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Simple sequence repeat (SSR) markers for molecular diversity and heterozygosity analysis in maize (*Zea mays* L.) inbred lines

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Abstract
An insight into the genetic diversity and relationships among the germplasm is important in any crop improvement programme. An effort was made to study the genetic diversity, polymomorphism and heterozygosity, among 10 maize inbreds using ten simple sequence repeats (SSR), spread across six maize chromosome *viz,* 1, 3, 4, 5, 6 and 8 during *Kharif* 2015. DNA from two week old young fresh healthy leaves was isolated using CTAB method and subjected to SSR marker analysis. A total of 14 alleles were detected across 4 polymorphic loci ranging from 2 (*bnlg* 1917) to 5 (*bnlg* 589) alleles per locus and an average of 3.50 allele. Mean value of polymorphic information content (PIC) was 0.49 which ranged from 0.26 (bnlg 1917) to 0.73 (bnlg 589). The mean value for expected heterozygosity (He) was 0.55 which varied from 0.32 (bnlg 1917) to 0.76 (bnlg 589), respectively. The Jaccard's dis-similarity index was obtained ranged from 0.00 to 1.00.The Jaccard's similarity coefficient divided maize inbred lines into five heterotic groups, where, Cluster I, III, IV and V contained one genotype each, and Cluster II- contained six genotypes. The study revealed that SSR markers are useful tool to explore the molecular diversity among the maize genotypes, as they are not influenced by the environment which can help breeders for selection of diverse parental lines which is useful for hybridization programme in heterosis breeding for maize yield improvement.

Keywords: CTAB, Genetic diversity, Heterozyogosity, Maize, PIC, Simple Sequence Repeats (SSR), Polymorphism, UPGMA.

Introduction
Maize (*Zea mays* L.) is most widely distributed and versatile food crop of the world, grown in tropical, sub-tropical and temperate regions and being high productive among other cereals, also known as Queen of cereals [27]. Since maize is cheap, it becomes the dominant food and main source of dietary energy and protein for the poor, particularly in rural and underprivileged segments of the society especially in our country. For exploiting heterosis [28] the potential of hybrid breeding in maize many breeders are trying to harness hybrid vigour to improve the yield and quality traits of maize in order to cope up with the increasing demand. While many maize inbreds in the past they have been developed from a limited number of elite lines and elite line synthetics, a practice that heightens the risk of decreased genetic diversity in commercial maize production fields [12].

Information on genetic diversity present in germplasm assists in the selection of parent and accelerates the technique on the genetic gain. Better understanding on the genetic diversity ensures the breeder in planning crosses for hybrid and line development, in assigning lines to heterotic groups and in plant variety protection [21]. The developments during the past three decades in the DNA marker technology are enormous and an array of DNA markers is made available as a tool to assess the genetic diversity in plants and animals. Genetic diversity studies using molecular markers reveal patterns of diversity in crops that are obscured by the complexities of pedigree records. With the initiation of molecular markers, the study of genetic variability 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 [14], are widely used in maize, as these markers are mapped, PCR-based, genetically co-dominant, hyper-variable, highly polymorphic [9], robust, reproducible and amenable to automation [22]. Several studies have profoundly used SSR markers to evaluate the molecular profiling of maize for drought tolerance [18], disease resistance [29], qualitative characters [20], genetic diversity [11] and genetic purity [1]. Molecular markers are definitely a powerful tool to delimit heterotic groups and to assign inbred lines into existing heterotic groups [17].

It is widely believed that the level of genetic distances between two inbred lines has an influence on the performance of resulting hybrids [30].Several attempts have been made so far to characterize maize landraces using SSR markers. A study was made to measure the genetic diversity among the 10 maize inbreds using microsatellite markers (SSR) for the selection of diverse lines as parents for single cross hybrid production.

Materials and Methods

Plant material

A total of 10 inbred lines belonging to diverse origin were used as plant material collected from the Department of Genetics and Plant Breeding, Sam Higginbottom University of Agriculture Technology and Sciences, Allahabad, India (Table 1). The seedlings were raised in pots and maintained in a laboratory condition till the 3-5 functional leaf formation during *Kharif*, 2015 in the Department of Genetics and Plant Breeding. These lines were early to intermediate in maturity, yellow in seed color and flint to dent in texture and resistant to major diseases and stem borers. 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 [25], 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 65ºC in water bath for 45 minutes with occasional swirling. Emulsified the mixture with equal volume of chloroform iso-amyl alcohol (24:1) and then centrifuged at 10,000 rpm for10 minutes. Added 10 µLRNase A after transferring upper aqueous layer in fresh 50 ml centrifuge tube and incubated at 37ºC 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 electrophoresed on 0.8% (w/v) agarose gels for quantification, stained with 5x gel loading dye and photographed under UV trans-illuminator attached to gel documentation system (Fig 1).

