African Journal of Biotechnology Vol. 8 (3), pp. 375-379, 4 February, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Genetic diversity of Pakistani maize genotypes using chromosome specific simple sequence repeat (SSR) primer sets

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Accepted 30 October, 2007

For improvement of maize crop presence of genetic diversity in the germplasm is very important. This study was conducted to determine genetic diversity among 17 Pakistani maize genotypes using 10 simple sequence repeat (SSR) primer sets. All the amplification products were in the range of <250-750 bp. To estimate the genetic diversity among the genotypes, bivariate data matrix was generated and genetic distances were calculated. High degree of genetic polymorphism was observed among the maize genotypes with overall genetic distance ranged from 0 - 100% and average genetic distance ranged from 0 - 81%. A total of 196 alleles were amplified with an average of 1.56 alleles per-microsatellite/genotypes locus. SSR primer sets p-umc1354, p-umc1984, p-umc1186, p-umc1325, p-umc2281, p-umc1586, p-umc1824 and p-umc1154 amplified 2.90, 1.06, 2.00, 1.35, 1.50, 1.50, 1.00 and 1.18 loci per genotype, respectively. Two SSR primer sets p-mmc0411 and p-umc1184 amplified none of the genomic DNA of 17 genotypes. Two genetically most diverse comparisons (population 9804 vs pahari and population 9804 vs CGCW) were identified and recommendations have been made to use these comparisons of maize genotypes in further breeding programs.

Key words: *Zea mays*, SSR markers, genetic diversity.

INTRODUCTION

Maize is an annual, cross-pollinated plant. It is an angiosperm and monocot. There are various subspecies of maize based on their use as food including flour corn - *Zea mays* L. subsp. *mays* Amylacea Group, popcorn - *Zea mays* L. subsp. *mays* Everta Group, Dent corn - *Zea mays* L. subsp. *mays* Indentata Group, Flint corn - *Zea mays* L. subsp. *mays* Indurata Group and sweetcorn - *Zea mays* L. subsp. *mays* Saccharata Group.

Maize has 10 chromosomes (2 n=2 x=20). The total genetic length of these chromosomes is roughly 1500cm. Many maize chromosomes have chromosomal knobs, or highly repetitive heterchromatic regions which stain darkly on chromosome spreads. Along with the standard complement of chromosomes, maize can also have several unusual chromosomes.

To improve genetic diversity of local germplasm, it is important to know the extent of already existing genetic variability in the material. To estimate genetic diversity in maize germplasm, various kinds of markers can be used. In the past morphological traits were used as markers for assessing genetic diversity but these markers are often influenced by the environment, and are unreliable. Later, bio-chemical and cytological markers were used to monitor germplasm biodiversity. But these markers were limited in number and hence cannot be used to study complete genome of the species (Islam and Shepherd,

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Table 1. Seventeen Pakistani maize genotypes used for genetic diversity analysis using simple sequence repeat (SSR) primer sets.

S/N	Genotype
1	Sarhad yellow
2	Sarhad white
3	Population 2004 BS
4	Population 9804
5	CCCY
6	EV 32ES
7	EV 9806
8	Jalal
9	WD 3 x 6
10	Pahari
11	CGCW
12	Kisan
13	Azam
14	Ghauri
15	EV9801
16	Population 9801
17	Population 9805

1991). More recently, DNA based markers have enhanced the utilization of biotechnology in crop improvement (Miller et al., 1989). These DNA markers, when tightly linked to genes of interest, can be used to select indirectly for the desirable allele and this represents the simplest form of Marker Assisted Selection (MAS). Molecular markers are also used to study the level of genetic diversity among different cultivars/closely related species/gene bank accessions (Mullis, 1990). Molecular markers have also been very valuable for improving the understanding of genome structure and function and have allowed the isolation of genes of interest through cloning (Devos and Gale, 1992).

