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Assessment of Polymorphic AFLP Markers in *Triticum durum* and *Aegilops* sp.

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Abstract: Compared to other Polymerase Chain Reaction (PCR) based molecular markers, the Amplified Fragment Length Polymorphism (AFLP) marker system probably requires more technical expertise for the sequential enzymatic reaction steps. In addition, the reproducibility of the AFLP marker patterns is much more dependent on the quality and the purity of the DNA samples to be processed. The present study describes the optimization of reaction conditions and determination of the most polymorphic selective amplification primer sets in a comparison of winter and spring type Turkish durum wheat varieties. AFLP products resulted in about 100 bands, and the number of polymorphic AFLP loci obtained ranged from 6 to 32 per selective primer combination. Among the nine *Aegilops* accessions, at a single combination, 137 polymorphic loci were observed.

Key Words: Aegilops, AFLP, DNA fingerprinting, durum wheat

Buğdayda ve Aegilops'ta Polimorfik AFLP Belirleyicilerinin Değerlendirilmesi

Diğer Polimeraz Zincir reaksyonu (PZR) tabanlı moleküler belirleyicilere kıyasla, Çoğaltılmış Parça Uzunluk Polimorfizm (ÇPUP) belirleyici sistemi birbirini izleyen enzim reaksiyonlarından ötürü daha çok teknik deneyim gerektirmektedir. Bununla birlikte ÇPUP belirleyicilerinin tekrarlanabilir sonuçlar üretebilmesi DNAnın saflığına ve kalitesine bağlıdır. Bu çalışma reaksiyon koşullarının optimizasyonunu ve en polimorfik seçici çoğaltma aşamasındaki primer setlerinin bir kışlık ve bir yazlık Türk durum buğdayı çeşitinde saptanmasını içermektedir. ÇPUP ürünleri 100 civarında bant vermiştir. Seçici çoğaltma primer setlerinde polimorfik ÇPUP lokusları 6 ile 32 arasında değişmiştir. Dokuz *aegilops* akseyonunda ise bir primer kombinasyonunda 137 polimorfik lokus gözlenmiştir.

Anahtar Sözcükler: Aegilops, ÇPUP (AFLP), DNA parmakizi, durum buğdayı

Introduction

Various DNA fingerprinting techniques have been successfully developed and used for the estimation of genetic diversity in plant species. Most of the recent marker techniques are PCR-based. Such markers with no prior sequence information requirement have been significantly

improved in the past decade. They include random amplified polymorphic DNA (RAPD; by Williams et al., 1990 (1)), arbitrarily primed PCR (AP-PCR; by Welsh and McClelland, 1990 (2)), and DNA amplification fingerprinting (DAF; by Caetano-Anolles et al., 1991 (3)). As an alternative to these markers, AFLP was developed by Zabeau and Vos (4,5). The AFLP marker system, like Restriction Fragment Length Polymorphism (RFLP), is based on the detection of restriction fragments. Unlike locus specific Simple Sequence Repeat DNA markers (SSRs) or microsatellites, AFLP markers are capable of detecting non-specific but many independent loci, with reproducible amplification (6). The AFLP marker sytem is likely to produce a high level of length polymorphism by detecting a large number of loci in a single amplification reaction. This powerful DNA fingerprinting technique is highly informative, reliable, reproducible, fast and cost effective. The AFLP bands are scored as presence/absence of bands among a set of genotypes.

The AFLP system is composed of four successive enzymatic reaction steps on genomic DNA samples: digestion of DNA with two restriction endonucleases, and ligation of two specific adaptors to the restriction fragments, followed by pre-selective amplification and selective amplification PCRs. (5). The primers designed for pre-selective amplification are complementary to the adaptors with one extra nucleotide added to the 3' ends. In this way, DNA fragments flanking a nucleotide at the restriction site, which is complementary to the pre-selective amplification primer, can be re-amplified selectively. For the following selective amplification, the primers used usually contain three extra nucleotides added to the 3' ends. In this subsequent selective amplification, the pre-selective amplified DNA fragments containing nucleotides complementary to the two additional 3' end nucleotides are amplified, and thus further selectivity is obtained.

There are many applications of AFLP markers, e.g., genetic relationship studies (7-10). AFLP can also be used for plant improvement in breeding (11-14). In this study on two relatively distant cultivars, one being spring and the other being winter type, the number of polymorphic AFLP loci is reported. Also, a high level of polymorphism at an AFLP selective primer combination on the *Aegilops* wheat samples is observed.

Materials and Methods

Genetic Materials

'Tunca-79' (winter/facultative type) was obtained from the Ministry of Agriculture and Foresty, Central Research Institute for Field Crops, Ankara. 'Ege-88' (spring type) and wild types were obtained from the Aegean Agricultural Research Institute, İzmir.