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 mMTris-HCl (pH 8.3), 50 mMKCl, 2 mM $MgCl_2$, 0.2 mM each dNTP 0.02 unit/µLTaq DNA polymerase, 0.4 µM each primers and 25 mg template DNA. Amplification reactions were performed following the programme: pre-denaturation at 94 ºC followed by 2 minute of denaturation at 94ºC, 48-58ºC (depending on primers) for 1minute of annealing (decrease of 1oC in each cycle), 72ºC for1 minute for extension; 32 cycles of repetition. A final extension at 72ºC 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 trans-illuminator 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 [24]. Determination of polymorphic information content (PIC) was done [2].

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

For a single locus, an unbiased estimate of heterozygosity (H) [19] was calculated using the formula as given below.

$$H = 2n\left(1 - \sum_{i=1}^{m} P_i^2\right) / (2n - 1)$$

Where, *pi* 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 [13] as follows.

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

Where, N11 is number of bands present in both genotypes, N10 is number of bands present in one genotype (lane) and N01 is number of bands present in other genotypes.

Based on the genetic similarity coefficients obtained with un-weighted 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

The experimental results revealed that out of 10 SSR primers 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 5. A total number of 14.00 alleles were detected, from polymorphic loci ranging from 2 (*bnlg* 1917) to 5 (*bnlg* 589) alleles per locus and an average of 3.50 alleles per locus (Table 2). The values were somehow close agreement with previous studies using SSR marker on maize inbred lines [31]. While studying 124 maize landraces (bulked DNA samples [23] 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 outcrossing crops like maize [34]. 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 [7]. 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 *bnlg589* (Fig. 2). As the microsatellite is co-dominant, heterozygote produces two

bands indicates the amplification of two loci and could be readily identified [6]. 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 [15]. The molecular weight of polymorphic bands amplified by SSR primers on 10 maize inbred lines were ranged from 250-800bp. In a study, [4] found that the molecular weight of band obtained from amplification of SSR products were ranged from 91-288bp while [8] detected a ranged of 250-800bp 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 0.73. The PIC value for polymorphic loci ranged from0.26 (bnlg 1917) to 0.73 (bnlg 589) with an average of 0.49 (Table 2). One marker namely bnlg 589 expressed PIC which is more than average indicating that it was 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 [3], 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 [32].

Expected heterozygosity (H*e*)

The expected heterozygosity for SSR primers used in this study varied from 0.00 to 0.76. Concomitant with the higher number of alleles, the polymorphic SSR loci has high gene diversity or expected heterozygosity, ranges from 0.32 (*bnlg* 1917) to 0.76 (*bnlg* 589) with an average of 0.55 (Table 2). 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 [33]. 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 589 with high expected heterozygosity (0.76) is highly informative and have potentiality to discriminate the maize inbred lines studied. Also, it was earlier reported [16] also found that a mean value of heterozygosity of 0.54 in maize lines. Similarly, the results are in close proximity with the findings reported by others in studies of maize inbred lines with SSR primers [26].

Genetic distance and cluster formation

The genetic relationship between the maize inbred lines was measured through Jaccard's dis-similarity matrix (Table 3). The genetic similarity index marks the closeness relationship among the genotypes. The dis-similarity index ranged from 0.00-1.00 indicating the presence of substantial variability among the inbred lines studied. The lowest genetic distance (0.00) was observed between the inbred lines CML 41 and HKI 193-2, POP31Q and CML 359 manifested highest genetic distance (1.00) making lowest degree of similarity between them. Genetic distance of 0.24 to 0.78 was also reported of genetic similarities of 0.16 to 0.88 [5].

Dendogram

A dendogram is a graph representation of genetic relationship, based on the genetic similarity coefficients obtained with UPGMA, clustered 10 maize genotypes into 5 heterotic groups (Figure 5) with the genetic similarity coefficient of 0.65. Cluster I contained one genotype Early yellow, while cluster II consisted of six genotypes CML 359, LM 13, CM 124, DMRQPM 28, CML 41 and HKI193-2. Cluster III however consisted of one genotype CM 129, Cluster IV with POP 445, while Cluster V contained only POP 31 inbred. Similarly, the results were well supported by the findings of earlier researchers [10] and [35] who reported more heterotic groups for 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.

