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Rapid Characterization of Garlic Clones with Locus-Specific DNA Markers

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Abstract: Maintenance of redundant garlic (*Allium sativum* L.) accessions is expensive due to the necessity of yearly regenerating garlic accessions in germplasm centers. Therefore, rapid characterization of garlic accessions is important for avoiding duplicated genotypes. For this purpose we developed several locus-specific polymerase chain reaction (PCR)-based DNA markers, and tested them for the characterization of garlic clones that were previously analyzed using amplified fragment length polymorphism (AFLP) and randomly amplified polymorphic DNA (RAPD) markers. Among 25 garlic clones, 4 locus-specific markers generated 24 polymorphic alleles. Garlic accessions within a group determined by AFLP and RAPD analyses generally had the same alleles as the locus-specific markers. In addition, several alleles of these locus-specific markers were associated only with certain AFLP and RAPD groups, which can allow rapid assessment of newly collected garlic accessions; therefore, these locus-specific markers can be used as another tool for the rapid characterization of garlic germplasm collections.

Key Words: *Allium sativum* L., genetic characterization, DNA markers, germplasm

Sarımsak Klonlarının Lokus Spesifik DNA Markırlarıyla Hızlı Tanımlanması

Özet: Gen bankalarında sarımsak klonlarını muhafaza etmek için her yıl yeniden üretilmesi gerektiğinden aynı genotipe sahip sarımsak klonlarını gen bankalarında muhafaza etmek pahalıdır. Bu nedenle gen bankalarında aynı genotipe sahip sarımsak klonlarının muhafazasını önlemek için sarımsak klonlarının hızlı olarak tanımlanması önemlidir. Bu amaçla PCR'a (polimeraz zincir reaksiyonu) dayalı lokus spesifik DNA markırları geliştirildi ve bu markırlar daha önceden AFLP (çoğaltılmış parça uzunluğu farklılığı) ve RAPD (rasgele çoğaltılmış DNA farklılığı) markırları ile analizi yapılmış olan sarımsak klonlarının tanımlanmasında denenmiştir. Dört lokus spesifik markır 25 sarımsak klonu arasında 24 farklılık gösteren allel üretmiştir. Daha önce AFLP ve RAPD analizleri tarafından belirlenen gruplardaki sarımsak klonları genellikle lokus spesifik markırların aynı allellerine sahip olduğu belirlenmiştir. Buna ek olarak, bu lokus spesifik markırların bazı allelleri sadece belirli AFLP ve RAPD grupları ile ilişkili olduğu belirlenmiştir. Bu özellik yeni toplanan sarımsak klonlarının daha hızlı tanımlanmasına izin verecektir. Bu nedenle, bu lokus spesifik markırların sarımsak gen kaynaklarının hızlı olarak tanımlanmasında başka bir araç olarak kullanılabilecektir.

Anahtar Sözcükler: *Allium sativum* L., genetik tanımlama, DNA markırları, gen kaynağı

Introduction

Garlic (*Allium sativum* L.) is cultivated and clonally propagated worldwide. It is also maintained in germplasm collections by re-propagating vegetatively. For the maintenance of garlic genotypes in germplasm centers, regeneration of garlic accessions is required every year, but this is an expensive and labor intensive task. In order to reduce maintenance expenses, only unique and diverse garlic accessions need to be identified, and core collections need to be established.

Garlic clones can be exchanged freely among collectors and farmers from different countries, and a popular garlic clone may be cultivated under different names in different regions of the world; therefore, the

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introduction of duplicated garlic accessions to germplasm centers from those countries can easily occur. Indeed, the presence of duplicated accessions was detected by genetic diversity studies based on DNA markers (Bradley et al., 1996; Ipek et al., 2003). Volk et al. (2004) reported that 64% of the U.S. National Plant Germplasm System's garlic collection held at the Western Regional Plant Introduction Station in Pullman, Washington, USA, and 41% of commercial garlic collections were duplicates. Detection and elimination of those duplicated accessions in germplasm collections can reduce maintenance costs significantly.

