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Anjali Kumari
Department of Plant Breeding and Genetics, Bihar Agricultural University, Sabour, Bhagalpur, (Bihar), India

Sweta Sinha
Department of Molecular Biology and Genetic Engineering, Bihar Agricultural University, Sabour, Bhagalpur, (Bihar), India

Kumari Rashmi
Department of Plant Breeding and Genetics, Bihar Agricultural University, Sabour, Bhagalpur, (Bihar), India

SS Mandal
Department of Plant Breeding and Genetics, Bihar Agricultural University, Sabour, Bhagalpur, (Bihar), India

Sanjay Sahay
Department of Horticulture, Bihar Agricultural University, Sabour, Bhagalpur, (Bihar), India

Correspondence
Sweta Sinha
Department of Molecular Biology and Genetic Engineering, Bihar Agricultural University, Sabour, Bhagalpur, (Bihar), India

Genetic diversity analysis in maize (*Zea mays* L.) using SSR markers

Anjali Kumari, Sweta Sinha, Kumari Rashmi, SS Mandal and Sanjay Sahay

Abstract
A set of eight maize genotypes was subjected to genetic diversity analysis using 22 SSR primers. Out of 22 SSR primers, 9 primers were polymorphic and displayed clear size differences. A total of 19 polymorphic alleles were generated through the 9 polymorphic primers with an average polymorphism information content and genetic diversity value of 0.297 and 0.373, respectively. The phi064 and phi053 was found as the best marker for identification of genotypes as revealed by PIC values (0.367). By using UPGMA cluster analysis, the eight genotypes were grouped under 3 clusters. The genetic distances based on SSR marker analysis varied from 0.21 - 0.64. The distances measured based on SSR primers showed that the BML7 was most diverse genotype. The variability detected among the genotypes using SSR markers in this study could be utilize for the exploitation of heterosis and development of genetically diverse populations in maize breeding improvement programs.

Keywords: Genetic diversity, Maize, SSR.

Introduction
Maize (*Zea mays* L.) is the world's third most important cereal after wheat and rice and known as "Queen of Cereals". It has worldwide significance as human food, animal feed and for a large number of many other industrial products like glucose, starch, oil etc. It is a major food crop and main source of dietary energy and protein for the most food insecure people in the world. Maize was domesticated in Mexico, from where it was introduced to other regions of the world with diverse agro-climatic conditions. Therefore, a wide diversity is found within and among the maize genotypes at both phenotypic and genotypic level. The knowledge of genetic diversity in maize is important for understanding of the genetic structure and subsequently helps the breeder in choosing desirable parents to conduct breeding program (Al-Badeiry *et al.,* 2014) [1]. The more diverse genotypes can be used to produce superior hybrids/ lines, segregating population with high variability and introgression of desirable traits/genes. Therefore, there is a need to evaluate the available genotypes for the extent of genetic diversity.

Genetic diversity can be assessed by common morphological traits or molecular markers. Molecular markers have become the study of choice for the plant genetic diversity. Over the last three decades, the advent of molecular markers has revolutionized the entire scenario of plant and animal sciences. Molecular markers offer several advantages over traditional morphological traits, as molecular markers are not environmentally influenced. Among the different molecular markers such as RAPD, ISSR, SSR, AFLP etc, SSR have been successfully employed in maize genetic diversity studies because of their reproducibility, multi-allelic nature, co-dominant inheritance, relative abundance and good genome coverage (Senior *et al.,* 1998; Selvi *et al.,* 2003; Bantte and Prasanna, 2003; Ranatunga *et al.,* 2009; Shehata *et al.,* 2009; Nepolean *et al.,* 2013; Sserumaga *et al.,* 2014) [2, 3, 4, 5, 6, 7, 8]. Therefore, in the present study, an attempt has been made to determine the extent of molecular diversity among eight maize genotypes using twenty two SSR markers which could serve as a basis in the identification of genetically distant genotypes.

Materials and methods
Plant material and DNA extraction
The plant materials consisted of eight genotypes of maize (Table 1). The genomic DNA was extracted from fresh leaf tissue of the maize genotypes following CTAB method described by Doyle and Doyle (1990) [9]. Quantity and quality of DNA was checked by agarose gel eletrophoresis (0.8%). After quantification, the DNA was diluted to a concentration of 40 ng/µl for SSR analysis.