DNA Isolation

Genomic DNA was isolated from 15-day old seedlings, after 2 days of dark treatment. The isolation was carried out using 200 mg of each individual plant by a slight modification of the

CTAB method (15), in which the volumes were adjusted so that the purification could be performed in 2 mL microcentrifuge tubes. Also the extraction buffer contained 2% CTAB instead of 1%.

Restriction Enzyme Digestion of Genomic DNA

Different levels of genomic DNA (300-600 ng) and restriction endonucleases (3-6 units) were employed. DNA was digested with two different levels of *Eco*RI and *Tru*I, (*Tru*I is isoschizomer of MseI, MBI Fermentas Inc. Lithuania) in a 40 µL final reaction volume containing, 1 x Universal Buffer (Stratagene, CA) and 0.1 µg Bovine Serum Albumin (BSA) (MBI Fermentas, Inc., Lithuania) for 3 h of incubation at 37°C.

Ligation of Adaptors

*Eco*RI and *Mse*I enzyme (or *Tru*I) restriction sites (E and M, respectively) of genomic DNA fragments were ligated to the doubled stranded (DS) adaptors: EDS adaptor and MDS adaptor, carrying *Eco*RI and *Mse*I complementary ends, respectively. The sequences of the DS adaptors were as developed by Vos et al., (5). The single strands of each adaptor were separately annealed together by cooling to RT slowly, after denaturation at 95°C for 5 min. The ligation reaction mixture contained 20 µL of restriction digest aliquot, 3 or 6 µM of EDS adaptor, 30-60 µM of MDS adaptor, 100 units of T4 DNA ligase (New England Biolabs, MA), 0.2x T4 DNA ligase buffer (New England Biolabs, MA), 0.1 µg BSA and 0.2 mM of ATP. The reaction volume was completed to 25 µL with PCR grade water. The ligation was performed for 5 h at 37°C.

Pre-selective Amplification PCR

Pre-selective amplification primers were referred to as Ecs+A and Mcs+A, both having "A" at the 3' ends of the restriction sites as an extra base ("E" and "M" refer to the *Eco*RI site primer and MseI site primer, respectively; "cs" refers to the common sequence). The pre-selective amplification reaction mixture contained 5 µL ligation reaction products as PCR template, 75 ng of each of the pre-selective amplification primers, 0.2 mM dNTPs (MBI Fermentas Inc., Lithuania), PCR buffer (10 mM Tris-Cl, pH 8.3, 50 mM KCl), 2.5 mM $MgCl_2$, and 1.5 units of Taq polymerase (MBI Fermentas Inc., Lithuania). The reaction volume was 50 µL. The cycling conditions were 20 cycles of three steps as 94°C for 30 s, 60°C for 30 s, 72°C for 1 min.

Labeling Reaction

The labeling reaction contained 5 ng of *Eco*RI adaptor binding selective primers, 0.2 units T4 DNA kinase, 1x T4 DNA kinase buffer (MBI Fermentas Inc., Lithuania), and 0.05 µL of [γ^{33}P]-ATP (3000 Ci/mmol) (Institute of Isotopes Co., Ltd., Hungary) in a final volume of 0.5 µL per selective amplification PCR. The labeling reaction was performed for 10-100 sets of selective-amplification PCRs at 37°C for 1 h. The enzyme was inactivated by 10 min of incubation at 70°C.

Selective Amplification PCR

The selective amplification primers tested were Ecs+AAA; Ecs+ACC; Ecs+ACT; Ecs+AGC; Ecs+AGG; Ecs+ATC; Ecs+ATG; Ecs+ATT; Mcs+AAG; Ecs+AGA; Mcs+ACT; Mcs+ATA; and Mcs+AAT. All of the oligonucleotides were custom synthesized by Research Genetics, Inc., AL, USA. The reaction mixture per PCR contained 5 µL of 1:20 diluted pre-selective amplification product as a template, 0.5 µL labelled E site selective primer, 3 ng M site selective primer, 0.2 mM dNTPs (MBI Fermentas Inc., Lithuania), PCR buffer (10 mM Tris-Cl, pH 8.3, 50 mM KCl), 3.0 mM $MgCl_2$, and 1 unit of *Taq* DNA polymerase (MBI Fermentas Inc., Lithuania). The reaction volume was 20 µL. The cycling conditions were 11 cycles of three steps of denaturation at 94ºC for 30 sec, annealing at 65ºC (-0.7ºC/each cycle) for 30 sec, extension at 72ºC for 1 minute and additional 24 cycles of three steps of denaturation, annealing and extension at 94°C for 30 s, 56°C for 30 sec, and 72°C for 1 min, respectively.