Conclusion

Based on the present results the authors report that the SSR technique was efficient in detecting polymorphism and determining genetic divergence among the maize genotypes used in the investigation. However, more number of markers should be used to assess their consistency. The SSR markers largely separated the inbred lines into 5 cluster's, which signifies that these maize inbreds; HKI 193-2, CM-129, POP 31, Early Yellow and POP 445 are highly diverse, as they belonged to different clusters, thus heterotic segregates would be obtained when crossing among these diverse maize lines is made for achieving a quantum jump in present maize grain and also fodder yield of the country, without compromising the nutritional security for millions of the rural masses at large.

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Table 1: Characteristics and sources of maize inbred lines used in the present investigation.

S.N.	Maize lines	Parent code	Pedigree	Kernel Texture	Maturity	Resistance /tolerance	Quality Protein Maize	Center developed
1	Early yellow	(P_1)	[P30/P45/2/M162W/MSR]	F	Early	Resistance to E. turgidium.	No	CIMMYT, Mexico
2	POP- 445	(P_2)	S2PoolC21SEY90C1	D	Early	Drought tolerance	No	CIMMYT, Mexico
3	POP -31 Q2	(P_3)	P31DMR#1-55-2-3-2-1-BBBBBBB	F/SD	Early	Resistance to Downey mildew	No	CIMMYT, Mexico
4	CML -359	(P_4)	SA3C4HC(16X25)-2-4-3-1-B	F	Intermediate	Resistant to Lodging	No	CIMMYT, Mexico
5	CM 129	(P_5)	G32c19#32-1#1-5-1#b1#b1-2-b1-b1#b1	F	Early	Resistant to Lodging	No	VPKAS, Almora, Uttarakhand
6	LM 13	(P_6)	JCY3-7-1-2-2-1-1 f	F	Late	Resistant to Lodging	No	PAU, Ludhiana
7	CM- 124	(P_7)	(P47/Mp704)-3-1-8-2-4-1-4-b1-b1#b1	F	Early	Resistant to Lodging	No	IIMR, New Delhi
8	DMRQPM28	(P_8)	Shakti (SO) HE25# Chain cross bulk 50% F-#-⊗-1-3-B-4-BB	F	Intermediate	Resistant to Lodging	Yes	IIMR, New Delhi
9	CML- 41	(P_9)	Pob36C5HC223-1-1-2-3-#-1-1-##	SD	Intermediate	Resistant to Lodging	No	CIMMYT, Mexico
10	HKI-193-2	(P_{10})	CYO162-B-1-1-B	F	Late	Resistant to Lodging	Yes	Karnal, Haryana

F-Flint, D-Dent, SD-Semi dent

Table 2: 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	Heterozygosity (H*e*)	Ta (^{o}C)
1	umc 1044	1.03	CA(8)	F:CACCAACGCCAATTAGCATCC R:GTGGGCGTGTTCTCCTACTACTCA	1	250	0.00	0.00	48
2	bnlg 1811	1.04	AG(16)	F:ACACAAGCCGACCAAAAAAC R: GTAGTAGGAACGGGCGATGA	1	260	0.00	0.00	52
3	bnlg1832	1.05	AG(15)	F: GCGCCCACAACAAGTAAATT R: CCTCATTGTAAGGGGCAGAA	1	260	0.00	0.00	57
4	bnlg1523	3.02	AG(17)	F: GAGCACAGCTAGGCAAAAGG R: CTCGCACGCTCTCTCTTCTT	1	270	0.00	0.00	56
5	bnlg 1019	3.04	AG(28)	F: ACCATAGTTGGACGGACCAC R:ACCACAACACAGACGAGCAC	4	387-800	0.52	0.59	58
6	bnlg 1917	4.10	AG(26)	F: ACCGGAACAGACGAGCTCTA R: TTTGCTTCCAACTCACATGC	2	244-260	0.26	0.32	51
7	bnlg 589	4.10	AG	F: ACCGGAACAGACGAGCTCTA R:GCGACAGACAGACAGACAAGCGCATTGT	5	280-560	0.73	0.76	56
8	umc2063	5.03	(AGG)4	F: GGACTGAAGCGTGGAATGTTCT R: ATCGCAATCTGAGACCACTTGTT	1	250	0.00	0.00	58
9	umc1859	6.06	(TC)8	F: ATATACATGTGAGCTGGTTGCCCT R: GCATGCTATTACCAATCTCCAGGT	1	211	0.00	0.00	55
10	umc1592	8.01	CA	F: GACCATATGTGCTCCAAAACCTTC R: AAGCTTCTTCGGTCTTTGTAGGGT	3	240-325	0.47	0.56	58
Polymorphic markers				Total	14.0		1.98	2.23	
				Average	3.50		0.49	0.55	

