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Abstract: Simple Sequence Repeat (SSR) DNA markers or Microsatellites are di-, tri-, tetra-nucleotide tandem repeats containing loci of eukaryotic genomes. It is demonstrated that these loci are very polymorphic due to the change in the number of repeating units among the individuals of populations. Each SSR locus can easily be amplified by using a polymerase chain reaction (PCR) knowing the DNA sequence flanking the repeat region specifically. The only limiting feature of the application of these markers is the need for prior sequence information for developing primers in locus-specific PCR amplification. This limitation is alleviated for the economically important species and the ones closely related, since primer sequences of the SSR DNA markers and the amplification conditions are available in the literature. However, when the reported PCR amplification conditions were applied, with the 2 wheat SSR markers, we found that contaminating bands appeared on the gels. This is most likely due to different laboratory conditions. In this study, we report the optimization of PCR conditions for WMS30 and WMS46 of wheat. The most important parameters were found to be the annealing temperature and Mg^{2+} ion concentrations.

Key Words: *Triticum durum*, microsatellites, Simple Sequence Repeat DNA (SSR) markers, differentiation, DNA fingerprinting

Buğday Basit Dizilim Tekrar DNA Belirleyicilerinin PZR Çoğaltma Optimizasyonu

Özet: Basit Dizilim Tekrar (BDT) DNA belirleyicileri ya da Mikrosatellitler, ökaryot genomlarında bulunan di-, tri-, tetra-nükleotit tekrar üniteleridir. Bu belirleyici lokuslarının popülasyonların bireyleri arasında oldukça polimorfik olmaları, tekrar ünitelerinin sayılarındaki değişimden kaynaklanmaktadır. Her BDT lokusu, polimeraz zincir reaksiyonu (PZR) kullanılarak ve tekrar ünitelerinin dışındaki bölgelerin DNA dizisinin bilinmesi sayesinde özgün bir şekilde çoğaltılabilir. Bu belirleyicilerin kullanılmasındaki tek kısıtlayıcı faktör, lokusa özgün primerlerin tasarımı için DNA dizilimlerinin bilinmesi gerekliliğidir. Ekonomik önemi olan organizmalar ve bunlara genetik olarak yakın olan diğerleri için bu kısıtlama, primer dizilerinin ve PZR koşullarının yayınlanmış olması nedeni ile azalmıştır. Bununla birlikte rapor edilen PZR amplifikasyon koşulları çalışmamızda uygulandığında (buğday mikrosatelitlerinden WMS30 ve WMS46 markörleri ile) bazı kontaminasyon bantları görmüş bulunuyoruz. Bunun nedeni büyük olasılıkla farklı laboratuvar koşullarından kaynaklanmaktadır. Bu çalışmada buğday mikrosatelitlerinden WMS30 ve WMS46'nın PZR koşularının optimizasyonu sunulmaktadır. En önemli parametrelerin primer bağlanma ısısı ve Mg^{2+} iyonu derişimi olduğu bulunmuştur.

Anahtar Sözcükler: *Triticum durum*-mikrosatelitler-Basit Dizilim Tekrar DNA (BDT) belirleyicileri

Introduction

SSRs are highly polymorphic, abundantly present and quite randomly distributed loci containing tandem repeats with a core unit length of 2-5 nucleotides in eukaryotic genomes. These features of microsatellites make them favorable markers to be used for genetic linkage mapping and DNA fingerprinting for many applications. They are especially powerful for species presenting low levels of polymorphism in general, such as wheat (1-4). The polymorphism of Simple Sequence Repeat DNA markers (SSRs) was first demonstrated in soybean by Akkaya et al. in 1992 (5). Since then, isolations of microsatellites and their applications in genome analysis of many different plant species have rapidly accumulated. The early studies are the work of Wu and Tanksley, 1993 (6) in rice; Senior and Heun, 1993 (7) in maize; Lagercrantz et al., 1993 (8) in Brasicca; Saghai-Maroof et al., 1994 (9) in barley; Bell and Ecker, 1994 (10) in Arabidapsis; Röder et al. 1995 (3) in wheat.