Separation of AFLP Markers

The selective-amplification reactions were stopped by the addition of formamide containing stop solution and denatured for 10 min at 94°C prior to loading onto 4.5% of denaturing polyacrylamide gel (4.2% acrylamide, 0.3% N, N' methylene-bis-acrylamide, 8 M urea, and TBE (90 mM Tris base, 90 mM Boric acid, 2 mM EDTA)). The electrophoresis was performed until the bromophenol blue dye reached the bottom of the gel. The dried gels were exposed to Kodak Bio-Max/MR film for several days.

Analysis of Polymorphisms

The banding patterns were analyzed directly on the autoradiograph and reanalyzed on the enlarged scanned image by eye. The polymorphisms were scored as the absence or the presence of the bands for every selective primer combination.

Results

The selective amplification primers were chosen from among the ones of Heun (16) shown to be useful specifically for wheat. During the optimization of the several stepwise reaction conditions, one unit of each enzyme per 100 ng DNA in a 40 µL restriction volume resulted in better digestion. Typical AFLP banding patterns are presented in Figure 1.

The 36 different selective primer combinations were tested on the two presumably genetically distant cultivars of durum wheat: 'Ege-88', a spring type, and 'Tunca-79', a winter type. The numbers of polymorphic bands obtained are presented in Table 1. In the comparison of a spring and a winter type cultivar, the most polymorphic combination was obtained with primers Ecs+ACC/Mcs+ATA, resulting in 32 polymorphic loci. Since Turkish durum wheat cultivars originate from a narrow genetic pool (17), a low number of polymorphic bands (such as 6 obtained with the primer combination Ecs+AAA/Mcs+ATA) is to be expected. When the *Aegilops* accessions were analyzed with the primer combination of Ecs+AGC/Mcs+ACT, 137

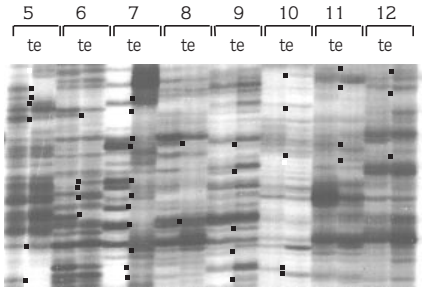

Figure 1. A section of AFLP banding patterns. 5-12 are various selective primer combinations as presented in Table I. The letters "t" and "e" refers to the durum cultivars, 'Tunca-79' and 'Ege-88', respectively. The dots indicate the polymorphic loci present in the section.

Table 1. Number of polymorphic AFLP bands, comparing two genotypes, 'Tunca-79' and 'Ege-88', with 36 different selective amplification primer combinations.

No	Primer combinations	Polymorphic bands
1*	Ecs+AAA/Mcs+AAG	17
2	Ecs+AAA/Mcs+ACT	12
3	Ecs+AAA/Mcs+ATA	6
4	Ecs+AAA/Mcs+AAT	14
5*	Ecs+ACC/Mcs+AAG	22
6*	Ecs+ACC/Mcs+ACT	22
7*	Ecs+ACC/Mcs+ATA	32
8*	Ecs+ACC/Mcs+AAT	24
9*	Ecs+ACT/Mcs+ACT	19
10*	Ecs+ACT/Mcs+ATA	23
11	Ecs+ACT/Mcs+AAT	8
12	Ecs+ACT/Mcs+AAG	13
13	Ecs+AGC/Mcs+AAG	9
14	Ecs+AGC/Mcs+ACT	11
15	Ecs+AGC/Mcs+ATA	11
16*	Ecs+AGC/Mcs+AAT	24
17*	Ecs+AGG/Mcs+AAG	23
18*	Ecs+AGG/Mcs+ACT	17
19*	Ecs+AGG/Mcs+ATA	20
20*	Ecs+AGG/Mcs+AAT	24
21	Ecs+ATC/Mcs+AAG	7
22	Ecs+ATC/Mcs+ACT	10
23*	Ecs+ATC/Mcs+ATA	24
24	Ecs+ATC/Mcs+AAT	11
25*	Ecs+ATG/Mcs+ACT	28
26	Ecs+ATG/Mcs+ATA	14
27*	Ecs+ATG/Mcs+AAT	18
28*	Ecs+ATG/Mcs+AAG	17
29*	Ecs+ATT/Mcs+AAG	17
30*	Ecs+ATT/Mcs+ACT	18
31*	Ecs+ATT/Mcs+ATA	28
32*	Ecs+ATT/Mcs+AAT	18
33	Ecs+AGA/Mcs+AAG	4
34	Ecs+AGA/Mcs+ACT	8
35	Ecs+AGA/Mcs+ATA	5
36	Ecs+AGA/Mcs+AAT	9

