the genetic variation and grouping of maize inbred lines at molecular level for maize improvement.

MATERIALS AND METHODS

Plant material and experimentation

Eighteen maize inbred lines were received from Department of Plant Breeding & Genetics, Tirhut College of Agriculture, Dholi, Muzzaffarpur, Dr. R.P.C.A.U., Pusa, Samastipur (Bihar) developed by different centers are presented in (Table 1). Plants were raised in Maize Research farm in randomized complete block design (RCBD) in triplicates, having plot size of 1.5 x 4.0 = 6 m^2. Each plot consisting of two rows of 4m length spaced at 75 cm row to row and 20 cm plant to plant, respectively. All recommended agronomic practices were followed during the crop raising period.

DNA extraction and quantification

Genomic DNA was isolated from young leaves of maize by CTAB method described by Doyle and Doyle (1987). Purification of the isolated DNA was done to remove RNA, proteins and polysaccharides, which are considered to be the

major contaminants in the DNA precipitate. Inclusion of CTAB in the DNA extraction buffer helps elimination of polysaccharides. RNA was removed by RNAase treatment and proteins were removed by phenol-chloroform extraction. The absorbance (optical density) of the purified DNA sample obtained after the purification step was recorded by UV Varian-Make Spectrophotometer at 260 nm in a quartz cuvette in order to quantify the purity of DNA.

Microsatellite primers, polymerase chain reaction (PCR) and electrophoresis

Thirty four SSR primers were obtained from Eurofins mwg/ operon (Table 2). The primer vials were centrifuged before and after the addition of 1X TE buffer to the vials. Mentioned volume of the TE buffer was added to each vial so as to obtain the desired concentration of the primer stock solution (100 μM). The primer stock solutions were diluted accordingly to 10 μM for further use to prepare PCR reaction mixture. Diluted primers were stored at -20ºC. Standardization of genomic DNA has been attempted to optimize the PCR reaction for amplification. A method designed to reveal the effects and interactions of specific reaction components simultaneously using a few reactions was adopted. In this method, the most important components of reaction such as concentration of primer and template DNA, which were likely to affect the PCR process, were arranged in orthogonal array (Primer concentration- 0.2, 0.5, 1.0, 1.0, 2.0μM and DNA concentration 2.3, 4, 5ng / μl). The other components of PCR process were kept constant. The amplified products were separated electrophoretically on 0.8 per cent agarose gel in 0.5 x TBE buffer, visualized and photographed over a UV light in gel doc after staining with ethidium bromide (EtBr).

Scoring and analysis of bands

Clear visible bands were coded in a binary form by denoting '0' and '1' indicated the absence and presence of bands, respectively in each genotype. The data were used for further calculations. To measure the informativeness of the SSR markers, the polymorphism information content (PIC) for each marker was calculated according to the formula given by Anderson *et al.* (1993).

$$PIC_i = 1 - \sum_{j=1}^{k} P^2 ij$$

Where,

k is the total number of alleles detected for a locus of a marker and Pi the frequency of the ith allele in the set of 19 varieties investigated.

The PIC value for each marker was used to justify the polymorphic information, and the mean PIC value for a group of individuals implies the genetic diversity within the group. Both the PIC for each marker and mean PIC for each group were determined. To describe the genetic relationship, microsatellite data were used to estimate the genetic distance based on Jaccard similarity coefficient and cluster analysis was done by using NTSYS-pc version 2.1m.

RESULTS AND DISCUSSION

Microsatellite (SSR) markers are useful for different applications in maize breeding and characterization due to their high level of polymorphism and easy handling (Kumari *et al.,* 2005) and are used to evaluate genetic diversity. Polymorphism in SSR is generally believed to be the result of replication error (Moxon and Willis, 1999), which occurs at a rate higher than the mutation in a non-repetitive DNA (Wierdl *et al*., 1997). The present study addressed the utility of SSR markers in revealing the extent of genetic diversity at the molecular level among 18 maize inbred lines. A total of 13 SSR primer pairs were used for the purpose of screening inbred lines, which were earlier identified in the genomic regions of chromosome 2, 2, 3, 3, 8, 5, 7, 8, 8, 9, 10, 10 and 3 of the maize genome; they are presented in (Table 2). A total of 26 loci were assigned to the 13 SSR primer pairs. (Fig 1-18). Altogether 110 alleles were detected among the eighteen maize inbred lines with an average of 8.46 alleles per locus (Table 3). The number of alleles per locus ranged from seven in the cases of nc133, umc1161, umc1367 and umc1196 to ten in the cases of, phi029, umc1332, phi034 and umc1266. This revealed significant differences in allelic diversity among various microsatellite loci. Many studies have also reported remarkable