True seed-producing garlic clones have been discovered (Etoh and Simon, 2002; Simon and Jenderek, 2003). Use of these true seed-producing garlic clones in breeding programs will facilitate the development of many new garlic genotypes worldwide. Introduction of genotypes from these breeding programs and from new germplasm collection projects will likely increase the number of garlic accessions held in germplasm centers; therefore, the development of methods for quick and reliable characterization of garlic accessions will become more important in the future.

Genetic characterization of garlic clones maintained in germplasm collections has been accomplished using various molecular markers, such as isozymes, AFLPs, and randomly amplified polymorphic DNA (RAPD) markers. Isozymes are co-dominant markers, but the number of isozyme markers is quite limited in garlic and some are stage-specific (Pooler and Simon, 1993; Ipek et al., 2003). On the other hand, RAPD markers are abundant (Maass and Klaas, 1995; Bradley et al., 1996; Ipek et al., 2003) and RAPD is an easily applicable marker system for the genetic characterization of garlic, but this marker system's reproducibility has been criticized (e.g., Karp et al., 1996). Recently, AFLP markers have been used for genetic characterization and mapping of garlic clones (Ipek et al., 2003; Lampasona et al., 2003; Volk et al., 2004; Ipek et al., 2005, Ipek et al., 2008). AFLP markers were found to be numerous and reproducible in garlic (Ipek et al., 2003; Ipek et al., 2006), and ideal for the differentiation of closely related accessions; however, this method can be costly and time consuming for the genetic characterization of large garlic collections. Therefore, additional marker systems for the quick and reliable characterization of a large number of garlic accessions are needed. Simple sequence repeats (SSR) was proven to be a very powerful marker technique for this purpose in other plant species, such as olive (*Olea europaea* L.) (Sarri et al., 2006); however, to the best of our knowledge large numbers of SSR markers have not yet been developed for garlic

The present study describes the use of locus-specific markers for quick and reliable characterization of garlic clones, and to organize large germplasm collections. For this purpose we developed locus-specific markers using the sequences of garlic genes available from GenBank at NCBI, and analyzed a core garlic collection that was previously analyzed with RAPD and AFLP markers.

Materials and Methods

Plant Materials

The study included 25 diverse garlic clones that were previously analyzed by Ipek et al. (2003) (Figure 1). In our previous study, genetic relationships of 48 garlic clones were analyzed using AFLP and RAPD markers, and 10 arbitrary groups were identified. For the current study, 25 garlic clones representing these 10 groups were selected from those 48 garlic clones for analysis.

Sampling and DNA Extraction

Leaf sampling and DNA extraction were performed according to the methods described by Ipek et al. (2003).

Locus-Specific Marker Analysis and Sequencing

For the generation of locus-specific markers, primer pairs were designed from garlic DNA sequences available from GenBank at NCBI, as described by Ipek et al. (2005). Four primer combinations resulted in size polymorphisms in the PCR products of the genotypes and, therefore, were used in PCR analysis for genetic assessment (Figure 1). The primers and their sequences were as follows: As-SST-1-F 5'-TGG ACA ATG ATG AGT ACA TGT CAG TCG C-3' and As-SST-1-R 5'-CAG ATA ATT TTG ATT ACA GAG AAT TTG CTG TCA ACT T-3' for the ASLSM1 marker; As-Alliinase-F 5'-CTC AAC TCA TCC ATG GAC TCG TCA TCT CT-3' and As-Alliinase-R 5'-GAT CGT ACG TTA GAT CGA TGT GTG C-3' for the ASLSM2 marker; As-Chtn-F 5'-CAG CAA CAG GCT ATG CTG TAG C-3'and As-Chtn-R 5'-GAA TGA GTT TGC AGC TGC TAT GAA GG-3' for the ASLSM3 marker; As-Leafy-F 5'-CGA GAA ACT CAC GGC ACT GCT C-3'; and As-Leafy-R 5'-TCC TCG TCG GTG AAC GCT ATG G-3' for the ASLSM4 marker.

