APPLICATION POSSIBILITIES OF AFLP FINGERPRINTING TECHNIQUE IN MAIZE DNA PROFILING AND PLANT VARIETY PROTECTION

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As a contribution to DUS testing within the system of protection of plant breeders' rights (PBR), the AFLP molecular system has been used in this study to produce DNA fingerprinting profiles. DNA polymorphism and genetic distance of nine agronomicaly important maize genotypes has been investigated using the AFLP technique. Two specific adapters, two preselective primers and twenty selective primers were utilized for DNA amplification. The selective primers were GC rich, each having a 3-mer selective sequence at 3' termini. Ten double stranded primer combinations were made out of the twenty primers but only five of them turned out to be reliable. Out of 253 amplified DNA fragments, 177 were polymorphic (70%). The CGA/GAG (B) primer combination has proved to be the most polymorphic (44 polymorphic fragments have been recorded) revealing the polymorphism rate of 81.5%. Genotypes g1 and g7 were most distinct (GD=55% and GD=79%, respectively) and genotypes g1, g4 and g8 were closest (GD=55% in all cases). The paper discusses possible uses of AFLP DNA profiling technique to achieve a unique fingerprinting pattern of agronomicaly important maize genotypes.

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INTRODUCTION

Plant variety protection and patents are means by which intellectual property protection can be conferred upon the end product of research investment in plant breeding. In order for cultivars to be nationally or internationally registered, varieties must pass test for the criteria of distinctness, uniformity and stability (DUS) (BAILEY, 1983).

Traditionally, morphological data have been used to define the parameters of certification and DUS tests. However, many of the characters used are multigenic, quantitative or continuous and their expression can be altered by environmental factors, which necessitates replication in observation. There is a growing interest in the use of biochemical and DNA-based methods that could provide more rapid, cost effective as well as sharply defined and reproducible genotypic descriptions.

Following LAW *et al*. (1998), it may be assumed that molecular data could be used as a source of additional characters, along with all of the presently used morphological characters.

Up to date a variety of molecular markers technique are available for detecting DNA polymorphisms in plant materials. These markers could be hybridization-based or PCR-based and they include: RFLPs, microsatellites e.g. SSRs (TAUTZ, 1989; AKKAYA *et al*., 1992; MORGANTI and OLIVIARI, 1993), minisatellites (JEFFREYS *et al*., 1988; BROUN *et al*., 1992, STOCKTON *et al*., 1992), RAPDs (WILLIAMS *et al.* 1990), AP-PCRs (WELSH and MCCLELLAND, 1990) and DAFs (CAETANO-ANOLLES *et al*., 1991; SEN *et al*., 1997). The AFLP molecular system was developed by VOS *et al*. (1995) as a PCR-based method which produces "fingerprint" patterns of different lengths that are characteristic and proved to be highly reproducible i.e. applicable for a single organism (MCGREGOR *et al*., 2000). According to DONINI *et al*. (1997), the AFLP technique in maize and wheat demonstrate high fidelity and appreciable levels of polymorphism between varieties. In this paper we describe a possible application of AFLPs in statutory distinctness testing of maize genotypes so as to reveal their DNA fingerprinting profiles.

The objective of this research was to evaluate the potential use of AFLP marker system in maize genotypes identification through evaluating their DNA fragment polymorphisms and revealing their genetic distance.

MATERIALS AND METHODS

Plant DNA and enzymes. - Genomic DNA isolation of nine maize genotypes [1. NS832D (g1); 2. NS92D (g2); 3. NS260692/5 (g3); 4. NS106D (g4); 5. 250128/2(g5); 6. 220019/2 (g6); 7. NS53D (g7); 8. 240212/31(g8); 9. 240236/3 (g9)], was performed after SAGHAI-MAROOF *et al*. (1984) extraction procedure

with minor modifications. Restriction enzymes were chosen to fit the crop species studied. In digestion reaction, *MseI/PstI* restriction enzyme combination was used. Both enzymes recognized the specific restriction sites and cut genomic DNA in fragments of different lengths. Three kinds of fragments were revealed at the end of the digestion reaction. Fragment type *MseI/PstI* was most appropriate.

AFLP adapters and oligonucleotides. - Oligonucleotides were used as AFLP adapters and primers without further purification. All of them were synthesized in and purchased from Applied Biosystems.

AFLP adapters were double-stranded and consisted of a core sequence and an enzyme-specific sequence. The bodies of the adapters for both restriction enzymes were identical, except that their cohesive ends were specific and corresponded to the respective enzymes.