Table 3: Distance (dissimilarity) matrix of 10 maize inbred lines

Genotypes	Early yellow	POP-445	POP31 Q2	CML359	CM 129	LM 13	CM 124	DMR QPM-28	CML 41	HKI 193-2
Early yellow	0	0.78	0.89	0.45	0.55	0.45	0.45	0.45	0.45	0.45
POP 445		0.00	0.78	0.63	0.71	0.63	0.63	0.63	0.63	0.63
POP 31 Q2			0.00	1.00	0.95	1.00	1.00	1.00	1.00	1.00
CML 359				0.00	0.32	0.00	0.00	0.00	0.00	0.00
CM 129					0.00	0.32	0.32	0.32	0.32	0.32
LM 13						0.00	0.00	0.00	0.00	0.00
CM 124							0.00	0.00	0.00	0.00
DMR QPM-28								0.00	0.00	0.00
CML 41									0.00	0.00
HKI193-2										0.00

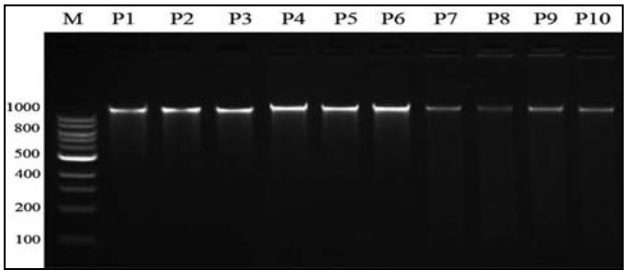

Fig 1: DNA quantification image using 0.8% agarose gel

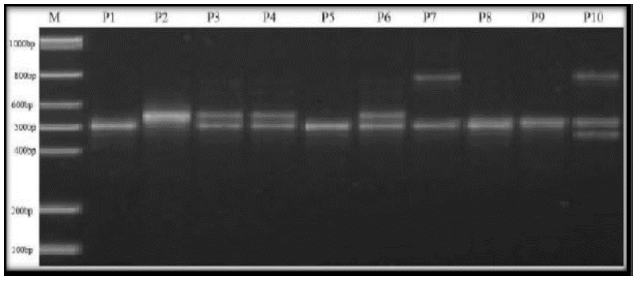

Fig 2: SSR profiling for bnlg 1019 primer (M:marker ladder;P1-P10 :10 Maize inbred lines)

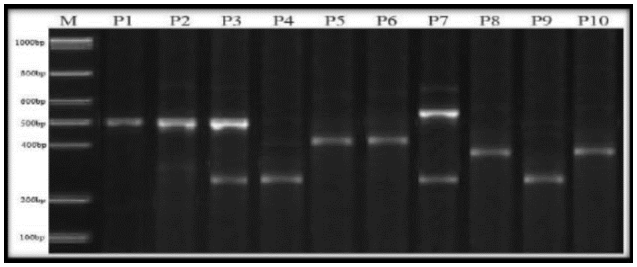

Fig 3: SSR profiling for bnlg 589 primer (M:marker ladder;P1-P10 :10 Maize inbred lines)

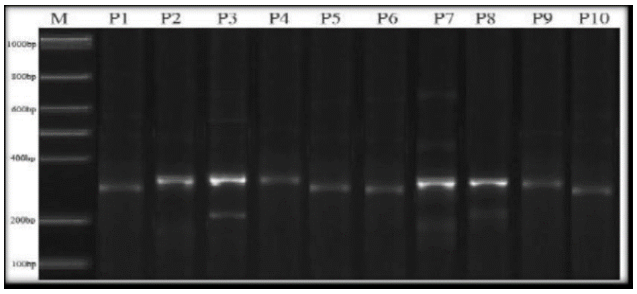

Fig 4: SSR profiling for umc 1592 primer (M:marker ladder;P1-P10 :10 Maize inbred lines)

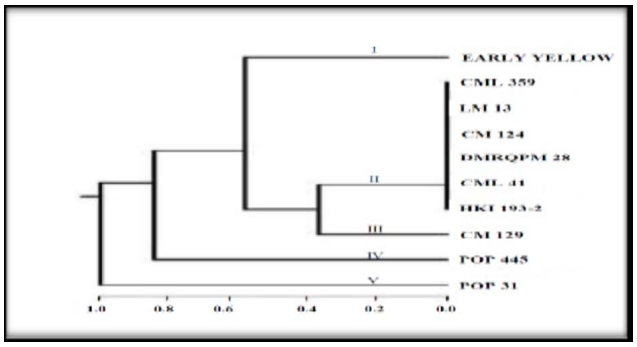

Fig 5: Dendogram showing clustering of 10 maize inbreds using UPGMA based on Jaccard's coefficients from SSR profiling

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