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# DNA MICROSATELLITE ANALYSIS FOR TOMATO GENETIC DIFFERENTIATION

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Commonly used method for determination of the genetic diversity among the populations is the test for genetic differentiation. DNA microsatellite markers are usually used to investigate the genetic structure of natural populations. The aim of this study was to evaluate the applicability of eight DNA microsatellite loci (LECH13, LE21085, LEMDDNa, LEEF1Aa, LELEUZIP, LE20592, TMS9 and LE2A11) in genetic differentiation of six morphologically different tomato varieties (var. *grandifolium* from subsp. *cultum*; var. *cerasiforme* - red and yellow, var. *pruniforme* and var. *pyriforme* from subsp. *subspontaneum*; and var. *racemigerum* from subsp. *spontaneum*). The fragment analyses was performed using *Applied Biosystems* DNA analyzer *(ABI 3130*) and *GeneMapper® Software program.* The data were analysed using the specific program *Power Marker Software*. The average number of detected alleles was 3,625. Also, the average PIC value for all 8 DNA microsatellites loci was 0,3571. The genetic differentiation test in the researched tomato subspecies showed minor differentiation for locus LELEUZIP (- 0,0009), modest differentiation for locus LECH13 (0,0896), locus LEMDDNa (0,0896) and locus LE21085 (0,0551) and major differentiation for locus LE2A11 (0,7633), locus LEEF1Aa (0,6167), locus TMS9 (0.4967) and locus LE20592 (0,4263). On the other hand, in the estimated tomato varieties, locus LE21085 (0,0297), locus LECH13 (0,0256) and locus LELEUZIP (0,0005) showed minor differentiation, locus LEMDDNa (0,1333) showed modest differentiation, while locus TMS9 (0,5929), locus LEEF1Aa (0,5006), locus LE2A11 (0,4013) and locus LE20592 (0,2606) showed

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major differentiation. The eight DNA microsatellite loci can be applicable solution for tomato genetic differentiation. The overall results suggest that these microsatellite loci could be used in further population genetic studies of tomatoes.

*Key words:* DNA microsatellites, genetic differentiation, *Lycopersicon esculentum*, subspecies, varieties.

## INTRODUCTION

The cultivated tomato is second most consumed vegetable of the world. Worldwide, tomatoes are an important source of vitamins, minerals, phenolic antioxidants and anti-oxidant lycopene having anti-cancer characteristics (WILLCOX *et al.,* 2003). Over 75.000 accessions of cultivated and wild species of tomato are maintained in Genbank around the world (LARRY and JOANNE, 2007) but relative and absolute differentiation of these accessions need microsatellite markers (STR). The genome of tomato has been sequenced by the Tomato Genome Consortium in 2012. According to BREDEMEIJER *et al.* (1998) the microsatellite markers are especially suitable for a species such as tomato which has a low level of variation detected by other types of markers.

Microsatellite markers also called short tandem repeats (STRs) or simple sequence repeats (SSRs) because their polymorphism is based on the variation in the number of repeats of a simple DNA sequences are nowadays a tool of choice to address population genetics and demographic questions. Around 1.4 million STRs were generated from tomato genome (IQUEBAL *et al.*, 2013). The microsatellites play significant role in mapping, variety development, trait improvement and variety identification.

Traditionally, the variety characterization is based on morphological characteristics, but it is very difficult to distinguish varieties with very similar morphological characteristics and identification of the cultivars is essential for maintaining cultivar integrity and Plant Breeders' Rights (IQUEBAL *et al.*, 2013). Limited researches have been reported in variety identification of tomato using STR DNA markers (VOSMAN *et al.*, 2001). BREDEMEIJER *et al.* (2002) evaluated the suitability of 20 microsatellites markers for varietal identification and discrimination of the most common tomato varieties (>500) grown in Europa and it was shown that the database could be used in a reproducible way for variety discrimination and identification. According to SARDARO *et al.* (2013), from 27 STR markers, only 11 were able to discriminate 47 varieties. In the other research, 12 markers could differentiate 34 varieties (SRIVASTAVA *et. al*., 2011).

The test for genetic differentiation is widely used method for determination genetic diversity among the populations. The estimation of genetic differentiation (θ) based on the variance of allele frequencies among populations was proposed by WEIR and COCKERHAM (1984) and is most widely used estimator (cited approx. 7.000 times, source: Web of Science) (HOLSINGER and WEIR, 2009; EXCOFFIER, 2007). This estimator provides a nearly unbiased estimate of $F_{ST}$ at moderate population sample size (n = 15, 20 and 25) and small number of loci (10) and can also have negative values (WILLING *et al.*, 2012).