Among DNA markers, most commonly used are Polymerase Chain Reaction (PCR), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP) and Single Nucleotide Polymorphism (SNIP). Among these techniques, PCR based assays are more commonly used because they are easier, cheaper and faster than RFLP, AFLP and SNIPS. There are various kinds of PCR based assays viz., Randomly Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR), Allele-Specific Amplification (ASA), Cleavage Amplification Polymorphic Sequences (CAPS), etc. SSR/microsatellites are small DNA sequences, usually 2-6 bp in length and are dispersed throughout the genome of eukaryotes. These PCR based assays are efficiently being used to study genetic diversity of commercial crops like maize wheat, barley etc. (Cao et al., 1998; Sun et al., 1998).

At present SSRs are the most promising molecular markers which are able to identify or differentiate genotypes within a species. SSRs are ubiquitously interspersed in eukaryotic genomes and can find applications as highly variable and multi allelic PCR based genetic markers (Brown et al., 1996). The application of SSR techniques to finger print plant species was first reported by Akkaya et al. (1992). The high level of polymorphism and easy handling has made SSRs extremely useful for different applications in crop improvement (Stachel et al., 2000). The present study was carried out in order to study genetic variation at DNA level among various Pakistani maize genotypes using linkage group (chromosome specific SSR primer sets) and finding genetically most diverse genotypes of maize which can further be utilized in hybridization programs to create genetically diverse germplasm of maize.

MATERIAL AND METHODS

Plant material

Seventeen maize genotypes (Table 1) obtained from Maize Botanist at Cereal Crop Research Institute, Pirsabak, Pakistan were used to study the level of genetic polymorphism using DNA based markers (Simple Sequence Repeat). The plants were grown in green house of the Institute of Biotechnology and Genetic Engineering, NWFP Agricultural University Peshawar, Pakistan, during the year 2006.

DNA isolation

Total genomic DNA was isolated from maize plants according to the method of Weining and Langridge, 1991. Three young fresh leaves of 10 cm length were collected in eppendorf tubes and subsequently frozen in liquid nitrogen. Leaf material was then crushed with knitting needle to make a fine powder. Five hundred micro-liter extraction buffer (1% SDS, 100 mM NaCl, 100 mM Tris, 100 mM Na_2EDTA, pH 8.5 by HCl) was added to each eppendorf tube containing the crushed leaf material and was thoroughly mixed. Equal volume (500 µl) of phenol ; chloroform : isoamyl alcohol (ratio of 25:24:1) was added and tubes were then shaken until a homogenous mixture was obtained. Samples were then centrifuged at 5000 rpm for 5 min in bench centrifuge. The aqueous phase was transferred to a fresh tube. One tenth volume (50 µl) sodium acetate (pH 4.8) and an equal volume (500 µl) isopropanol were added in the tube and were mixed gently to precipitate the DNA. Samples were centrifuged at 5000 rpm for 5 min to pellet the DNA. After discarding the supernatant, the pellet was washed with 70% ethanol. Pellet was dried at room temperature for an hour and re-suspended in 40 µl of TE buffer (10 mM Tris, 1 mM Na_2 EDTA, pH 8.0). To remove RNA, DNA was treated with 40 µg RNAse-A at 37°C for 1 h. After RNase treatment, DNA samples were stored at 4°C. To use in Polymerase Chain Reaction (PCR) 1:5, dilution of DNA was made in double distilled de-ionized and autoclaved water.

Polymerase chain reaction (PCR)

All PCR reactions were carried out in 25 µl reaction containing 1 µl total genomic DNA solution, 0.25 µM of each primer, 200 µM of each dATP, dGTP, dCTP, dTTP, 50 mM KCl, 10 mM Tris, 1.5 mM $MgCl_2$ and 2.5 units of Taq DNA polymerase (Devos and Gale, 1992). Amplification conditions were: an initial denaturation step of

Table 2. Locus name, linkage group, sequence and annealing temperature of ten SSR primers sets used to study genetic diversity analysis in Pakistani maize genotypes.