AFLP primers were single-stranded and consisted of three parts: core sequence, enzyme-specific sequence and selective extension. The selective extensions of the two preselective AFLP primers were each represented by base C or G. The selective AFLP primers had three bases representing the selective extension. The following ten selective primer combinations were made using different combinations of the bases: CGA/GAG(M9/P3-**B**); CGC/GAA(M9/P1-**A**); CCC/GTG (M11/P7); CCC/GGG (M11/P12); CTT/GTT (M8/P8); CCC/GGA (M11/P9); CGA/GAA (M9/P1); CGA/GTG (M9/P7-**C**); CGA/GTT (M9/P8-**D)** and CGA/GGA (M9/P9-**E**).

Modification of DNA and template preparation. - Templates for AFLP reactions were generated using the enzyme combination *MseI/PstI* following the protocol:

- Genomic DNA digestion with the restriction enzymes: Genomic DNA (0.5 μg) was incubated for 12 hours at 37°C with 5U restriction enzyme in 40μl total volume of buffer R/L 5X (8 μl) and BSA 50ng/μl.

- Ligation reaction: Adapter *PstI* 5 pMol, adapter *MseI* 50 pMol, Ligase 3U/μl, ATP 100 mM and buffer R/L 5X (2 μl) in 10 μl total volume.

- Combine 40 μl digestion + 10 μl ligation solution, incubate for 3 hours at 37°C

- After ligation, the reaction mixture was diluted 10 x with 10mM Tris-HCl, 0.1 mM EDTA pH 8.0 and stored at –20°C.

The adapters were prepared by adding equimolar amounts of both strands and they were not phosphorylated.

AFLP reactions. - AFLP fingerprinting amplification reactions were performed after VOS *et al.* (1995) using DNA templates for the enzyme combination MseI/PstI and appropriete AFLP adapters and primers.

Amplification reactions were generated in two steps. The first step, preamplification, was performed with two preselective primers having a single selective nucleotides, C or G. After the preamplification step, the reaction mixture was diluted 50 fold with 10 mM Tris-HCl, 0.1 mM EDTA pH 8.0 and used as template for the second amplification reaction, the selective amplification.

Gel analysis. - PCR products of the AFLP procedure steps were checked on 2% agarose gels in 10 μl aliquots.

Amplification reaction products were mixed with 15 μl volume of formamide dye (98% formamide, 10 mM EDTA pH 8.0, bromo phenol blue and xylene cyanol as tracking dyes). The resulting mixtures as well as the Low Ladder DNA Marker were denatured by heating for 3 minutes at 99°C and then quickly cooling on ice. Each sample (3 μl) was loaded on 5% denaturing (sequencing) polyacrylamide gel. Gel matrix was prepared using 5% acrylamide (40% acrylamide-bis-acrylamide, 19:1 ratio), 7.5 M urea in 50 mM Tris-HCl , 50 mM boric acid and 1 mM EDTA (pH 8.0). The polymerization process of 60 ml gel solution was catalyzed with 250 μl of 10 % ammonium persulfate and 30 μl of TEMED and the gel was then run on the S2S vertical electrophoresis unit, 35 cm W x 45 cm L, (OWL Scientific, USA).

One liter of prewarmed (40° C) 1 x TBE was used as running buffer for the upper tank of the VE unit. Na acetate at 500 mM final concentration was added to the lower tank.

Electrophoresis was performed at constant power 80 W, for about 2 hours.

After electrophoresis, gels were visualized by the silver staining method (SAMBROOK *et al.*, 1989).

Band scoring and data analyses. - Due to cost consideration and relatively large amount of DNA required, the AFLP analysis was repeated in only one out of the ten primer combinations, so the reproducibility could not be taken into consideration. The intensity of banding was not taken into account in general scoring. AFLP profiles for each genotype were constructed by scoring 0 or 1 for absence or presence of each fragment, respectively, and the final data sets included both polymorphic and monomorphic fragments. Due to faint signals, bands above 700 bp were disregarded.

Statistical analyses were performed using software NTSYSpc (Numerical Taxonomy and Multivariate Analysis System) v. 2.0. For measuring similarity among individuals (based on Jaccard coefficient), dendrograms were generated according to the UPGMA (Unweighted Pair Group Method using Arithmetic Averages) procedure.

RESULTS

Using the different primer combinations, specific AFLP fingerprinting profiles were obtained for each of the nine maize genotypes. Of the ten primer combinations used, five were reliable. Out of the total of 253 amplified fragments, 177 were found to be polymorphic. The fragment length varied from 100 to 700 bp. The number of polymorphic fragments of each primer combination varied from 26 to 44 revealing the average polymorphisms of 70% (Table 1). B (M9/P3) primer combination gave the most polymorphic banding pattern. With the highest level of polymorphism (81.5%) and 44 DNA polymorphic fragments achieved, it proved to be the most distinctive combination.