The objective of this study was to survey the applicability of eight DNA microsatellite loci in genetic differentiation of six morphologically different tomato varieties of *Lycopersicon esculentum* Mill.

## MATERIALS AND METHODS

*Plant material*

The plant material used in this research was obtained from GenBank of Agricultural Institution in Skopje. According to BREZHNEV (1964), analysed tomato varieties belong to three subspecies of *Lycopersicon esculentum* Mill. (var. *grandifolium* from subsp. *cultum* Brezh.; var. *cerasiforme* (with red fruits), var. *cerasiforme* (with yellow fruits), var. *pruniforme* and var. *pyriforme* from subsp. *subspontaneum* Brezh. and var. *racemigerum* from subsp. *spontaneum* Brezh.).

*DNA isolation and PCR conditions*

DNA was isolated from the leaves of 10 individual plants per each tomato variety using Promega's Wizard ® Genomic DNA purification kit. DNA was also extracted from pooled seeds of each variety using the modified CTAB method (DOYLE and DOYLE, 1987 and CULLINGS, 1992, MISKOSKA-MILEVSKA *et al.*, 2011a). DNA quality was checked in 0,8 % agarose gel, stained with ethidium bromide.

The optimization of PCR conditions for amplification of eight microsatellite loci (LECH13, LE21085, LEMDDNa, LEEF1Aa, LELEUZIP, LE20592, TMS9 and LE2A11) was performed using appropriate primers, produced by Operon, Huntsville, AL.

The selection of eight microsatellite loci was based on the number of alleles and diversity index from previous studies (ARESHCHENKOVA and GANAL, 1999; BREDEMEIJER *et al.*, 1998; VOSMAN *et al.*, 2001; ALVAREZ *et al*., 2001; BREDEMEIJER *et al*., 2002; HE *et al*., 2003; VILLALTA *et al.*, 2005; GARCIA-MARTINEZ *et al.*, 2006).

*Table 1. General data for microsatellite loci and primers used in his study*

| Locus | Repeat motif | Primer sequences (5′-3′) |
|---|---|---|
| **LECH13** | $(TA)_{6-1}(GA)_4$ | F: M13-taa caa tca aaa gaa ctt cgc |
| | | R:atc ccc tta ttg att aca tcc |
| **LE21085** | $(TA)_2(TAT)_{9-1}$ | F: M13-cat ttt atc att tat ttg tgt ctt g |
| | | R: aca aaa aaa ggt gac gat aca |
| **LEMDDNa** | $(TA)_9$ | F: M13-att caa gga act ttt agc tcc |
| | | R: tgc att aag gtt cat aaa tga |
| **LEEF1Aa** | $(TA)_8(ATA)_9$ | F: M13-aaa taa tta gct tgc caa ttg |
| | | R: ctg aaa gca gca aca gta ttt |
| **LELEUZIP** | $(AAG)_{6-1}TT(GAT)_7$ | F: M13-ggt gat aat ttg gga ggt tac |
| | | R: cgt aac agg atg tgc tat agg |
| **LE20592** | $(TAT)_{15-1}(TGT)_4$ | F: M13-ctg ttt act tca aga agg ctg |
| | | R: act tta act tta tta ttg cca cg |
| **TMS9** | $(GATA)_{26}$ | F: M13-ttg gta att tat gtt cgg ga |
| | | R: ttg agc caa ttg att aat aag tt |
| **LE2A11** | $(ATCT)_{5-1}$ | F: M13-aat ttt gta agg aga aga cgg |
| | | R: tca tat tct tca cac caa agg |

F - Forward primer (5'-3') R - Reverse primer (5'-3') M13 tail:5′-cac gac gtt gta aaa cga c-3′

Some general data for selected microsatellites and appropriate primer pairs are given in Table 1. PCR products were separated on 2 % agarose gel, stained with ethidium bromide and photographed under UV light by using G-Box system (Sygene).

The DNA extraction and optimization of the PCR conditions were done in the Laboratory for biochemistry and molecular biology within the Department of Biochemistry and Genetic Engineering at the Faculty of Agricultural Sciences and Food – Skopje (MISKOSKA-MILEVSKA *et al*., 2012).

*Data analyses*

The PCR products were analysed using *Applied Biosystems* DNA analyzer (*ABI 3130*) and *GeneMapper®Software program.* The statistical analyses were performed using the specific computer program *Power Marker Software. Power Marker* software program enable making test for genetic differentiation (θ). The genetic differentiation among populations is quantified according to WEIR and COCKERHAM (1984). The estimation of genetic differentiation (θ) is based on the variance of allele frequencies among tomato varieties (subspecies) for each of the eight DNA microsatellites.