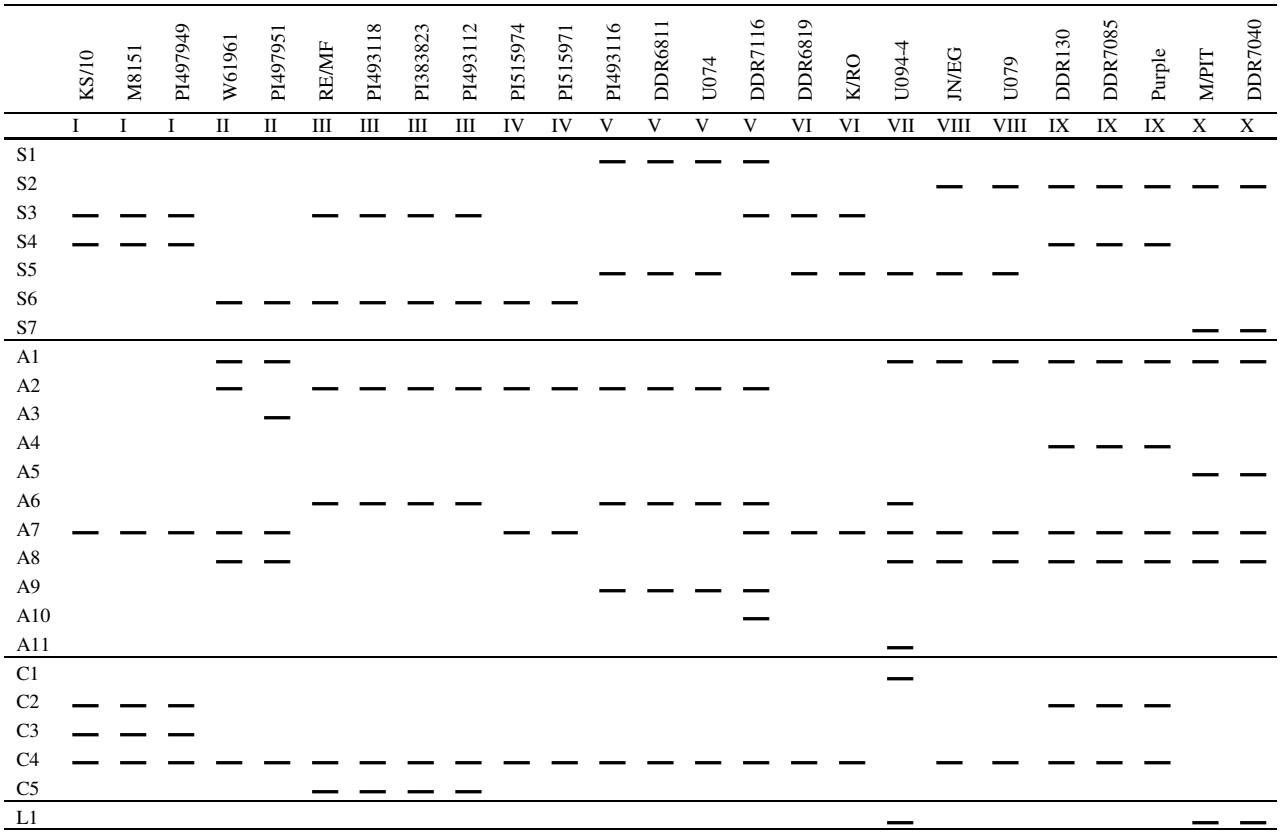

Figure 1. Graphical representation of the banding profiles of polymorphic locus-specific alleles in 25 garlic clones. S1-S7, A1-A11, C1-C5, and L1 are polymorphic alleles of ASLSM1, ASLSM2, ASLSM3, and ASLSM4 markers, respectively. Roman numerals denote groups assigned by Ipek et al. (2003). This graphic does not reflect the actual size of each polymorphic allele.