Locus	Linkage group (Chrom.)	Primer sequence (5′ - 3′)	Annealing temp (°C)
*xp-umc*1354	1	Forward: GATCAGCCCGTTCAGCAAGTT Reverse: GAGTGGAGGCGGAGGATCTG	54.8
*xp-umc*1824	2	Forward: ATCGTGCTTAAGCGGTTATAGGAAT Reverse: TGCACATGCTTTGTATAAGATGCC	54
*xp-umc*1184	3	Forward: AGAAGAAGAGGAGGTTCCATGACC Reverse: GCATGTTTCCCTTTCACCTCC	54.8
*xp-umc*2281	4	Forward: CAATGATTGGAGCCTAACCCCT Reverse: ATGATGATCTGCAGAGCCTAGTCC	55.1
*xp-umc*1325	5	Forward: ATATTGTACAGGAGCAGCTGGGAC Reverse: GGAGGTCATGCGTGTAAATAGGTC	57.4
*xp-umc*1186	6	Forward: TCAAGAACATAATAGGAGGCCCAC Reverse: AGCCAGCTTGATCTTTAGCATTTG	54
xp-mmc 0411	7	Forward: CGATGCAAGAGTGTCAAGTA Reverse: ACTCCCTAGTGCAAAAATCA	48.3
*xp-umc*1984	8	Forward: CTCTGGCCTCTGATACCAGTTGAT Reverse: CATCCTCCTGCAGCTGTTAACTC	57.4
*xp-umc*1586	9	Forward: TAGGAGATGAGCTCGTCGGATAAG Reverse: GAGGATGAGGAGGATGGCAATGGT	57.4
*xp-umc*1154	10	Forward: GTAGAGATCGATTCGCTAACCTGC Reverse: AGTTGTTCCGTTCCGTCCTTATC	55.4

4 min at 94°C, followed by 40 cycles each consisting of a denaturation step of 1 min at 94°C, annealing step of 1 min, and an extension step of 2 min at 72°C. The last cycle was followed by 10 min extension at 72°C. Annealing temperatures of all the primers along with their linkage groups and sequences are given in Table 2. All amplification reactions were performed using GeneAmp PCR System 2700 (Applied Biosystem) programmable thermocycler. The amplification products were electrophoresed on 1.5% agarose/TBE gel, and visualized by staining with ethidium bromide under ultra-violet (U.V) light and photographed using gel documentation system "Uvitec".

Statistical analysis

For statistical analysis, every scorable band was considered as a single locus/allele. The loci were scored as present (1) or absent (0). Bi-variate 1-0 data matrix was generated. Genetic distances were calculated using "Unweighted Pair Group of Arithmetic Means" (UPGMA) procedure described by Nei and Li (1979).

GD=1-dxy/dx+dy-dxy, where GD = Genetic distance between two genotypes, dxy = total number of common loci (bands) in two genotypes, dx = total number of loci (bands) in genotype 1 and dy = total numbers of loci (bands) in genotype 2.

RESULTS

During the present study, 10 simple sequence repeat (SSR) primer sets were used to detect and estimate genetic polymorphism based on co-dominant marker system and to find out the most diverse maize genotype for future breeding programs.

Microstallites displayed a high level of polymorphism in the present study. A total of 196 alleles were amplified with an average of 1.56 alleles per-microsatellite /genotypes locus. The highest number of alleles per locus/ genotype was detected using SSR primer set p-umc1354, showing 43 alleles with an average of 2.90 alleles per genotype. The lowest allele number per locus among the homologous chromosomes was observed using SSR primer set p-umc1824 and SSR primer set p-umc1984, showing a total of 12 and 18 alleles with an average of 1.00 and 1.06 alleles per genotype, respectively. Total genomic DNA isolated from 17 maize genotypes did not produce any useful polymorphism using SSR primer sets p-mmc0411 and p-umc1184. It may be concluded that either the site recognized by the primer sets was absent in the 17 genotypes or poly-merase chain reactions (PCR) did not work properly.