Table 1. Number of amplified and polymorphic fragments; their length and percentages of polymorphism of five AFLP selective primer combinations

Primer combination	Primer	Number of amplified fragments	Number of polymorphic fragments	Fragment length (bp)	Polymorphism (%)
A	M9/P1	42	26	127-440	61.9
B	M9/P3	54	44	115-520	81.5
C	M9/P7	49	38	100-700	77.5
D	M9/P8	54	35	135-650	64.8
E	M9/P9	54	34	102-500	63
Total		253	177		
Average		50.6	35.4		70

The existence of variations in discrimination power among the primer combinations (Table 1) was due to variations in the number of polymorphic fragments detected by the primer combinations.
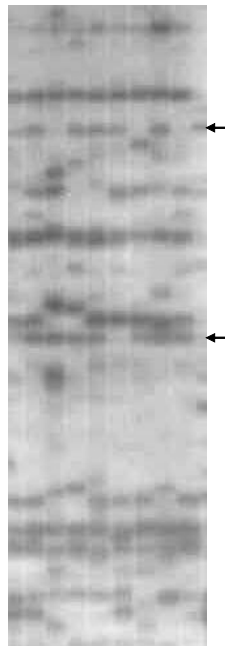

Fig. 1. Partial AFLP fingerprint profile of nine maize genotypes: 1. NS832D (g1); 2. NS92D (g2); 3. NS260692/5 (g3); 4. NS106D (g4); 5. 250128/2(g5); 6. 220019/2 (g6); 7. NS53D (g7); 8. 240212/31(g8); 9. 240236/3 (g9), obtained using primer pair B (M9/P3). The arrows indicate polimorfic bands

Regarding the discrimination power efficacy of primer combination, combination A was the poorest one, producing only 26 polymorphic fragments. Primer combination C was more powerful than D and E (38, 35 and 34 polymor-

phic fragments, respectively). Primer combination B was most polymorphic (44 polymorphic fragment), therefore it possessed the most powerful discrimination level.

Genomic DNA polymorphisms isolated from the different maize genotypes were scored on the basis of the presence (+) or absence (-) of the polymorphic *AFLP* fragments (Fig. 1). According to these data, using *Jaccard* coefficient of similarity, genetic distances among the maize genotypes for the five primer combinations were evaluated and presented in the matrix.

Table 2. Percentage of genetic distance (Jaccard coefficient of similarity) of nine maize genotypes based on five AFLP selective primer combinations

	g1	g2	g3	g4	g5	g6	g7	g8	g9
g1	-	75	71	55	68	77	79	55	70
g2	0.25	-	73	65	69	70	83	60	67
g3	0.29	0.27	-	70	71	73	69	72	67
g4	0.45	0.35	0.30	-	67	70	79	55	65
g5	0.32	0.31	0.29	0.33	-	76	70	64	69
g6	0.23	0.30	0.26	0.30	0.24	-	81	73	69
g7	0.21	0.17	0.32	0.21	0.30	0.19	-	81	73
g8	0.46	0.40	0.28	0.45	0.36	0.27	0.19	-	68
g9	0.30	0.33	0.33	0.35	0.31	0.31	0.27	0.32	-

The results of *Jaccard* coefficients were shown below and the values of genetic distance among the genotypes above the diagonal (Table 2).

The genetic distances (GD) among the genotypes ranged from 55% and 83%. Minimum GDs (55%) were found between genotypes g1, g4 and g8, the maximum GD value (83%) between g2 and g7. The average GD value among all genotype pairs was 70%.

According to the results (Table 2) obtained from the amplification profiles (Fig. 1), relationships among the maize genotypes were evaluated and clustered in UPGMA dendrogram (Fig. 2) indicating common origin of the genotypes and coincidence of their total genetic distance.

The genotypes formed two groups in the dendrogram (A and B). Group A involved genotypes g3 and g9 that shared the same genetic distances (67%). Genotype g6 and g7 were independent but associated with group A, with GDs of 77% and 79%, respectively. Group B involved 5 genotypes of which three (g1, g4, g8) were genetically very close (each with GD=55%). Genotypes g2 (GD=67%) and g5 (GD=65%) were independent but associated with group B. Genotypes g1 and g7 turned out to be most distinct, genotypes g1, g4 and g8 were closest.