Table 1: List of maize inbred lines and their source used in the present investigation:

Sl. No.	Name of maize inbred lines	Abbreviation of Inbred lines	Source
1.	CML-165	CML-165	TCA, DHOLI
2.	CML-169	CML-169	TCA, DHOLI
3.	CML-373	CML-373	TCA, DHOLI
4.	CML-161	CML-161	TCA, DHOLI
5.	(CML 161/CML-165)-BBB-11-BBB/CML-193	CML-BBB/ 193	TCA, DHOLI
6.	(CML 161×CML 451)-B-16-1-BB-2-B/CML 193	CML -2-B- 193	TCA, DHOLI
7.	WNC DMR 11 R 27290	WNC 27290	TCA, DHOLI
8.	WNC DMR 11 R 4776	WNC 4776	TCA, DHOLI
9.	WNC DMR 19 RYS FWS 1813	WNC 1813	TCA, DHOLI
10.	S99S1 YQ-BBB-5-BB-B/CML 193	S99S1- 193	TCA, DHOLI
11.	S99TLYQ(HG-AB)-BBB-54-BBB-54-BBB/CML-193	S99TLYQ	TCA, DHOLI
12.	Pool 18 Seq(C5×G 118)HS#47-1-2-1-1-B/CML 193	Pool 18- 193	TCA, DHOLI
13.	Pool 33 C 23(SubTINTY FQPM)-B-57-BB/CML 193	Pool 33- 193	TCA, DHOLI
14.	Pool 34 C 24(SubTINTY DQPM)-B-20-BB/CML-193	Pool 34 -193	TCA, DHOLI
15.	PoP 61 C1 QPM TEYE-51-2-1-2-B-1-13/CML-193	PoP 61 -193	TCA, DHOLI
16.	PoP-61	PoP-61	TCA, DHOLI
17.	LM-13	LM-13	TCA, DHOLI
18.	HKI-586	HKI-586	TCA, DHOLI