Table 1: List of maize genotypes taken for the study

Sl. No.	Name of genotypes	Sources
1	LM13	PAU, Ludhiana
2	VQL1	ALMORA, Uttarakhand
3	SML1	BAU, Sabour
4	CML451	CIMMYT, Mexico
5	DTPYC9	CIMMYT, Mexico
6	BML7	ANGRU, Hyderabad
7	CLQRCY44	CIMMYT, Mexico
8	CLO2450	CIMMYT, Mexico

SSR analysis

Eight genotypes were subjected to molecular diversity analysis using twenty two maize SSR primers spread across 10 chromosome (Table 2). The PCR amplification were carried out in 10 µl reaction volume containing 1 × PCR buffer, 0.2 mM dNTPs, 0.2 µM each forward and reverse primer, 0.5 U Taq DNA Polymerase (Xcelris, India) and 40 ng of template DNA using a thermal cycler (Eppendorf, USA). Touchdown-PCR reactions were performed as follows: 4 min pre-denaturation at 94 °C, followed by 94 °C for 30 sec, 60 °C for 40 sec, and 72 °C for 40 sec in the first cycle, then decreasing the annealing temperature by 1 °C/cycle for 10 cycles, followed by 94 °C for 30 sec, 55 °C for 40 sec, and 72 °C for 40 sec for 25 cycles and ending with 5 min of elongation at 72 °C. The amplified PCR products along with 100bp DNA ladder as molecular marker were resolved in 2% agarose gel stained with ethidium bromide (0.5µg/ml). Gel was visualized under UV light and documented in gel documentation system (Uvitec gel doc system, UK).

Table 2: List of primers used in the study

S.No.	Primer code	Primer sequence (5'- 3')	Chromosome number	Repeat type
1	phi002-F	CATGCAATCAATAACGATGGCGAGT	1	AACG
	phi002-R	TTAGCGTAACCCTTCTCCAGTCAGC		
2	phi056-F	ACTTGCTTGCCTGCCGTTAC	1	CCG
	phi056-R	CGCACACCACTTCCCAGAA		
3	phi064-F	CCGAATTGAAATAGCTGCGAGAACCT	1	ATCC
	phi064-R	ACAATGAACGGTGGTTATCAACACGC		
4	nc133-F	AATCAAACACACACCTTGCG	2	GTGTC
	nc133-R	GCAAGGGAATAAGGTGACGA		
5	phi029-F	TTGTCTTTCTTCCTCCACAAGCAGCGAA	3	AGCG
	phi029-R	ATTTCCAGTTGCCACCGACGAAGAACTT		
6	phi046-F	ATCTCGCGAACGTGTGTGCAGATTCT	3	ACGC
	phi046-R	TCGATCTTTCCCGGAACTCTGAC		
7	phi053-F	CTGCCTCTCAGATTCAGAGATTGAC	3	ATAC
	phi053-R	AACCCAACGTACTCCGGGCAG		
8	phi073-F	GTGCGAGAGGCTTGACCAA	3	AGC
	phi073-R	AAGGGTTGAGGGCGAGGAA		
9	phi006-F	AGGCGGCGTGCTGAACACCT	4	CCT
	phi006-R	CGCTTCATCTCCCGTGACAATG		
10	phi011-F	TGTTGCTCGGTCACCATACC	4	AGC
	phi011-R	GCACACACACAGGACGACAGT		
11	bnlg118-F	CTTCCAGCCGCAACCCTC	5	CT
	bnlg118-R	CCAACAACGCGGACGTGA		
12	nc130-F	GCACATGAAGATCCTGCTGA	5	AGC
	nc130-R	TGTGGATGACGGTGATGC		
13	phi024-F	ACTGTTCCACCAAACCAAGCCGAGA	5	CCT
	phi024-R	AGTAGGGGTTGGGGATCTCCTCC		
14	bnlg391-F	CAGATATCACAGCATCAGAAGATCA	6	CAA
	bnlg391-R	AAAATGTAAGAACTTGTTTGGGATT		
15	phi034-F	TAGCGACAGGATGGCCTCTTCT	7	CCT
	phi034-R	GGGGAGCACGCCTTCGTTCT		
16	phi014-F	AGATGACCAGGGCCGTCAACGAC	8	GGC
	phi014-R	CCAGCTTCACCAGCTTGCTCTTCGTG		
17	phi032-F	CTCCAGCAAGTGATGCGTGAC	9	AAAG
	phi032-R	GACACCCGGATCAATGATGGAAC		
18	phi065-F	AGGGACAAATACGTGGAGACACAG	9	CACTT
	phi065-R	CGATCTGCACAAAGTGGAGTAGTC		
19	phi050-F	TAACATGCCAGACACATACGGACAG	10	AAGC
	phi050-R	ATGGCTCTAGCGAAGCGTAGAG		
20	phi059-F	AAGCTAATTAAGGCCGGTCATCCC	10	ACC
	phi059-R	TCCGTGTACTCGGCGGACTC		
21	phi062-F	CCAACCCGCTAGGCTACTTCAA	10	ACG
	phi062-R	ATGCCATGCGTTCGCTCTGTATC		
22	phi063-F	GGCGGCGGTGCTGGTAG	10	TATC
	phi063-R	CAGCTAGCCGCTAGATATATACGCT		