The principal component analysis confirmed the results obtained by UPGMA (Fig. 3). Genotype g1 showed the highest relations with genotypes g8 and

g4, forming a group with similar genetic distances around 55%. The other group of closely related genotypes included g3 and g9 with GDs around 67%. There was a low homology between the two groups. Genotypes g6 (GD=77%) and g7 (GD=79%) formed a separate group. These genotypes were genetically close and independent from the first group.
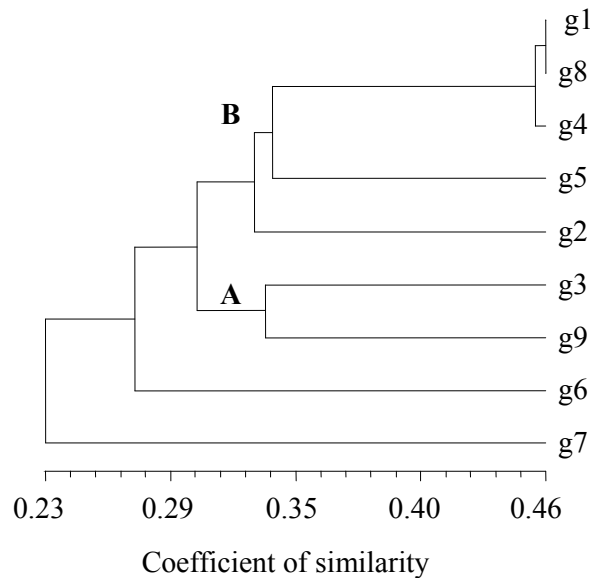

Fig. 2. UPGMA dendrogram (Jaccard coefficient of similarity) of the relationships among five primer combinations based on the genetic distance values of nine maize genotypes

DISCUSSION

The data presented in this paper confirm that the AFLP marker system could be successfully applied in DNA fingerprinting of maize genotypes in official tests. With dense banding profiles and high rates of DNA polymorphism in some DNA profiles (81.5%), the AFLP fingerprinting technique is a reliable genetic tool for achieving high discrimination rate among maize genotypes through determination of their genetic distances. This is in accordance with other authors who demonstrated AFLP suitability for different crops: hops (FLEISCHER *et al.*, 1999; PATZAK, 1999), sugar beet (SCHONDELMAIER *et al.*, 1996), rice (MAHESWARAN *et al.*, 1997), barley (BECKER *et al.*, 1995), wheat (VOS *et al.*, 1995) etc.

Only one primer combination is sufficient to obtain an informative banding pattern for any maize genotype, as reported by MCGREGOR *et al.* (2000).

The results showed variation in the number of polymorphic fragments detected by the primer combinations. This was due to the different levels of discrimination power among the primer combinations. The greater the number of po-

lymorphic bands detected by a primer combination, the higher the level of discrimination achieved (LAW *et al*., 1998).
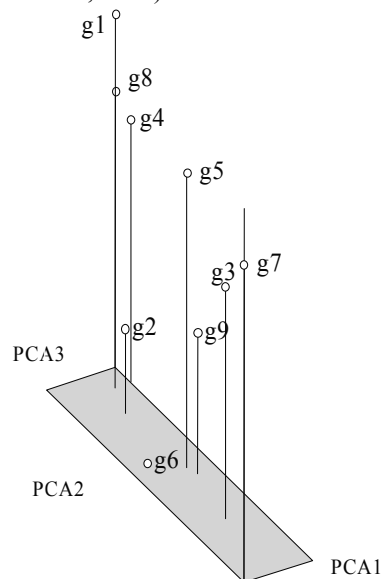

Fig. 3. Three-D graph of the first three factors calculated from Principal Component Analysis of the similarity matrix derived from the pair-wise comparisons of 9 maize genotypes out of 5 primer combinations

The discrimination power of the AFLP technique established in this work indicates the applicability of DNA profiling in DUS testing.

By choosing a correct primer combination, an appropriate number of polymorphic fragments and a suitable but stringent distinctness criterion, it is possible to apply this molecular system in the testing of other crops. The general procedure of the AFLP marker system in maize, as described in this paper, could serve as a useful framework.

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MOGUĆNOST PRIMENE AFLP FINGERPRINTING METODE U KARAKTERIZACIJI DNK PROFILA KUKURUZA I ZAŠTITI AUTORSKIH PRAVA

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I z v o d

U ovom radu korišćen je AFLP molekularni sistem u cilju ispitivanja mogućnosti njegove primene u karakterizaciji fingerprinting profila devet agronomski značajnih genotipova kukuruza. Za umnožavanje specifičnih DNK fragmenata korišćena su dva specifična adaptera, dva preselektivna prajmera i dvadeset selektivnih prajmera. Od ukupno 10 testiranih prajmer kombinacija, 5 je pokazalo značajnu polimorfnost. Od 253 umnožena fragmenta, 177 su bila polimorfna (70%). Prajmer kombinacija B (CGA/GAG) bila je najpolimorfnija (81,5%) a genotipovi g1(GD=55%) i g7 (79%) pokazali su najveću genetičku udaljenost. Na osnovu dobijenih rezultata zaključili smo da je AFLP molekularni sistem pogodna i pouzdana metoda za određivanje lične karte agronomski značajnih genotipova kukuruza i kao takva primenljiva u zaštiti autorskih prava.