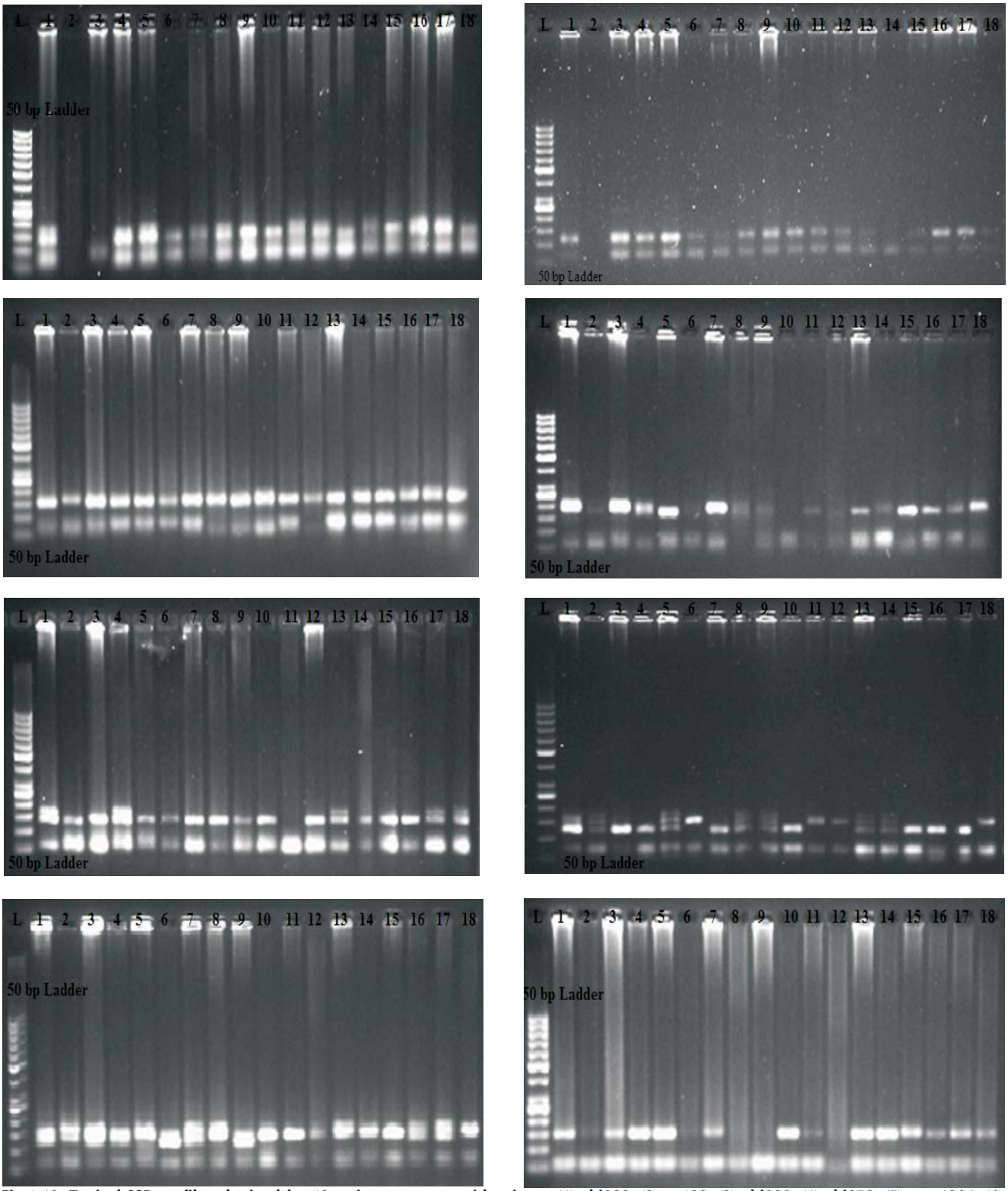

Fig 1-13: **Typical SSR profiles obtained for 18 maize genotypes with primers (1) phi083, (2) nc133, (3) phi029, (4) phi053, (5) umc1304, (6) umc1332, (7) phi034, (8) umc1161, (9) phi014, (10) phi065, (11) umc1367 (12) umc1196 and (13) umc1266.**

differences in allelic diversity among various microsatellite loci (Sserumaga *et al.,* 2014 and Yao *et al.,* 2007. The alleles revealed by markers showed a higher degree of polymorphism. The allelic polymorphic information content (PIC) varied from 0.805 in the case of umc1367 to 0.886 in the case of phi065 with an average of 0.849 per primer (Ranatunga *et al.*, 2009;