PCR analysis for ASLSM1, ASLSM2, and ASLSM3 was performed according to following thermal cycling conditions. Each 25 µl of PCR reaction contained 1.25 U of *Taq* DNA polymerase (Promega, Madison, WI, USA), the supplied 1× concentration of reaction buffer, 0.8 µM of each primer, dNTPs of 200 µM each, and 60 ng of template DNA. The reactions were heated to 95 °C for 2 min and exposed to 40 cycles of 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 2 min, then a final extension step of 5 min at 72 °C. After the 40 cycles, reactions were held at 4 °C. A Perkin Elmer model 9600 thermal cycler was used for amplifications. Amplified products were denatured at 90 °C for 4 min in an equal volume of formamide loading dye buffer and then immediately cooled on ice. Denatured PCR products were separated on 6% denaturing polyacrylamide sequencing gels containing 7.5 M of urea in 1× Tris-borate (TBE), 320 ml of 10% ammonium persulfate (APS), and 30 ml of TEMED by running 5-6-µl reactions at 25 W for 5-6 h. In order to visualize separated fragments, gels were stained using the Silver Sequence® DNA staining reagents kit, following the manufacturer's protocol (Promega). PCR cycling conditions for the primer combination of ASLSM4 were 2 min at 94 °C, 9 cycles of 45 s at 94 °C, 1 min at 64 °C (annealing temperature was reduced by 1 °C after each cycle), and 2 min at 72 °C, 35 cycles of 45 s at 94 °C, 1 min at 55 °C, and 2 min at 72 °C, and a final extension step of 5 min at 72 °C. ASLSM4 PCR products were separated with 4% Agarose SFR® (Amresco Inc., Solon, OH, USA) in 1× Tris-borate (TBE) buffer. Bands were visualized by staining with ethidium bromide (0.5 µg ml^{-1}) (Sigma, St Louis, MO, USA). For this marker we were able to detect polymorphism using a high resolution agarose gel; therefore, we utilized 4% Agarose SFR™ gel for easier visualization of polymorphisms than is possible with polyacrylamide gels.

Polymorphic locus-specific alleles were scored as (1) for the presence of an allele and (0) for the absence of an

allele. In order to find genetic relationships between the garlic accessions, obtained binary data were analyzed to develop a UPGMA dendrogram using the Dice coefficient (Dice, 1945) and the NTSYS-PC v.1.80 program (Rohlf, 1993), according to the procedure described by Ipek et al. (2003).

Bands of amplified ASLSM3 DNA fragments (alleles) were extracted from the polyacrylamide gel, cloned, and sequence characterized. Cloning and sequencing of these DNA fragments were performed according to procedures previously described by Ipek et al. (2006).

Results

Four primer combinations designed from the sequences available for the garlic genome from GenBank (NCBI) produced size polymorphisms among the garlic clones (Figures 1 and 2). The primer combination of ASLSM2 produced 11 alleles (bands of different sizes) among the 25 garlic clones. On the other hand, the primer combination of ASLSM4 produced only 1 polymorphic allele, which was present only in garlic clones clustered in AFLP groups VII and X (Figure 1). Other primer combinations, ASLSM1 and ASLSM3, produced 7 and 5 polymorphic alleles, respectively.

In order to verify if all amplified DNA fragments were the alleles of the same gene, amplified DNA fragments obtained for ASLSM3 markers were sequence characterized (Figure 3). The sequences of amplified DNA fragments for this marker on the polyacrylamide gel belong to the same gene and polymorphisms due to the deletions/insertions in the nucleotide sequences of ASLSM3. Although the development of single nucleotide polymorphism (SNP) markers was not the purpose of this study, many single nucleotide polymorphisms were observed among the sequences of ASLSM3 amplified DNA fragments.

A Dice similarity matrix was calculated using 24 polymorphic locus-specific alleles to develop a UPGMA dendrogram for visualizing the estimated relationships between the 25 garlic clones (Figure 4). According to the UPGMA dendrogram, 10 groups previously determined by a large number of AFLP and RAPD markers were also clearly identified by the 24 alleles of the 4 locus-specific markers. Garlic clones clustered in a group in this study were also clustered together in our previous study that used AFLP and RAPD markers (Ipek et al., 2003); however, tree topologies of UPGMA dendrograms based on the locus-specific markers and AFLP markers were not same, probably due to differences in the number of markers used in each study.