Band scoring and Data analysis

The clear and distinct bands amplified by SSR primers were scored visually for their presence (1) or absence (0) of the corresponding band among the 8 maize genotypes. The polymorphic information content (PIC) values of each primer were calculated using Power Marker 3.5. Similarity matrix was generated using the SIMQUAL function of NTSYS-pc version 2.02 (Rohlf, 1998) [10]. The Jaccard similarity

coefficients were used for cluster analysis and dendrogram was constructed using the Unweighted Pair-Group method (UPGMA) by SAHN clustering function of NTSYS software.

Results and Discussion

Twenty two SSR primers were used to assess molecular diversity and interrelationship in eight maize genotypes. All 22 SSR primers generated clear banding patterns but only 9 primers gave polymorphic profile. A total of 20 alleles were detected, out of which 19 were polymorphic through 9 polymorphic primers and displayed clear size differences (Table 3, Figure 1). The alleles ranged from 2 to 3 and the average number of alleles per locus among the genotypes was 2.22. Among the polymorphic markers, 7 produced 2 alleles each and 2 of them produced a maximum of 3 alleles each. The number of alleles did not vary greatly among genotypes used in the present study. Nepolean *et al.* (2013) [7] obtained a total of 111 polymorphic alleles with an average of 3.17 alleles per locus while Kumar *et al.* (2016) [11] reported a total of 59 alleles with an average of 2.62 alleles per locus. However, Patel *et al.* (2017) [12] detected total of 76 alleles with an average of 4.47 alleles per locus. The difference in the numbers of alleles obtained between studies may be due to the size of the samples under study, the methodologies used for detection of polymorphic markers which influence allelic differences or uniformity based on pedigrees. Polymorphism information content (PIC) value ranged between 0.195 and 0.367 with an average of 0.297 which is comparatively low as compared to polymorphism information content reported by Ranatunga *et al.* (2009) [5] (PIC= 0.53 to 0.99), Adeyemo *et al.* (2011) [13] (PIC = 0.17 to 0.84), Sserumaga *et al.* (2014) [8] (PIC = 0.16 to 0.91) and Patel *et al.* (2017) [12] (PIC = 0.46 to 0.86). But our results were comparable with the results of Kumar *et al.* (2016) [11] who used same set of primers on 13 maize genotypes and found PIC value ranged from 0.142 to 0.497 with an average value 0.304. A low PIC value in the present study was observed due to a high frequency of two alleles. The highest PIC value was observed in primers phi064 and phi053 (0.367) and the lowest PIC values were observed in primer nc130 and phi059 (0.195). Kumar *et al.* (2016) [11] has reported that primer phi050 showed maximum PIC value 0.497. PIC reveals the informativeness of the SSR loci and their ability to detect differences among the genotypes based on their genetic relationships. The primer phi064 and phi053 was found to be the most appropriate marker for genetic diversity study on the basis of highest PIC value of 0.367. The values of genetic diversity for each locus varied from 0.219 for nc130 and phi059 loci to 0.484 for locus phi064 and phi053 with a mean of 0.373. We found that SSR marker showed low allelic variants as well as the diversity values which may due to the narrow genetic base of the maize genotypes used in the present study or due to particularly use of tri/tetra/penta-nucleotide primers.