* number of polymorphic bands, 17 and above.

polymorphic bands were obtained (Figure 2). The same combination resulted in 11 polymorphic bands between the two cultivars. This is anticipated, since a higher level of polymorphism is expected among the wild types. The analysis of a larger number of more diverse samples (nine *Aegilops* wild types) can also explain the much higher number of polymorphic bands (more than 10-fold). Among the wild types, *A. cylindrica* shows the most distinct pattern. These preliminary results suggest that the AFLP markers used in this study are applicable for DNA fingerprinting of Turkish durum wheat cultivars for genotype identification and genetic relationship studies. AFLP markers can be used not only in marker assisted parental selection for breeding to widen the genetic diversity in cultivars, but also in cultivar certification studies.

Discussion

In the AFLP marker system, all the steps possess potential complications which can interfere especially in the DNA fingerprinting applications. The degree of the restriction digestion must be at the same level in all the samples. The contaminating phenolic compounds in DNA isolations may alter the enzyme digestion levels. The differences in the level of intactness of genomic DNA can also be crucial. Not all the DNA isolation methods from plants are applicable for AFLP studies. In our hands, the modified CTAB isolation method worked better, when compared to a short isolation method (18) successfully used for microsatellite markers. There is an indication that, when different plant organs are used as the source of genomic DNA, AFLP markers display different banding profiles (19). Other complications may arise from the PCR artefacts, such as various mismatches and the amplification of products of the same size belonging to different loci. The scoring for the absence or the presence of the bands can be affected by the gel electrophoresis conditions and by the contents of the gels. Thus, in genotyping with AFLP markers, the evaluation of reproducibility of the experiments is valuable.

In a recent important paper, AFLP is thoroughly evaluated in many important aspects, including reproducibility (20). In the study, for the polymorphism between two wild beta lines, a 0.2% genotyping error was found. This low error rate suggests a high level of reliability. The authors also claimed that the screening for polymorphic primer combinations prior to the fingerprinting of large number of samples is advantageous. In accordance with these suggestions, in the present study we assessed the most polymorphic combinations prior to the genotype identifications and genetic relationship studies of Turkish durum wheat cultivars by taking into account all the critical issues raised above to obtain reproducible and error-minimum AFLP profiles. The use of the most polymorphic selective primer combinations (Table I, marked with an asterisk) will reduce the labor for further studies, as opposed to the use of any selective amplification primers.

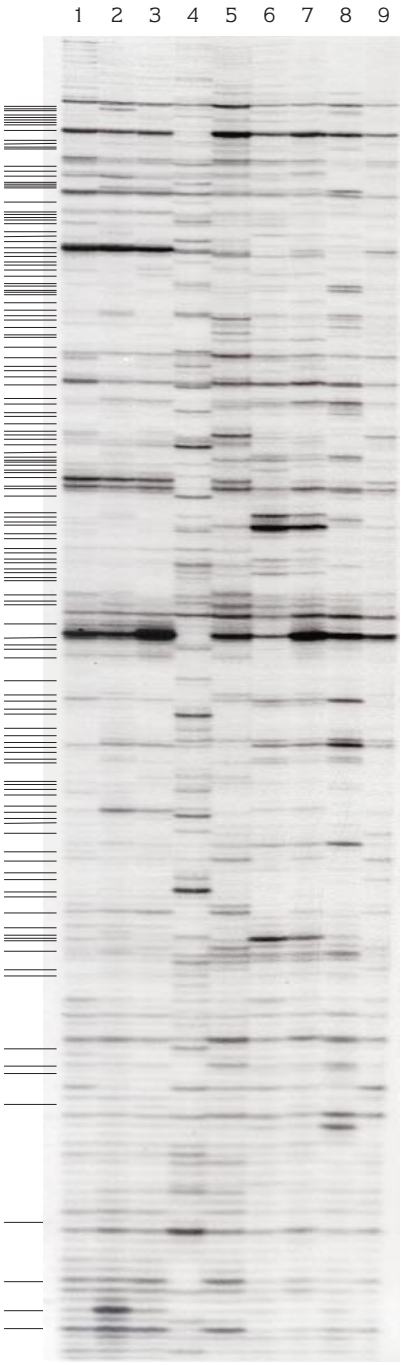

Figure 2. AFLP profile of nine *Aegilops* accessions with Ecs+AGC and Mcs+ACT, selective amplification primer set. The lane numbers 1-9 correspond to different species in the following order: *A. biuncialis, A. caudata, A. columnaris, A. cylindrica, A. ovata, A. speltoides, A. triaristata, A. triuncialis, A. umbellulata*. The bars on the left are indicating the polymorphic bands.

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