The most important advantage of microsatellites over other molecular markers such as RFLP is the availability of primer sequences in the literature. However, when amplifying the SSR markers reported, it may be necessary to optimize the PCR cycling conditions for each marker, since the reported conditions are tuned for a particular thermocycler, particular types of PCR tubes and brands of reaction components used. The different brands of components or thermocyclers, even the minor differences between the wall thicknesses of the PCR tubes, can be critical and may result in inadequate PCR amplification. Such, outcome can be very problematic during the evaluation of the polymorphism, especially for polyploid species. In this study, we report the tested and working conditions with 2 wheat SSR DNA markers, namely, WMS30 and WMS46 (2,3).

Materials and Methods

DNA isolation and PCR: Durum wheat seeds were obtained from the Ministry of Agriculture and Forestry, Central Research Institute for Field Crops, Ankara. DNA was isolated from half-cut seeds according to Plaschke (6) with some modifications indicated below. The half-cut seeds were immersed in liquid nitrogen and crushed in 1.5 mL microcentrifuge tubes using flame-rounded, 1 mL tips. The rest of the procedure and the materials used were the same as in (6), except for the phenol:chloroform:isoamyl extraction, performed twice prior to the ethanol precipitation of genomic DNA.

The PCR primer sets were custom synthesized by Iontek, Bursa, Turkey. The markers WMS30 and WMS46 were amplified at various primer annealing temperatures (T_a) and Mg^{2+} ion concentrations until clean PCR products were obtained. The amplification conditions were according to (6, 7) with the following modifications. PCR reactions were labeled with 0.1 µL of [α^{32}P]-dATP (3000 Ci/mmol). The reaction mixture, in addition to the radiolabeled nucleotide, was composed of 10 mM Tris-Cl, pH 9.3, 50 mM KCl, 1.50-2.25 mM $MgCl_2$, 1u

Taq DNA polymerase, 0.15 mM of each dNTP and 50 pmol of forward and reverse primers. Microsatellite loci were amplified in a final volume of 10 µL, using 50-60 ng of template DNA. PCRs were performed in the absence of mineral oil on Techne Genius thermocycler. The cycling reactions were hot-started for 2 min at 94ºC, followed by 32-35 cycles of 1 min denaturation step at 94ºC, 1-1.5 min primer annealing step at varying temperatures of 58, 59, 60 and 61ºC, and 1 min extension step at 72ºC.

The length polymorphism of the markers was detected on autoradiographs (BioMax-MR, Kodak) after the separation on a standard DNA sequencing gel at 55-60 watt constant power.

Results

The highest resolution gel electrophoresis method is preferred, namely the standard DNA sequencing gel electrophoresis, for the DNA fingerprinting studies in assessing the specific amplification performances of the reaction conditions. Thus, the PCR products were radioactively labeled by including [α^{32}P]-dATP in the reaction mixtures. Detection and determination of sizes of SSR alleles may be possible on the autoradiographs (1,4). The sizes of microsatellite alleles can be precisely determined in base pair units, when comigrated together with DNA sequence reaction products of a known-sequence DNA, i.e., M13mp18 (1,4).

Among the SSR primer sets selected for fingerprinting of Anatolian durum wheat varieties, some required optimizations. This was quite unexpected since the primers are locus-specific, thus allowing stringent annealing temperatures during PCR. The reported annealing temparature and the Mg^{2+} concentration are 60ºC and 1.5 mM, respectively for both of the markers, WMS30 and WMS46 (3). Under the conditions described in the literature, the PCR resulted in nonspecifically amplified bands in addition to the specific products (Figures 1 and 2). Minor changes in the annealing temperature and the Mg^{2+} ion concentration allowed locus-specific PCR amplification of these markers with our own reactants and tubes and on the Techne Genius thermocycler. The effect of annealing temperature on the specificity of PCR is shown in Figures 1 and 2. The absence of nonspecific PCR products in SSR loci amplification is the only way of assessing the polymorphism of polyploid species. The durum wheat samples used in this study are allo-tetraploid species. It is theoretically possible that a heterozygote locus can produce up to 4 different alleles in an individual plant. Observation of more than 1 band per locus in the autoradiograph for each plant complicates the conclusion about the homozygosity of that locus. Thus, especially for the applications of the microsatellite markers in the polyploid species, the specificity of the PCR becomes critical.

