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Determination of genetic diversity in watermelon [*Citrullus lanatus* (Thunb.) Matsum & Nakai] germplasm

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Abstract

The genetic diversity and relatedness of 90 watermelon (*Citrullus lanatus* var. *lanthus* and *Citrullus. lanatus* var. *citroides*) accessions were molecularly characterized using 30 sequence-related amplified polymorphism (SRAP) marker combinations. The accessions were collected from Turkey (59 accessions) and other regions of the world by the United States Department of Agriculture (USDA) and the molecular data were analysed using UPGMA (Unweighted Pair Group Method Analysis). The SRAP combinations were highly polymorphic (97%) with 87 polymorphic bands determined among 201 amplified fragments (43%). The UPGMA analysis characterized five major clusters (A, B, C, D and E). While minimum genetic similarity among groups A, B and C was high (83%), group E was the most distant with 63% genetic similarity. Principal component analysis (PCA) was performed and used to produce a two-dimensional plot from which two main groups could be distinguished. Based on the analyses, genetic diversity of watermelons was very low and Turkish watermelons were not distinct from other countries' accessions. These results could be used for generation of a core collection of watermelon by elimination of redundant accessions and for watermelon breeding by helping to identify useful, genetically distinct lines. In addition, the study indicated that SRAP markers are useful for analysing genetic diversity in crops like watermelon which have low variability.

Keywords: Core collection; gene banks; genetic variation; molecular markers; SRAPs; watermelon.
Abbreviations: AFLP-amplified fragment length polymorphism; CAPS- cleaved amplified, polymorphic sequences; PCA-principal component analysis; PI-plant introduction; RAPD-random amplified polymorphic DNA; RFLP-restriction fragment length polymorphism; SRAP- sequence-related amplified polymorphism; SSR- simple sequence repeat; TAE- Tris acetate EDTA buffer; UPGMA- unweighted pair group method analysis; USDA- United States Department of Agriculture.

Introduction

The genus *Citrullus* belongs to the *Cucurbitaceae* family which includes about 118 genera and 825 species. *Citrullus* is a member of the subfamily *Cucurbitoidae*, tribe *Benincaseae,* subtribe *Benincasinae* (Dane and Liu, 2007). The genus has four diploid species (2n=22) which are grown in Africa, Asia and the Mediterranean (Levi et al., 2001a). *Citrullus lanatus* (Thunb.) Matsum. et Nakai. is found in tropical and subtropical climates worldwide and consists of two cultivated watermelons: *C. lanatus* var. *lanatus* and *C. lanatus* var. *citroides* (Bailey) Mansf. which is known as preserving melon or citron because of its fruit rind. The rind is used in preserves, jellies, conserves and to make pickles (Dane et al., 2004; Dane and Liu, 2007). *Citrullus colocynthis* (L.) Schrad. grows in northern Africa, southwestern Asia and the Mediterranean (Dane et al., 2004) . This species, also known as bitter gourd, is a drought- resistant perennial and is used as a medicinal plant in the pharmaceutical industry (Dane et al., 2007). The other two wild species are *Citrullus eccirrhosus* Cogn. and *Citrullus rehmii* De Winter. which are endemic to the Namid Desert and are annual plants (Sarı et al., 2008; Solmaz and Sarı, 2009). All *Citrullus* species originated from Africa with the origin of *Citrullus lanatus* in the Kalahari Desert. Watermelon has been cultivated for a long time in Africa and the Middle East. For example, it has been grown in Egypt for at least 4000 years (Huh et al., 2008; Gichimu et al., 2009). By the 10th century, watermelon was introduced to China and today China is the most important producer and consumer of watermelon in the world (Goda, 2007). By the 13th century, watermelon was dispersed to Europe. It reached North America in the 17th century and since then it has been cultivated in the Western Hemisphere (Levi et al., 2001b). With 3.8 million ton/year of production over 137,000 hectares, Turkey ranks second behind China in worldwide production of watermelon (Sari et al., 2008). Watermelon is grown throughout the country with highest consumption during the summer and fall. Watermelon is an economically and socio-economically important crop in Turkey, especially in coastal regions where much of cultivation occurs. Diversity among organisms is a result of DNA sequence variation and environmental effects. Differences at the gene or DNA sequence level can be detected by various molecular marker systems (isozymes, RFLP, AFLP, RAPD, SSR, SRAP, CAPS). Molecular markers have a wide variety of uses in plant molecular biology and biotechnology (Kumar et al., 2009; Kumar, 1999). The SRAP (Sequence-Related Amplified Polymorphism) technique was developed relatively recently and is based on amplification of open reading frames (ORFs) using PCR (Li and Quiros, 2001; Ferriol et al., 2003). Two types of primers are employed. The forward primer is 17 nucleotides (nt) long, and contains a fixed 14 nt sequence rich in C and G, and three selective bases at the 3' end. This primer binds to exonic regions,

Table 1. Number of polymorphic bands for each SRAP primer combination.

