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MOLECULAR DIVERGENCE OF MAIZE (*ZEA MAYS* L.) INBREDS AS REVEALED BY DNA MICROSATELLITES

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ABSTRACT
The present investigation was carried out to measure genetic relationship among maize inbred lines and to sort out diversified parent to develop hybrids with yield potential in further breeding programme. Analysis of ten simple sequence repeats (SSR) or DNA microsatellite markers, spread across maize genome, was done for early to intermediate ten maize inbred lines. For polymorphic markers, an average 4.25 alleles per locus was reported with a range of 2 to 6. The polymorphic information content (PIC) of four polymorphic SSRs varied from 0.32 (bnlg 1832) to 0.71 (bnlg 1523) with mean 0.52 while expected heterozygosity (He) was ranges from 0.40 (bnlg 1832) & 0.74 (bnlg 1523) with an average of 0.58. The Jaccard's dis-similarity index was obtained range from 0.00 to 0.55. The unweighted pair group method with arithmetic mean (UPGMA) generated dendogram using Jaccard's similarity coefficient divided maize inbred lines into six heterotic groups which could easily facilitated in screening of diverse parents. Thus, these DNA microsatellite markers have good potentiality to discriminate maize inbred lines at molecular level and, hence, the molecular profiles can definitely be used to select genetically diverse lines to produce high heterotic progenies to enhance grain yield in maize.

INTRODUCTION

Maize (*Zea mays* L.), being an important staple crop, is continuously generating seed entrepreneurship and attracting many investors compared to other cereals. With the uncovered of heterosis (Shull, 1914), many breeders are trying to harness hybrid vigour to improve the yield of the maize till the date. Hybrid seed development relies on having homozygous lines from different source populations and gene pools and evaluating their mean performance as well as their performance in various combinations (Troyer 2004; Koutsika-Sotiriou and Karagounis 2005). For several decades, maize breeders have focused on short-term breeding based on maize inbreds that have been developed from a limited number of elite lines and elite line synthetics, which has resulted in the development of a narrow genetic base for commercial maize hybrids (Darrah and Zuber, 1986; Soni and Khanorkar 2013). The relative amount and type of genetic variability involved in maize genotypes facilitated for choosing the most efficient breeding scheme for improving maize population (Kumar *et al*., 2013).

Information on genetic diversity present in germplasm assists in the selection of parent and accelerates the technique on the genetic gain. With the advent of molecular markers, the study of genetic variabilty at the DNA level have been made easy and has significantly increased accuracy in assessing molecular diversity and identifying maize cultivars. The microsatellite or simple sequence repeat (SSR) markers (Litt and Luty, 1989) are widely used in maize, as these markers are mapped, PCR-based, genetically codominant, hypervariable, highly polymorphic, robust, reproducible and amenable to automation (Dubreuil *et al*. 2006; Prasanna *et al*. 2010). Several studies have profoundly used SSR markers to evaluate the molecular profiling of maize for drought tolerance (Nepolean *et al*., 2013), disease resistance (Small *et al*., 2012; Lukman *et al*., 2013), qualitative characters (Song *et al*., 2004, Pandey *et al*., 2015), genetic diversity (Xia *et al*., 2004; Shehata *et al*., 2009; Mishra and Singh, 2012; Sserumaga *et al*., 2014; Elci and Hançer, 2015), genetic purity (Abakemal *et al*., 2014). Molecular genetic markers are definitely a powerful tool to delimit heterotic groups and to assign inbred lines into existing heterotic groups (Melchinger, 1999). It is widely believed that the level of genetic distances between two inbred lines has an influence on the performance of resulting hybrids (Pajic *et al*., 2010; Soni and Khanorkar 2013). Several attempts have been made so far to characterize maize landraces using SSR markers. In the present study attempts have been made to measure the genetic diversity among the 10 maize inbreds using microsatellite markers.