The PCR of the WMS30 locus, at T_a= 58ºC and 61ºC, with 1.5 mM Mg^{2+} failed to amplify 4 of the 5 samples tried. Only sample number 1 could be amplified under these conditions. Attempts were made to amplify the same locus at 59ºC annealing temperature, but with 2 mM of Mg^{2+} ion concentration, nonspecifically amplified bands were again observed. When the

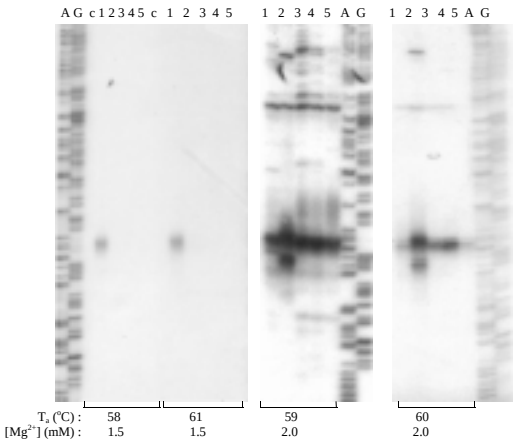

Figure 1. Autoradiograph of radiolabeled PCR products at WMS30 locus separated on DNA denaturing gels. PCRs were performed on the same durum wheat DNA samples (1-5) at different annealing temperatures indicated in the figure. A and G are the sequence reaction products of M13mp18. c is a negative control PCR (no DNA).

annealing temperature was raised to 60ºC, specific amplification products were obtained. Thus, the WMS30 locus PCR amplification optimized parameters can be reported as T_a= 60ºC and [Mg^{2+}] = 2.0 mM. The literature values were T_a= 60ºC and [Mg^{2+}+] = 1.5 mM (2,3). On the other hand, with WMS46 the SSR marker optimum annealing temperature and Mg^{2+} ion concentration were determined to be 58ºC and 1.5 mM, respectively (Figure 2). The literature conditions were 60ºC and 1.5 mM (3), at which we obtained similar product banding patterns as obtained in the cycling at the annealing temperature of 61ºC (Figure 2).

Discussion

This study reveals that it is critical to optimize the reaction conditions prior to large-scale application of each locus, such as DNA fingerprinting of hundreds of varieties. The need for optimization originates from the nature of PCR; since it is a very dynamic process, in each cycle available concentrations of the reactants change. The use of different enzymes can also introduce minor changes to the course of the reaction. The set temperature may be differently reflected internally in each thermocyler. The use of PCR tubes of different wall thickness can also affect the internal temperature.

Interestingly, as shown in Figure 2, a lower annealing temperature provides more specific amplification of the WMS46 locus. The reason for this is as follows. At elevated temperatures the nucleation of the primer hybridization becomes more difficult, thus, the nonspecific target sites with some mismatched base pairs compete with the specific target sites for the primer hybridization. The ratio of primers binding to the correct sites decreases allowing mismatched priming of the polymerization. Once the mismatched priming occurs, extension is also faster at

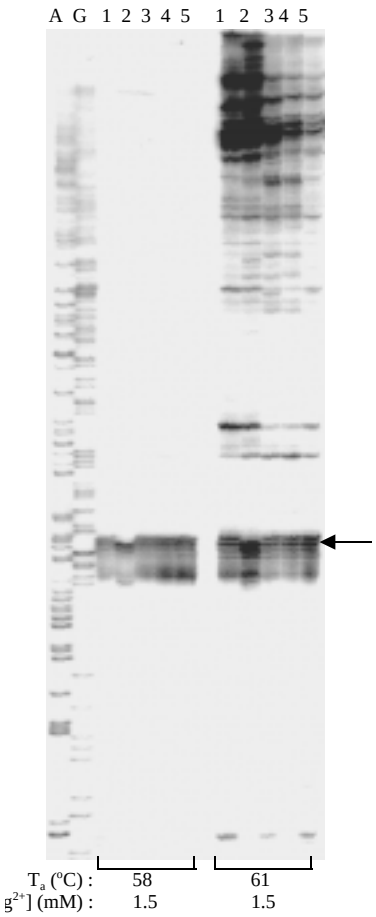

Figure 2. Autoradiograph of radiolabeled PCR products at WMS46 locus separated on DNA denaturing gels. PCRs were performed on the same durum wheat DNA samples (1-5) at different annealing temperatures indicated in the figure. A and G are the sequence reaction products of M13mp18. The arrow indicates the specifically amplified locus.

higher temperatures, and thus, there will be promotion of a nonspecific polymerization reaction. However, at 1 or 2°C lower annealing temperatures nucleation would take place easily and there will be no or little competition of nonspecific sites with a couple of mismatches for the correct target sites. Thus, a specific amplification will take place.

Acknowledgments

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