The range of genetic dissimilarity (estimated as genetic distance) was from 0 - 100%. Average genetic distances among the genotypes ranged from 0 - 81% (Table 3). Highest genetic distance (81%) was observed for two comparisons (Population 9804 vs Pahari and Population 9804 vs CGCW). The size of amplified fragments for 10 SSR primer sets used ranged from <250–750 bp. Maxi-

Table 3. Average estimates of genetic distances at DNA level among 17 Pakistani maize genotypes using 10 chromosome specific simple sequence repeat (SSR) primer sets.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1																
2	0.41															
3	0.40	0.48														
4	0.45	0.56	0.43													
5	0.26	0.50	0.55	0.63												
6	0.40	0.33	0.41	0.50	0.43											
7	0.17	0.00	0.00	0.33	0.25	0.00										
8	0.30	0.43	0.45	0.62	0.33	0.30	0.25									
9	0.43	0.38	0.39	0.58	0.44	0.48	0.25	0.20								
10	0.46	0.50	0.58	0.81	0.38	0.61	0.50	0.33	0.41							
11	0.46	0.57	0.46	0.81	0.33	0.63	0.38	0.33	0.40	0.14						
12	0.32	0.45	0.55	0.55	0.52	0.37	0.33	0.29	0.31	0.38	0.43					
13	0.26	0.50	0.49	0.23	0.41	0.56	0.38	0.44	0.44	0.61	0.58	0.33				
14	0.26	0.64	0.62	0.23	0.54	0.56	0.63	0.56	0.56	0.61	0.66	0.33	0.13			
15	0.25	0.50	0.60	0.57	0.38	0.54	0.38	0.52	0.60	0.50	0.56	0.50	0.52	0.40		
16	0.38	0.29	0.32	0.65	0.44	0.48	0.17	0.41	0.31	0.64	0.36	0.41	0.40	0.46	0.31	
17	0.43	0.50	0.58	0.33	0.42	0.48	0.38	0.62	0.69	0.69	0.60	0.54	0.26	0.33	0.58	0.56

1 = Sarhad yellow, 2 = Sarhad white, 3 = Population 2004 BS, 4 = Population 9804, 5 = CCCY, 6 = EV 32ES, 7 = EV 9806, 8 = Jalal, 9 = WD 3x6, 10 = Pahari. 11 = CGCW, 12 = Kisan, 13 = Azam, 14 = Ghauri, 15 = EV9801, 16 = Population 9801, and 17 = Population 9805.