MATERIALS AND METHODS

Plant materials

A total of 10 inbred lines including 4 lines from Department of Genetics and Plant Breeding, SHIATS and 6 lines from Chandra Shekhar Azad University of Agriculture & Technology, Kanpur were raised in pots and maintained in a laboratory condition till the 3-5 functional leaf formation during *Kharif*, 2014. These lines were early to intermediate in maturity, yellow in seed colour and flint to dent in texture. Young green and healthy leaf from each inbred lines were used for extraction of DNA.

DNA isolation

The CTAB method was used for genomic DNA isolation, as described by Saghai-Mahroof *et al*. (1984) with slight modifications. Shortly, 1g of young leaf sample of each inbred lines were crushed in liquid nitrogen to fine powder in mortar and pestle and transferred to 50ml centrifuge tube containing 10ml lysis buffer and vertexed for 2 minutes then incubated for 65^{0}C in water bath for 45 minutes with occasional swirling. Emulsified the mixture with equal volume of chloroform-isoamyl alcohol (24:1) and then centrifuged at 10,000 rpm for 10 minutes. Added 10 μL RNase A after transferring upper aqueous layer in fresh 50 ml centrifuge tube and incubated at 37^{o}C for 30 minutes. To the sample, mixed equal volume of phenol:chloroform:isoamylalchohol (25:24:1) and then centrifuged at 12,000 rpm for 5 minutes. Extracted DNA was elctrophoresed on 0.8% (w/v) agarose gels for quantification, stained with 5x gel loading dye and photographed under UV transilluminator attached to gel documentation system.

Polymerase chain reaction (PCR) conditions and electrophoresis for SSR analysis

A total of 10 SSR maize primers (Table 1) were used for PCR amplification of repeat sequences from the genomic DNA of each inbred. These primers were chosen from Maize Genome Database (http:/ /www. maizegdb.com) on the basis of bin location (to maximize genomic coverage).

The SSR-PCR reactions were performed on GeneAmp PCR system (Applied Biosystem® thermocycler, USA). Each 25μl of reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM each dNTP 0.02 unit/μL Taq DNA polymerase, 0.4 μM each primers and 25 ng template DNA.

Amplification reactions were performed following the programme: pre-denaturation at 94^{o}C followed by 2 minute of denaturation at 94^{o}C, 48-58^{o}C (depending on primers) for 1 minute of annealing (decrease of 1^{o}C in each cycle), 72^{o}C for 1 minute for extension; 32 cycles of repetition. A final extension at 72^{o}C for 2 minutes was performed. Amplification products were visualized by running on 2% agarose gel, following by ethidium bromide staining using 1X TAE buffer. Fragment size was measured using 100 base pairs (bp) molecular sizes ladder (New England Biosys). The bands of DNA were photographed under UV transilluminator with the help of Alpha-Imager Gel Documentation Software.

Data analysis

The banding pattern of each amplified PCR products of various marker systems were scored manually and the data set was assembled in Microsoft Excel spread sheet in format suitable for analysis by NTSYS pc 2.02 (Rohlf, 1998). Determination of polymorphic information content (PIC) was done according to Anderson *et al*. (1993).

$$PIC = 1 - \sum_{i=1}^{n} p_i^2$$

For a single locus, an unbiased estimate of heterozygosity (H) was calculated using Nei's (1978) formula as given below.

$$H = \frac{2n\left(1 - \sum_{i=1}^{n} p_i^2\right)}{(2n-1)}$$

Where, p_i is the frequency of the i^{th} allele in a sample from population and n is the number of alleles

Calculation of the genetic similarities among pair wise comparison of maize inbreds based on data from 10 primer pairs was performed using the method of Jaccard Similarity Coefficient (Jaccard, 1908) as follows.

$$J = \frac{N_{11}}{(N_{11} + N_{10} + N_{01})}$$

Where, N_{11} is number of bands present in both genotypes; N_{10} is number of bands present in one genotypes (lane) and N_{01} is number of bands present in other genotypes

Based on the genetic similarity coefficients obtained with unweighted pair-group method with arithmetic mean (UPGMA), dendogram was made to determine genetic relationships among the inbred lines studied using NTSYS pc 2.02.