Table 3: Primers showing polymorphism across maize genotypes

SSR locus	Approximate product size amplified (bp)	Total number of alleles	Total number of polymorphic alleles	PIC value	Gene diversity
phi064	75-100	2	2	0.367	0.484
phi053	170-200	2	2	0.367	0.484
nc130	140-170	2	1	0.195	0.219
bnlg391	70-110	2	2	0.359	0.469
phi034	100-150	2	2	0.250	0.297
phi014	90-400	3	3	0.286	0.354
phi065	150-180	2	2	0.332	0.422
phi059	145-160	2	2	0.195	0.219
phi063	100-200	3	3	0.323	0.406

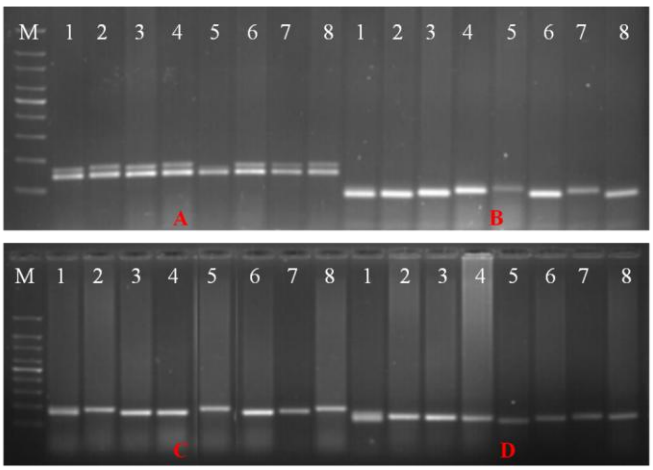

Fig 1: PCR amplification using SSR primers A) nc130 B) bnlg391 C) phi053 D) phi034 in eight maize genotypes. Lane M: 100 bp DNA ladder, Lane 1-8: SML1, CLQRCY44, CLO2450, VQL1, BML7, LM13, DTPYC6 and CML451

Cluster analysis

The dendrogram of eight maize genotypes were constructed using 19 polymorphic loci generated by SSR markers. The similarity coefficients ranged from 0.21 to 0.64 (Table 4). The similarity coefficient largest value 0.64 between the genotype CLQRCY44 and LM13, and CLO2450 and CML451 which shows high similarity between them and it may be expected that both of them may have derived from the same parents. The lowest similarity coefficient value 0.21 between the genotype BML7 and CLO2450, BML7 and VQL1, BML7 and LM13, and BML7 and CML451 showing that they are dissimilar from each other may be because they exhibit different morphological features and traits or different geographical origins or derived from different parents. However, the genotype BML7 shows highest similarity with DTPYC9 (0.38).

Table 4: Jaccard's Similarity coefficient matrix for 8 maize genotypes based on SSR marker analysis

	SML1	CLQRCY44	CLO2450	VQL1	BML7	LM13	DTPYC9	CML451
SML1	1.00							
CLQRCY44	0.47	1.00						
CLO2450	0.47	0.50	1.00					
VQL1	0.38	0.50	0.38	1.00				
BML7	0.24	0.31	0.21	0.21	1.00			
LM13	0.38	0.64	0.50	0.50	0.21	1.00		
DTPYC9	0.35	0.36	0.46	0.46	0.38	0.58	1.00	
CML451	0.47	0.50	0.64	0.38	0.21	0.29	0.27	1.00

The phylogenetic tree grouped the genotypes in three clusters on the basis of similarity coefficient values. In the cluster II had maximum four genotypes followed by cluster I containing three genotype and cluster III one genotype (Figure 2). The most diverse genotype was BML7. Therefore, highest diverse genotypes can be used as parents in breeding programme. Ranatunga *et al.* (2009) [5] grouped 45 maize genotypes into two major clusters which further subdivided into several sub clusters. Kanagarasu *et al.* (2013) [14] used ten SSR molecular markers for diversity analysis of 27 maize inbred lines and cluster analysis revealed five major clusters at 0.62 similarity coefficient. Patel *et al.* (2017) [12] constructed dendrogram using similarity coefficient values divided 8 genotypes in three divergent clusters. Kumar *et al.* (2016) [11] reported 13 genotypes grouped into five clusters. The clustering pattern indicated the existence of variability among the maize genotypes in present study.