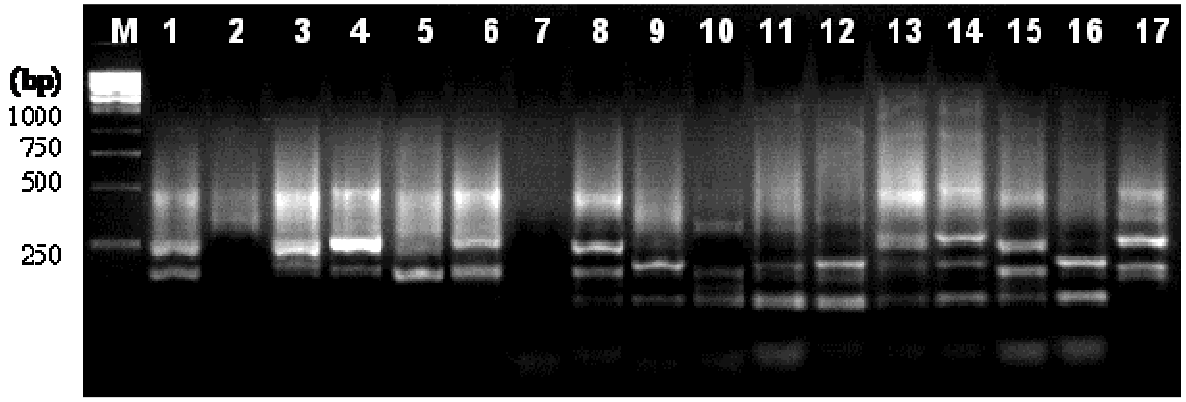

Figure 1. PCR Amplification profile of 17 Pakistani maize genotypes using SSR primer set p-umc1354. M = Molecular size marker (1 Kbp ladder). (1 = Sarhad yellow, 2 = Sarhad white, 3 = Population 2004 BS, 4 = Population 9804, 5 = CCCY, 6 = EV32ES, 7 = EV 9806, 8 = Jalal, 9 = WD 3x6, 10 = Pahari. 11 = CGCW, 12 = Kisan, 13 = Azam, 14 = Ghauri, 15 = EV9801, 16 = Population 9801, and 17 = Population 9805).

mum size (750 bp) of amplified fragment was observed for SSR primer set p-umc1186, while minimum size (<250 bp) was observed in p-umc1354 (Figure 1).

DISCUSSION

Genetic diversity is of prime importance for the survival, adaptation to certain agro-climatic conditions, success and improvement of any crop species. Unless there is not enough genetic diversity in the germplasm, it is practically not possible to increase the yield and other desirable characters of the crop, because selection for the improved genotypes depends on the availability of genetic variability within the breeding material.

There is a need to characterize the local germplasm of maize (Zea *mays* L.*)* using available methods. Phenotypic/morphological characterization (previously used) is easy and cheaper way to characterize the germplasm, but it has its own limitations, because very few morphological characters can be utilized for characterization. Similarly, biochemical characterization (Koebner and Shepherd, 1986; Rogovaski et al., 1991, Islam and Shepherd, 1992), though successful in many cases but because of limited number of biochemical marker loci

(Hart, 1987) the technique could not be used on wider scale. Recent development of molecular genetic technique, especially RFLP (Paterson, 1991), Simple Sequence Repeat (Roder et al., 1998) and RAPD (Williams et al., 1990) have transformed the opportunities of utilizing molecular biology for characterization of crop species. These molecular biology techniques have been used to characterize germplasm of various crop species including maize, wheat and barley.

The genomes of all eukaryotes contain a class of sequences, termed microsatellites or simple Sequence Repeats (SSRs) (Litt and Luty, 1989). The SSR technique gained rapid acceptability because of its codominant nature, reproducibility, and high information content (De Loose and Gheysen, 1995). Microsatellites with tandem repeats of a basic motif of <6 bp have emerged as important source of ubiquitous genetic markers for many eukaryotic genomes. These are ideal genetic markers for detecting differences between and within species. SSRs have been used extensively for designing primers which are not only highly polymorphic but also species specific (Pestova et al., 2000). Genetic diversity plays an important role in crop improvement and was demonstrated through SSR primer sets.

The current study strengthened earlier reports that SSRs can be used for estimation of genetic diversity in maize improvement (Liu et al., 2003; Xia et al., 2004; Legesse et al., 2006).

Conclusion and Recommendations

The present study indicates the validity of PCR technique for estimating genetic diversity among maize genotypes. The current data will enhance the breeding efficiency and will add the strength of Marker Assisted Selection. In the light of information about the genetic diversity in 17 Pakistani maize genotypes, it is suggested that the breeding programs with the help of DNA fingerprinting technology will be helpful to utilize the genotypes to produce cultivars/varieties by crossing them with different elite.

Genetic diversity plays a key role in crop improvement. Present study was aimed at identifying genetic diversity in 17 Pakistani maize genotypes using 10 chromosome specific Simple Sequence Repeat (SSR) primer sets. Range of amplified fragments was from <250-750 bp in size.

The degree of genetic polymorphism ranged from 0 - 100% indicating that these genotypes were genetically very diverse and possess a high amount of polymorphism. Average genetic distances ranged from 0 - 81%. Two comparisons (population 9804 vs pahari and population 9804 vs CGCW) showed maximum genetic distance (81%). It is recommended that these comparesons should be used in hybridization programs aimed atincreasing level of genetic polymorphism in maize germplasm.

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