RESULTS AND DISCUSSION

SSR polymorphism

Out of 10 pair of SSR used for genotyping of maize inbred lines, 4 marker loci reflected polymorphism across 10 maize inbred lines while remaining six primers were monomorphic showing only one allele in all the tested genotypes. The number of alleles scored across SSR loci ranged from 1 to 6. A total number of 17.00 alleles were detected from polymorphic loci ranging from 2 (bnlg 1832) to 6 (umc 1044 & bnlg 1523) alleles per locus and an average of 4.25 allele (Table 1). The values were somehow close agreement with previous studies using SSR marker on maize inbred lines (Singh and Choudhury, 2013). While studying 124 maize landraces (bulked DNA samples), Qi-Lun *et al*. (2008) also found 6.4 SSR alleles per locus (across 45 loci). High values of effective number of allele have been reported in case of highly and out-crossing crops like maize (Berg and Hamrick, 1997; Wasala and Prasanna, 2013). The occurrence of higher number of allele per locus in present study might be due to use of di-repeat type SSR marker as they are well known for yielding significantly higher number of allele per locus than the primer with longer repeat motif (Chou-kan *et al*., 2006).

Depending on the types of microsatellite loci (homozygotes or heterozygotes), either single band (homozygotes) or double band (heterozygotes) were noticed in each primers. More frequencies of double band in the present study were reported in primer bnlg 1523 (Fig. 1). As the microsatellite is co-dominant, heterozygote produces two bands indicates the amplification of two loci and could be readily identified (Bantte and Prasanna, 2003; Wu *et al*., 2010). The underlying causes for obtaining double bands may be residual heterozygosity (differential drift or fixation of alleles at loci that were heterozygous in the plant from which the line was derived), contamination of the line with pollen or seed of another genotypes, mutation at specific SSR loci, or amplification of similar sequences in different genomic regions due to duplications (Senior *et al*, 1998; Matsuoka *et al*, 2002; Liu *et al*, 2003). The molecular weight of polymorphic bands amplified by SSR primers on 10 maize inbred lines were

Table 1: SSR primers, with their respective sequence, no. of alleles, amplicon length (bp), Polymorphic Information Content (PIC), heterozygosity and annealing temperature (^{0}C) in 10 maize inbreds

SN	Primer	Bin	Repeats	Sequence (5′-3′)	No. of alleles	Amplicon length (bp)	PIC	Hetero zygosity (He)	Ta (°C)
1	umc 1044	1.03	CA(8)	F: CACCAACGCCAATTAGCATCCR: GTGGGCGTGTTCTCCTACTACTCA	6	250-390	0.59	0.62	48
2	bnlg 1811	1.04	AG(16)	F: ACACAAGCCGACCAAAAAACR: GTAGTAGGAACGGGCGATGA	1	260	0.00	0.00	52
3	bnlg 1832	1.05	AG(15)	F: GCGCCCACAACAAGTAAATTR: CCTCATTGTAAGGGGCAGAA	2	260-295	0.32	0.40	57
4	bnlg1523	3.02	AG(17)	F: GAGCACAGCTAGGCAAAAGGR: CTCGCACGCTCTCTCTTCTT	6	280-375	0.71	0.74	56
5	bnlg 1019a	3.04	AG(28)	F: ACCATAGTTGGACGGACCACR: ACCACAACACAGACGAGCAC	1	387	0.00	0.00	58
6	bnlg 589	4.10	AG	F: ACCGGAACAGACGAGCTCTAR: GCGACAGACAGACAGACAAGCGCATTGT	1	280	0.00	0.00	56
7	bnlg 1917	4.10	AG(26)	F: ACCGGAACAGACGAGCTCTAR: TTTGCTTCCAACTCACATGC	1	244	0.00	0.00	51
8	umc2063	5.03	(AGG)4	F: GGACTGAAGCGTGGAATGTTCTR: ATCGCAATCTGAGACCACTTGTT	3	250-295	0.46	0.54	58
9	umc1859	6.06	(TC)8	F: ATATACATGTGAGCTGGTTGCCCTR: GCATGCTATTACCAATCTCCAGGT	1	211	0.00	0.00	55
10	umc1592	8.01	CA	F: GACCATATGTGCTCCAAAACCTTCR: AAGCTTCTTCGGTCTTTGTAGGGT	1	240	0.00	0.00	58
Polymorphic markers				Total	17.00		2.08	2.30	
				Average	4.25		0.52	0.58	