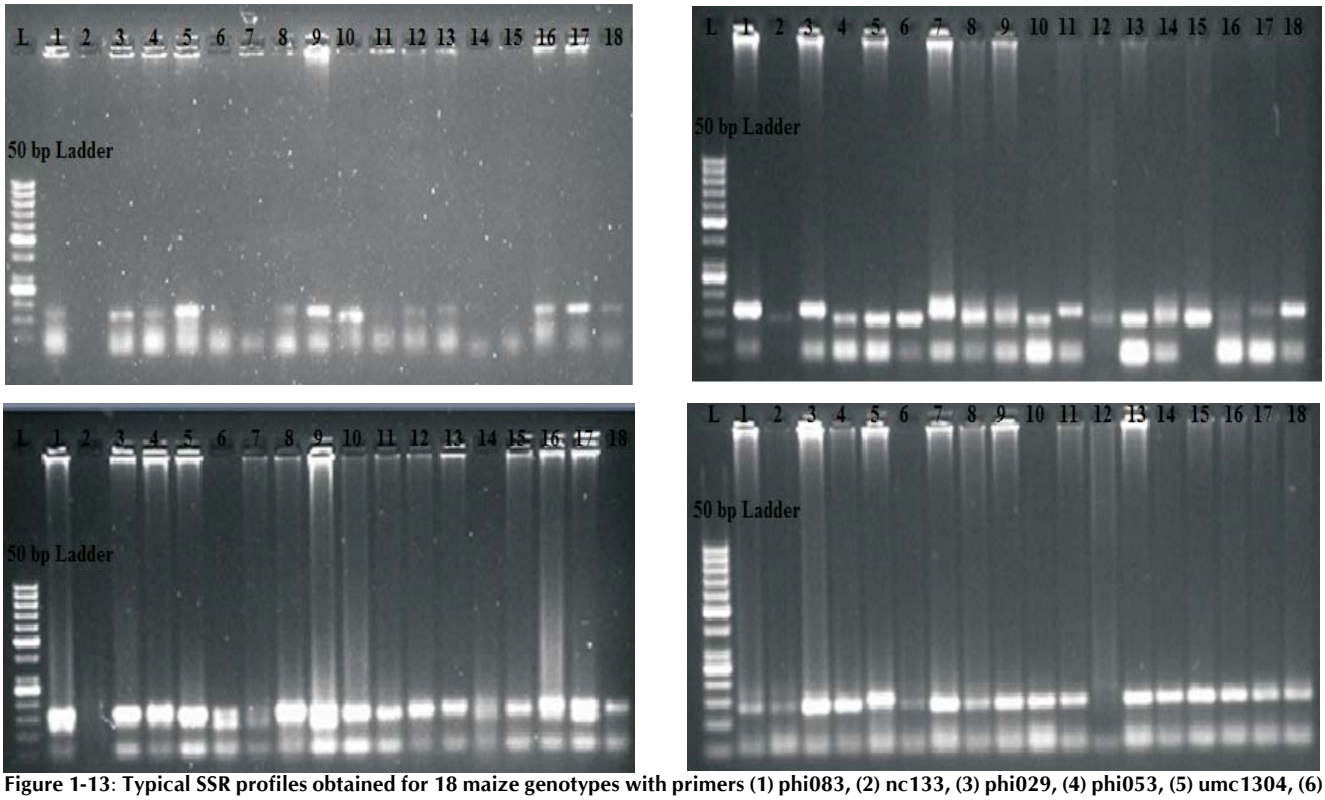

Figure 1-13: **Typical SSR profiles obtained for 18 maize genotypes with primers (1) phi083, (2) nc133, (3) phi029, (4) phi053, (5) umc1304, (6) umc1332, (7) phi034, (8) umc1161, (9) phi014, (10) phi065, (11) umc1367 (12) umc1196 and (13) umc1266.**

Table 2: List of thirteen primers utilized for amplification of maize genomic DNA extracted from eighteen maize genotypes used in the present study.

Sl.No.	Locus	Chromo some No.	Primer sequence (5′- 3′)	Repeat Motif	Annealing temp. (°C)
1.	phi083	2	(F) CAA ACA TCA GCC AGA GAC AAG GAC (R) ATT CAT CGA CGC GTC ACA GTC TAC T	AGCT	62
2.	nc133	2	(F) AAT CAA ACA CAC ACC TTG CG (R) GCA AGG GAA TAA GGT GAC GA	GTGTC	55
3.	phi029	3	(F) TTG TCT TTC TTC CTC CAC AAG CAG CGA A (R) ATT TCC AGT TGC CAC CGA CGA AGA ACT T	CCCTCT	57
4.	phi053	3	(F) CTG CCT CTC AGA TTC AGA GAT TGA C (R) AAC CCA ACG TAC TCC GGC AG	ATAC	61
5.	umc1304	8	(F) CAT GCA GCT CTC CAA ATT AAA TCC (R) GCC AAC TAG AAC TAC TGC TGC TCC	$(TCGA)_4$	62
6.	umc1332	5	(F) CCT CTT GCT TCC TCG TCA TGT ACT (R) AAG GAG CTG GAA CAT AAA ACA CCA	$(CGT)_5$	59
7.	phi034	7	(F) TAG CGA CAG GAT GGC CTC TTC T (R) GGG GAG CAC GCC TTC GTT CT	CCT	59
8.	umc1161	8	(F) GGT ACC GCT ACT GCT TGT TAC TGC (R) GCT CGC TGT TGG TAG CAA GTT TTA	$(GCTGGG)_5$	62
9.	phi014	8	(F) AGA TGA CCA GGG CCG TCA ACG AC (R) CCA GCT TCA CCA GCT TGC TCT TCG TG	GGC	66
10.	phi065	9	(F) AGG GAC AAA TAC GTG GAG ACA CAG (R) CGA TCT GCA CAA AGT GGA GTA GTC	CACTT	58
11.	umc1367	10	(F) TGG ACG ATC TGC TTC TTC AGG (R) GAA GGC TTC TTC CTC GAG TAG GTC	$(TGA)_4$	62
12.	umc1196	10	(F) CGT GCT ACT ACT GCT ACA AAG CGA (R) AGT CGT TCG TGT CTT CCG AAA CT	CACACG	62
13.	umc1266	3	(F) CAC AGG TAA AAG TAA ACG CAC ACG (R) CTC GTC ATT TTC AAC GTC CTC TTT	$(TGC)_4$	57