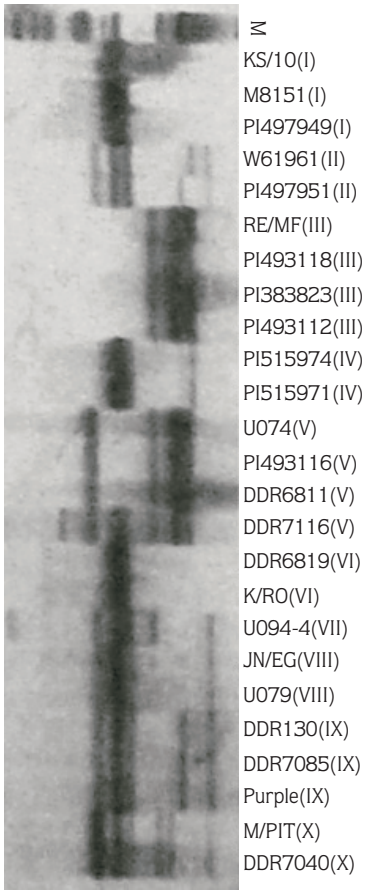

Figure 2. Part of the polyacrylamide gel of ASLSM2. M is molecular weight markers and Roman numerals within the parentheses denote the AFLP groups assigned by Ipek et al. (2003).

Discussion

The large number of allelic polymorphisms observed for 3 markers among the garlic clones supports earlier observations that garlic has a heterogeneous genome (Ipek et al., 2005). Based upon the broad phenotypic and molecular diversity found in wild garlic, it is likely that the broad range of genetic diversity in clonally propagated cultivated garlic is a result of multiple domestication events during the history of garlic cultivation. Furthermore, we can speculate that the accumulation of somatic mutations gave rise to additional allelic polymorphisms in the large garlic genome, as garlic clones have been propagated clonally for centuries.