Ta: annealing temperature

Table 2: Distance (dissimilarity) matrix of 10 maize inbred lines

Genotypes	R13-1-10	R13-1-17	CML439	Tarun83-1-3-2	POP31Q	DMR9047	TSK196	R13-1-1	TSK194	TSK197
R13-1-10	0.00	0.32	0.32	0.00	0.32	0.32	0.32	0.32	0.32	0.45
R13-1-17		0.00	0.00	0.32	0.00	0.45	0.45	0.45	0.00	0.32
CML439			0.00	0.32	0.00	0.45	0.45	0.45	0.00	0.32
Tarun83-1-3-2				0.00	0.32	0.32	0.32	0.32	0.32	0.45
POP31Q					0.00	0.45	0.45	0.45	0.00	0.32
DMR9047						0.00	0.45	0.45	0.45	0.55
TSK196							0.00	0.45	0.45	0.55
R13-1-1								0.00	0.45	0.55
TSK194									0.00	0.32
TSK197										0.00

ranged from 250-390bp. In a study, Al-Badeiry *et al*. (2014) found that the molecular weight of band obtained from amplification of SSR products were ranged from 91-288bp while Daniel *et al*. (2012) detected a ranged of 200-500bp for amplified band of SSR primers.

Polymorphic information content (PIC)

The polymorphic information content (PIC) value estimated for all SSR marker varied from 0.00 to 0.71. The PIC value for polymorphic loci ranged from 0.32 (bnlg 1832) to 0.71 (bnlg 1523) with an average of 0.52 (Table 1). Two marker namely umc 1044 and bnlg 1523 expressed PIC more than average indicating that they are highly informative to detect differences among the inbred lines. High average of polymorphism level indicates high genetic variation among maize inbred lines. Similarly, the PIC value of other studies of maize genotypes also varies according to the primer tested (for e.g. Singh and Choudhury, 2013; Elçi and Hançer, 2014), supporting high information and polymorphism detected by the SSR primers. Occurrence of high mean PIC in present study was particularly due to use of di-nucleotide primers. The dinucleotide SSR primers would result higher mean number of allele per locus and mean PIC value compared to the tri- and/or tetra-nucleotide primers (Adetimirin *et al*, 2008; Serumaga *et al*., 2014).

Expected heterozygosity (He)

The expected heterozygosity for SSR primers used in this study varied from 0.00 to 0.74. Concomitant with the higher number of alleles, the polymorphic SSR loci has high gene diversity or expected heterozygosity, ranges from 0.40 (bnlg 1832) to bnlg 1523 (0.74) with mean of 0.58 (Table 1). For any given number of allele, the expected heterozygosity would be highest when all the allelic frequencies are equal. Higher mean expected heterozygosity reflected the presence of high allelic variation in the marker loci and their distribution in landraces and it also revealed the presence of high level of polymorphism in the inbreds chosen. It also gives idea about the information available from the SSR marker and their potential to discriminate the maize lines based on their genetic relation. In present study, SSR loci 1523 with high expected heterozygosity (0.74) is highly informative and have potentiality to discriminate the maize inbred lines studied. Serumaga *et al*. (2014) reported a range of 0.18 to 0.92 for gene diversity with an average of 0.65. Also, Morales *et al*. (2010) reported a mean value of heterozygosity of 0.54. Similarly, the results are in close proximity with the findings reported by others in studies of maize inbred lines with SSR primers (Senior *et al*, 1998; Vaz Patto *et al*, 2004).