SRAP Primer Combination	Number of Polymorphic Bands
Me1-Em2	3
Me1-Em3	1
Me1-Em4	4
Me2-Em1	4
Me2-Em5	4
Me2-Em6	3
Me2-Em7	3
Me3-Em1	3
Me3-Em2	3
Me3-Em7	2
Me4-Em2	2
Me4-Em3	3
Me4-Em5	3
Me4-Em6	5
Me5-Em1	1
Me5-Em3	1
Me5-Em4	3
Me5-Em5	1
Me6-Em1	3
Me7-Em8	2
Me7-Em10	5
Me8-Em1	5
Me8-Em2	3
Me8-Em13	2
Me9-Em2	3
Me10-Em10	1
Me14-Em14	3
Me14-Em15	4
Me14-Em16	2
Me14-Em17	5

which tend to be rich in these nucleotides. The reverse primer is 18 nt long and contains a 15 nt sequence rich in A and T with three selective bases at the 3' end. This primer preferentially amplifies intronic regions and regions with promoters which are rich in these nucleotides. The observed polymorphism originates from variation in the length of these introns, promoters and spacers, both among individuals and among species. The goal of this study was to determine the genetic relationships among 90 watermelon accessions belonging to *C. lanatus* var. *lanthus* and *C. lanatus* var. *citroides* by using SRAP markers and genetic similarity analysis. Most (65%) of the accessions were of Turkish origin, however, accessions from ten other countries were also included for comparison.

Results

A total of 40 SRAP primer combinations were tested on 90 watermelon accessions. In all, 31 of the tested combinations produced amplification products, and 30 (97%) of these combinations were polymorphic. A total of 201 fragments were obtained from the 30 combinations, however, only 87 fragments were polymorphic among accessions. Thus, fragment polymorphism was 43%. The number of polymorphic fragements for each SRAP primer combination varied from one to five with an average of 2.9 polymorphic fragments obtained for each primer combination (Table 1). A genetic distance dendrogram of the 90 watermelon accessions was constructed using the Dice matrix and UPGMA (Supplementary Figure 1). The correlation between the distance matrix and the dendrogram, was determined with a Mantel test. According to this test, the correlation (r) value was 0.973 which indicated that the fit between the distance matrix and the dendrogram was very high. In the dendrogram, the 90 watermelon accessions fell into five groups (Supplementary Table 1) and the dendrogram scale varied from 0.68 to 1.00. Groups A, B and C were very similar with minimum similarity among these groups of approximately 95%. Group D was more diverse than groups A, B and C. Minimum similarity between groups A, B, C and D was ~83%. Group E was a very distant group and had minimum similarity of 63% with the other groups. Group A included 60 accessions and was the largest group. These 60 accessions were very closely related with each other with minimum similarity of ~97%. In fact, some of the accessions were genetically identical. As a result, it was determined that the 60 accessions in this group actually represented 56 distinct genotypes. In group B, 11 accessions clustered together and were very closely related to each other with ~96% minimum similarity. Two accessions in this cluster were identical, thus, this group contained 10 distinct genotypes. Group C also had 11 accessions and these were very similar to each other with ~95% minimum similarity. However, none of the accessions in this group were genetically identical. Group D consisted of seven accessions which showed more genetic diversity than the previous three clusters. Minimum similarity between these accessions was ~83% and none were identical. Group E included only one accession (PI 299379, a *citroides* accession). This accession was very distant compared to the other accessions with only 68% similarity with the accessions in Groups A, B, C and D. Principal component analysis (PCA) was carried out and the two-dimensional plot is shown in Figure 1. In this plot, watermelon accessions separated into two main groups with five accessions located outside of these main groups: PI 172786, PI 271779 (a *citroides* accession), PI 174106, PI 595200 and PI 299379 (*citroides*). The first and second axes for PCA explained 51% and 4% of the total variance, respectively.