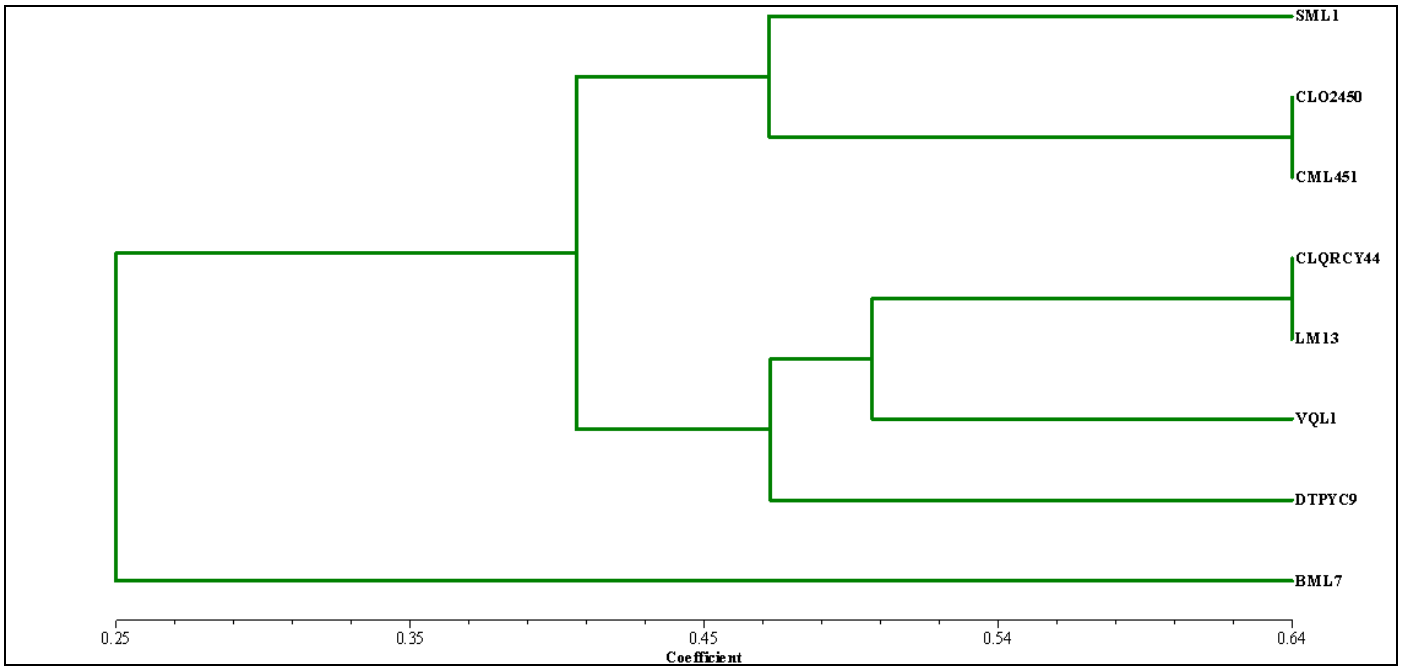

Fig 2: Dendrogram showing clustering of eight maize genotypes based on SSR marker analysis

Conclusion

This study indicated the existence of molecular diversity and interrelationship in eight maize genotypes. Polymorphism information content (PIC) values clearly showed that phi064 and phi053 was the best SSR marker for identification of genotypes. The genotype BML7 placed separately from remaining genotypes in cluster analysis through SSR markers. Thus, SSR markers could be used to characterize lines precisely at the molecular level, they can help maize breeders efficiently assign lines to heterotic groups and guide them in the choice of parents for the development of new hybrids. At the same time, SSR markers provide a means for distinctly identifying individual genotypes with their unique allelic pattern, an application that is becoming important in varietal protection.

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