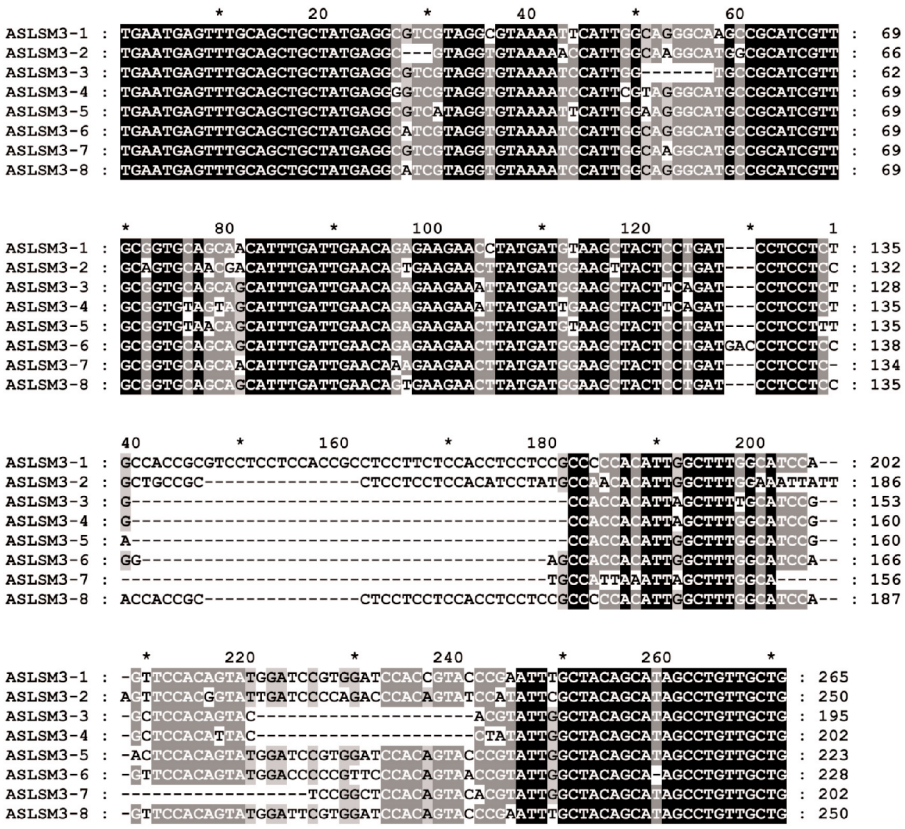

Figure 3. Sequence alignment of 8 amplified DNA fragments of ASLSM3. Black, dark gray, and light gray indicate 100%, 80%, and 60% identities, respectively. Sequence alignment was displayed using GENEDOC (Nicholas and Nicholas, 1997).

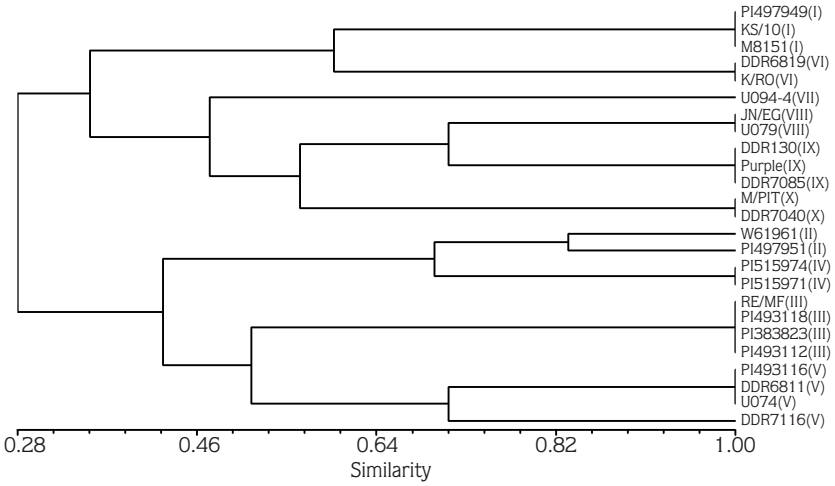

Figure 4. UPGMA dendrogram based on the Dice similarity matrix of 24 locus-specific markers. Roman numerals within the parentheses denote the AFLP groups assigned by Ipek et al. (2003).

The presence of more than 2 alleles of a gene in the diploid genome of garlic was observed in ASLSM2 and ASLSM3 (Figure 1), suggesting that one of the reasons behind the formation of the large garlic genome could be

duplications in its chromosomes. Ipek et al. (2005) found that a significant proportion of AFLP markers in a garlic mapping population was segregating according to the 15:1 ratio typical for duplicated loci and suggested that duplication may be common in the large garlic genome. Similarly, King et al. (1998) reported that about 21% of the RFLP loci were duplicated in another *Allium* species, onion (*A. cepa* L.). The authors also reported that the Alliinase gene in onion is present in multiple copies according to their mapping study that used RFLP markers (King et al., 1998).

In our previous study, we analyzed a diverse garlic collection using AFLP and RAPD markers (Ipek et al., 2003). Based on AFLP markers, 48 garlic clones in that collection were clustered in 10 groups by UPGMA analysis. AFLP polymorphisms within a group were less than 5% and garlic clones clustered in a group shared the same plant characteristics. These 48 garlic clones were also analyzed using RAPD markers and the same grouping was identified by RAPD analysis, but no RAPD polymorphisms within a group were observed in that study. Similarly, garlic clones clustered together in a group shared the same locus-specific alleles as in the current study (Figure 4).

In conclusion, locus-specific markers can be a very useful tool for evaluating existing garlic germplasm collections and for assigning newly acquired garlic genotypes to previously determined garlic groups. This assignment will provide quick information about the genetic relationships between recently acquired garlic genotypes and previously characterized garlic clones, and allows germplasm collectors to predict plant characteristics of newly acquired garlic genotypes. In this way, large garlic collections can be characterized efficiently. Twenty-four locus-specific markers were enough to assign 25 garlic clones to 10 groups and more locus-specific markers can be developed to increase the confidence level of the analysis.

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