Table 3. Analysis of primer pairs used for the amplification of genomic DNA extracted from eighteen maize inbred lines.

Sl. No.	Primers	No of locus	Size of alleles (bp)	No. of alleles	No. of unique alleles	Percentage of unique alleles	PIC	No. of entries having null alleles
1.	phi083	2	55.40-153.25	8	3	37.50	0.844	1
2.	nc133	2	55.40-123.06	7	2	28.57	0.836	3
3.	phi029	2	56.09-159.69	10	5	50.00	0.876	0
4.	phi053	2	55.40-205.55	8	3	37.50	0.864	5
5.	umc1304	2	55.409-152.80	9	4	44.44	0.858	0
6.	umc1332	2	74.43-158.32	10	4	44.44	0.883	0
7.	phi034	2	57.66-157.52	10	5	50.00	0.870	0
8.	umc1161	2	55.40-157.52	7	3	42.85	0.817	1
9.	phi014	2	64.92-160.46	8	3	37.50	0.830	1
10.	phi065	2	55.40-156.69	9	3	33.33	0.886	0
11.	umc1367	2	55.40-164.32	7	3	42.85	0.805	2
12.	umc1196	2	55.40-165.63	7	4	42.85	0.806	6
13.	umc1266	2	55.40-151.00	10	6	60.00	0.870	0

Table 4 : Estimates of thirty four SSR primer pairs based Jaccard's similarity coefficients among nineteen rice varieties used in the present study.

Inbred lines	CML-165	CML-169	CML-373	CML-161	CML-BBB /193	CML-2 -B-193	WNC 27290	WNC 4776	WNC 1813	S99S1 -193	S99T LYQ	Pool 18-193	Pool 33-193	Pool 34-193	PoP 61-193	PoP-61	LM-13
CML-169	0.076																
CML-373	0.133	0.161															
CML-161	0	0.06	0.205														
CML-BBB/193	0	0	0.114	0.151													
CML-2-B-193	0.033	0.054	0.046	0.073	0.135												
WNC 27290	0.088	0	0.025	0.055	0.09	0.076											
WNC 4776	0.033	0.029	0.027	0	0.027	0.047	0.225										
WNC 1813	0.1	0.032	0	0.027	0.029	0.078	0.129	0.193									
S99S1-193	0	0.04	0	0	0.035	0.029	0.074	0.033	0.217								
S99TLYQ	0.05	0	0	0	0.04	0.032	0	0.12	0.086	0.266							
Pool 18-193	0	0.047	0	0.038	0.086	0	0.041	0.038	0.2	0.2	0.25						
Pool 33-193	0.052	0.047	0	0.038	0.086	0	0.041	0.08	0.09	0	0.153	0.166					
Pool 34-193	0.062	0.055	0	0	0	0.037	0.047	0	0	0	0.09	0	0.375				
PoP 61-193	0	0.625	0	0.047	0	0	0	0	0	0	0	0.125	0	0			
PoP-61	0.142	0	0	0.045	0	0	0	0	0	0	0	0	0	0	0		
LM-13	0.142	0	0	0	0.105	0	0.05	0.045	0	0	0	0	0	0	0	0.2	
HKI-586	0.071	0	0	0	0	0	0.052	0.047	0	0	0	0	0	0	0	0	0