Discussion

In this study, the genetic diversity of 90 watermelon (*Citrullus lanatus* var. *lanatus*) accessions was examined with SRAP markers. The SRAP marker combinations were very polymorphic (97%) on the accessions and amplified 201 DNA fragments of which 43% were polymorphic. In a previous study, Levi and Thomas (2007) tested 41 SRAP markers on 24 watermelon cultivars. In that study, 33 of 41 SRAP markers were polymorphic (80%). Aiping et al. (2008) observed 39% SRAP band polymorphism in Chinese watermelons (var. *lanatus).* Thus, our results are in agreement with previous studies that used SRAP markers in watermelon and indicated that the SRAP marker system is efficient for detection of polymorphism in crops, like watermelon, which have low levels of polymorphism. Although our accessions originated from 11 different countries, genetic diversity was limited. This may be because domestication of watermelon occurred outside of its center of origin and, therefore, genetic variation is narrow. Previous studies also indicated a lack of genetic diversity among watermelon cultivars. For example, Levi et al. (2001b) found little genetic diversity among 46 watermelon accessions of *C. lanatus* var. *lanatus* and 12 *Citrullus* sp. using RAPD markers. They detected high genetic similarity values among watermelon cultivars (92% to 99%) and *C. lanatus* var. *lanatus* (88% to 95%) but lower similarity values among *C. lanatus* var. *citroides* (65% to 82.5%). These results are in

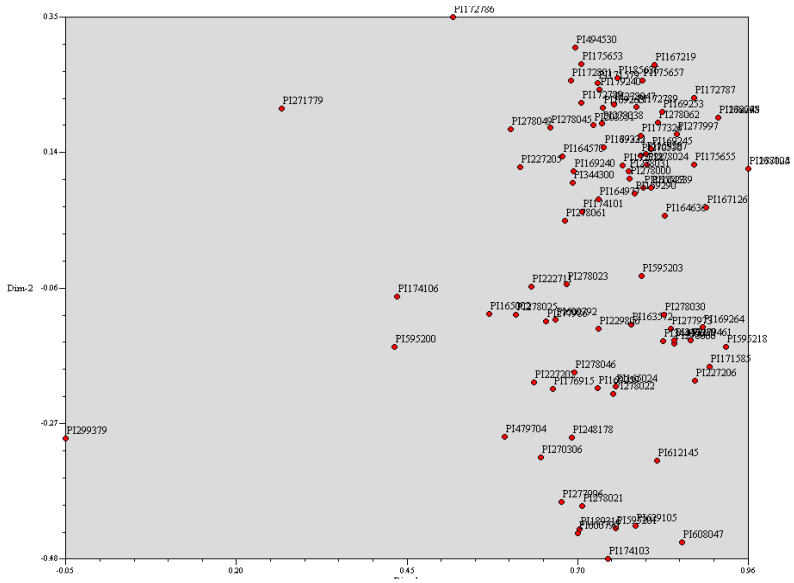

Fig 1. Relationships among 90 watermelon accessions as determined by principal component analysis

agreement with our study which also indicated that the two *citroides* accessions were more diverse than any of the watermelon accessions with a similarity value of only 68%. Similarly, Mujaju et al. (2010) found higher levels of genetic diversity between two major groups of sweet watermelons (*C. lanatus* var. *lanatus*) and cow-melons (*C. lanatus* var. *citroides*) from Zimbabwe using RAPD markers. Solmaz et al. (2010) assessed RAPD diversity in 303 accessions of *Citrullus* species and *Praecitrullus fistulosus* collected from different geographical regions In that study, genetic diversity was found to be very low within Turkish *C. lanatus* var. *lanatus* accessions. Interestingly, Solmaz and Sari (2009) also performed morphological characterization of Turkish watermelon genetic resources (*C. lanatus*) and found excessive morphological diversity among the accessions. Thus, it is clear that morphological and molecular genetic diversity are distinct factors and must be considered separately in germplasm characterization. This is especially important for crops like watermelon which have limited molecular genetic diversity. In general, genetic variation did not depend on origin of the watermelon accessions in our study. For example, the Turkish accessions did not cluster together but were intermixed with ones from other countries. Jarret et al. (1997) used SSRs to determine molecular genetic relationships among morphologically different species of watermelon from distinct geographic areas and also did not find a consistent correlation between geographic origin and genetic relationships. In addition, the intermixing of accessions in the dendrogram and PCA analyses indicated that Turkish watermelons are neither more nor less diverse than those from other countries. The watermelon accessions with higher than average diversity as seen in the PCA (Figure 1) were two accessions from Turkey (PI 172786 and PI 174106) and one from the USA (PI 595200). Depending on their morphological characteristics, these accessions may be of particular interest for breeders who wish to increase genetic diversity in watermelon.