Genetic distance and cluster formation

The genetic relationship between the maize inbred lines was measured through Jaccard's dis-similarity matrix (Table 2). The genetic similarity index marks the closeness relationship among the genotypes. The dis-similarity index ranged from 0.00-0.55 indicating the presence of substantial variability among the inbred lines studied. The lowest genetic distance (0.00) was observed between the inbred lines R13-1-10 & Tarun 83-1-3-2; and R13-1-17, CML 439, POP31Q & TSK 194 pointed out that the presence of similarity between these lines is high degree with SSR, while DMR 9047, TSK 196, R13-1-1 and TSK 197 manifested highest genetic distance (0.55) marking lowest

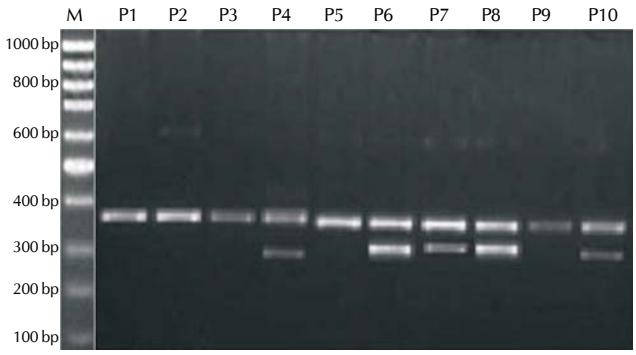

Figure 1: SSR profiling for bnlg1523 primer (M: Marker ladder; P1-P10: Maize inbred lines)

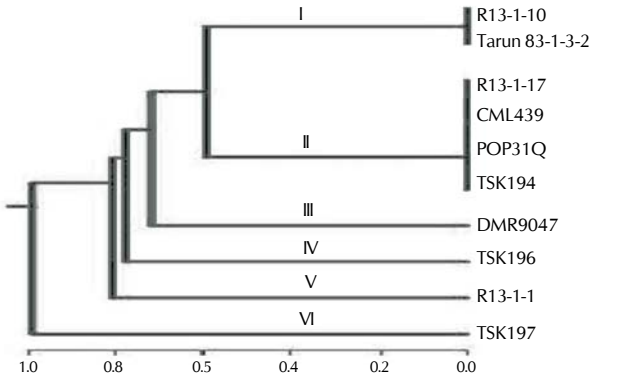

Figure 2: Dendogram showing clustering of 10 maize inbreds using UPGMA based on Jaccard's coefficients obtained from SSR profiling

degree of similarity between them. Genetic distance of 0.24 to 0.78 was reported by Al-Badeiry *et al*. (2014) while Babu *et al*. (2012) reported a range of genetic similarities of 0.16 to 0.88. However, the present result somehow deviated from these result.

A dendogram, a graph of genetic relationship, based on the genetic similarity coefficients obtained with UPGMA, clustered 10 maize genotypes into 6 heterotic groups (Figure 2) with the genetic similarity coefficient of 0.55. Cluster I included R13-1-10 and Tarun 83-1-3-2 as they have 100% similarity. In addition, cluster II comprised R13-1-17, CML 439, POP31Q and TSK 194 while remaining clusters (III to VI) contained solitary genotypes. The SSR markers largely separated the inbred lines into different cluster signifies that these inbreds are highly diverse, thus heterotic segregates would be obtained when crossing between these inbred is made. The SSR primers used were unable to differentiate the inbred *viz*. R13-1-10, Tarun 83-1-3-2, R13-1-17, CML 439, POP31Q and TSK 194. Singh and Choudhury (2013) reported 2 main clusters along with 2 sub-groups while using six SSR primers in eight maize inbreds. Similarly, Efendi *et al*. (2015) reported six heterotic groups for 51 maize inbred at genetic similarity coefficient of 0.35. The grouping of the 10 maize inbred based on SSR profiling was partially congruent with their pedigree, which might be due to small number of SSR primers used in study as well as broad genetic base of the source population.

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