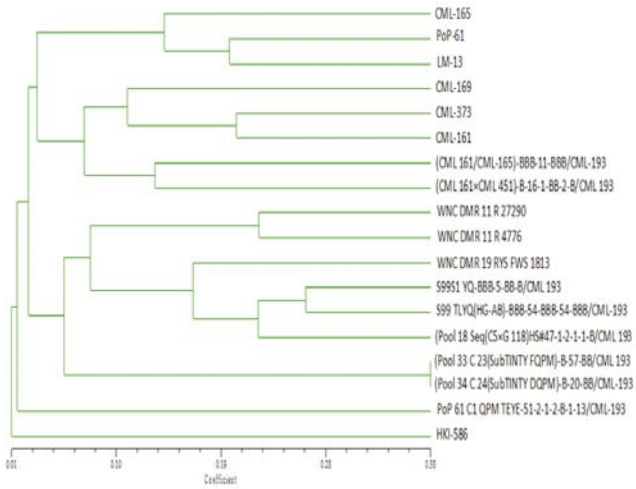

Figure 14: Dendrogram based on average Jaccard's similarity coefficient for thirteen SSR primer pairs among 18 maize inbred lines.

Gupta and Singh, 2010; Babu *et al.*, 2012) (Table 3). Null alleles are known to arise as a consequence of sequence changes at the primer binding site(s). Occurrence of null alleles was also noticed in various inbred lines for a particular locus, whenever an amplification product could not be detected in a specific SSR primer pair combination. Null alleles were also detected (Nikolic *et al.*, 2015; Mukesh *et al.*, 2016; Kumari *et al.*, 2005). Presence of stutter bands was also detected in the present study. Stutter bands indicated the presence of minor products amplified in PCR that had lower intensity than the main allele and normally lacked or had extra units. Such bands were observed in the case of tri-nucleotide SSR sequence detected by primer pairs umc1304 and umc1332. The SSR loci with di-nucleotide repeat motifs, in general, tended to detect greater number of alleles as revealed by phi029, umc1332, phi034 and umc1266. Stutter bands were probably produced by the slippage of the polymerase amplification and the factors that influenced the proportion of stutter band to the main allele were the repeat number, number of PCR cycles, length and the characteristics of the repeat sequence. Considering the number of alleles generated by different primer pairs in conjunction with the level of polymorphism detected in the present study, the primers umc1367, umc1196, umc1161, phi014, nc133, phi083, umc1304, phi053, phi034, umc1266, phi029, umc1332 and phi065 appeared to be the most informative primers for the purpose of molecular characterization of maize inbred lines under evaluation.

Since, a change in the number of repeats leads to generation of allelic variants because of variation in the size of the SSR allele, the total repeat count of tri-nucleotide SSR loci was found to be associated with the number of alleles. Larger the repeat number involved in the SSR locus, the larger was the number of identified alleles. (Alam, M.S. and Alam, M.F., 2013) observed increase in number of alleles with the repeat number of the microsatellites used and their relative distance from the centromere, which were not dependent on the motif of microsatellites.

Allelic diversity data was used to produce a dendrogram in order to elucidate the relationship among eighteen inbred lines. The similarity coefficient was estimated on the basis of Jaccard's coefficient (Rohlf, F. J., 2000). The estimates of similarity coefficients, ranging from 0 to 1, indicated a considerably greater extent of variation among the maize inbred lines under evaluation in the present study (Table 4). The large range of similarity coefficient revealed by SSR markers provided greater confidence for the assessment of genetic divergence and interrelationship among the maize inbred lines. A perusal of similarity coefficients clearly reflected that a very high degree of similarity exists between maize genotypes Pool 33-193 and Pool 34-193, whereas CML-165 and HKI-586 found more diverse, may be used in breeding programme to generate more recombinants. Further the phenon line divided all other sub-clusters into mono-genotypic clusters (Fig. 19). The presence of unique alleles in the set of inbred lines indicated that these materials are useful for plant breeders and geneticists as a rich source of genetic diversity for purposeful utilization in maize breeding programme . Pattern of clustering and sub clustering indicated that the genotypes with distinct DNA profiles are likely to be contain the novel genes and are likely to unique and agronomically useful, may be exploited for maize improvement programme.

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