Materials and methods

Plant materials

A total of 90 watermelon accessions collected from different regions of Turkey and the world by the USDA-ARS Plant Germplasm Inspection Station, Beltsville, Maryland, USA were molecularly characterized in this study. The accessions were self-pollinated to remove potential heterogeneity due to seed mixtures. The watermelon accessions are listed in Supplementary Table 1. In all, 59 of the accessions were from Turkey, 11 accessions were from the US, four accessions were from India, five accessions each were from Japan and West Africa, two accessions were from South Africa, and one accession each was from West Azerbaijan, Iran, Philippines, Guatemala and Zaire. Ten seeds of each accession were planted in a peat and perlite mixture in seedling plates and germinated under optimal conditions in a growth chamber at Izmir Institute of Technology, Urla, Izmir, Turkey for molecular analysis.

DNA extraction and SRAP analysis

Total genomic DNA was extracted from young leaves with the CTAB-DNA extraction protocol (Fulton et. al., 1995) and Promega Wizard Genomic DNA Purification Kit. The DNAs were quantified on the Nanodrop ND-1000 spectrophotometer. All genomic DNAs were stored at -20 °C in TE buffer. PCR amplifications were carried out using 40 SRAP marker combinations (Tables 1 and 2) including 11 forward primers (ME1, ME2, ME3, ME4, ME5, ME6, ME7, ME8, ME9, ME10 and ME14) and 13 reverse primers (EM1

Table 2. Primer sequences for the SRAP primers used in the analysis.

SRAP Forward Primers	SRAP Reverse Primers
Me1: 5′-TGAGTCCAAACCGGATA-3′	Em1: 5′-GACTGCGTACGAATTAAT-3′
Me2: 5′-TGAGTCCAAACCGGAGC-3′	Em2: 5′-GACTGCGTACGAATTTGC-3′
Me3: 5′-TGAGTCCAAACCGGAAT-3′	Em3: 5′-GACTGCGTACGAATTGAC-3′
Me4: 5′-TGAGTCCAAACCGGACC-3′	Em4: 5′-GACTGCGTACGAATTTGA-3′
Me5: 5′-TGAGTCCAAACCGGAAG-3′	Em5: 5′-GACTGCGTACGAATTAAC-3′
Me6: 5′-TGAGTCCAAACCGGTAG-3′	Em6: 5′-GACTGCGTACGAATTGCA-3′
Me7: 5′-TGAGTCCAAACCGGTTG-3′	Em7: 5′-GACTGCGTACGAATTATG-3′
Me8: 5′-TGAGTCCAAACCGGTGT-3′	Em8: 5′-GACTGCGTACGAATTAGC-3′
Me9: 5′-TGAGTCCAAACCGGTCA-3′	Em10: 5′-GACTGCGTACGAATTTAG-3′
Me10: 5′-TGAGTCCAAACCGGGAC3′	Em13: 5′-GACTGCGTACGAATTGGT-3′
Me14:5′-TGAGTCCAAACCGGCTA-3′	Em14: 5′-GACTGCGTACGAATTCAG-3′
	Em15: 5′-GACTGCGTACGAATTCTG-3′
	Em16: 5′-GACTGCGTACGAATTCGG-3′

EM2, EM3, EM4, EM5, EM6, EM7, EM8, EM10, EM13, EM14, EM15 and EM16; Li and Quiros, 2001; Zhongxu et al., 2003; Lin et al., 2005). Each 20 mL PCR mixture consisted of 2 µl 10X PCR buffer, 20-50 ng DNA templates, 2 µl Mg2+, 0.7 µl dNTP, 2µl forward primer, 2 µl reverse primer and 0.3 µl Taq DNA polymerase. Amplifications were performed in an ABI 9700 Thermocycler with the following PCR program: 5 min initial denaturation at 94 °C; five cycles of three steps: 1 min denaturation at 94 °C, 1 min annealing at 35 °C and 1 min elongation at 72 °C; followed by 35 cycles with the annealing temperature increased to 55 °C; and a final elongation step of 10 min at 72 °C and hold at 4 °C. Amplification products were separated by electrophoresis through 2% agarose and metaphor agarose gels in TAE buffer.

Data analysis

SRAP primer amplification fragments were scored as present (1) or absent (0). The NTSYS-pc version 2.2 (Numerical Taxonomy Multivariate Analysis System, Exeter Software, Setauket, N.Y.) software program was used for distance matrix and dendrogram construction. First Dice similarity index was used to compute similarity coefficients for each pair of accessions. Then UPGMA (Unweighted Pair Group Method with Arithmetic Averages) and SHAN clustering programs were used to produce a genetic distance dendrogram. In order to analyze the correlation between the distance matrix and the dendrogram, a Mantel test was done (Mantel, 1967). Principal component analysis (PCA) was also performed in NTSYS.

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