## RESULTS AND DISCUSSION

The microsatellite markers are polymorphic both among tomato species and cultivars. One important goal of population genetic studies is to estimate the amount of genetic differentiation among populations. The use of DNA-based markers offers another approach for population level genetic analyses. The microsatellite markers are routinely used to investigate the genetic structuring of natural populations. Most statistics that describe the genetic differentiation from genetic markers rely solely on the allele identity information (HARDY *et al*., 2003). WEIR and COCKERHAM (1984) proposed an unbiased estimator (θ) of genetic differentiation that has been widely used.

The eight analysed microsatellite primer sets gave good amplification across the six tomato varieties and were used for molecular characterization of all six tomato varieties. Based on the received results the largest number of alleles (6) in investigated tomato varieties were found for locus LEEF1Aa and locus LE20592. Only two alleles were found for locus LE21085, locus LEMDDNa and locus TMS9. In the researched tomato varieties, for all microsatellite loci, the average number of detected alleles was 3,625. The number of alleles detected per microsatellite locus ranged from 2 to 6 is low in comparison with values for microsatellite loci in other crops such as grapevine, soybean and maize, but in agreement with previous researches on tomato microsatellite loci (BREDEMEIJER *et al.,* 1998; ALVAREZ *et al*., 2001; BREDEMEIJER *et al*., 2002; HE *et al*., 2003; MAZZUCATO *et al.,* 2008).

The informativeness of polymorphic DNA markers can be quantitatively measured by a statistic called the polymorphism information content or PIC. In this research, PIC-test showed different informativeness for analysed DNA microsatellites in the researched tomatoes. In investigated varieties, the lowest average PIC value was determined for locus LEMDDNa (0.0601), and the highest average PIC value was determined for locus LEEF1Aa (0,7552). The average PIC value for all 8 DNA microsatellite loci was 0.3571 and according to classification of BOTSTEIN (1980) they belong to the group of modest informative markers.

Obtained data from the test for genetic differentiation in the analysed tomato subspecies (Table 2) showed major differentiation for locus LE20592 (0,4263), locus TMS9 (0,4967),

LEEF1Aa (0,6167) and locus LE2A11 (0,7633), modest differentiation for locus LE21085 (0,0551), locus LECH13 (0,0896) and locus LEMDDNa (0,0896) and minor differentiation for locus LELEUZIP (- 0,0009). In general, all microsatellites loci showed major differentiation (0.3907) in the analysed tomato subspecies.

*Table 2. Obtained results from the test for genetic differentiation in the analysed tomato subspecies*

| Locus | θ |
|---|---|
| **LECH13** | 0,0896 |
| **LE21085** | 0,0551 |
| **LEMDDNa** | 0,0896 |
| **LEEF1Aa** | 0,6167 |
| **LELEUZIP** | -0,0009 |
| **LE20592** | 0,4263 |
| **TMS9** | 0,4967 |
| **LE2A11** | 0,7633 |
| **Total** | 0,3907 |

In the investigated tomato varieties, locus LE20592 (0,2606), locus LE2A11 (0,4013), locus LEEF1Aa (0,5006) and locus TMS9 (0,5929) showed major differentiation, locus LEMDDNa (0,1333) showed modest differentiation, while locus LE21085 (0,0297), locus LECH13 (0,0256) and locus LELEUZIP (0,0005) showed minor differentiation (Table 3). In general, all microsatellites loci showed major differentiation (0,2568) in the analysed tomato varieties.

*Table 3. Obtained results from the test for genetic differentiation in the analysed tomato varieties*

| Locus | θ |
|---|---|
| **LECH13** | 0,0256 |
| **LE21085** | 0,0297 |
| **LEMDDNa** | 0,1333 |
| **LEEF1Aa** | 0,5006 |
| **LELEUZIP** | 0,0005 |
| **LE20592** | 0,2606 |
| **TMS9** | 0,5929 |
| **LE2A11** | 0,4013 |
| **Total** | 0,2568 |

According to BREDEMEIJER *et al.* (1998) the values of gene diversity associated with each of the tomato microsatellite markers were ranged from 0,06 to 0,74 in the analysed set of 16 tomato cultivars and it was possible to distinguish all 16 cultivars with a selection of four microsatellites markers (locus LEEF1Aa, locus LEE11, locus LE21085 and locus LELE25). Also, according to BREDEMEIJER *et al.* (2002) the values for the genetic diversity of the tomato

microsatellite markers was ranged from 0,01 to 0,70 in more than 500 different European tomato varieties.

The differentiation statistics, as estimated from microsatellite allele frequencies, are still expected to be one of the most valuable tools for studying moderately structured populations. In general, all eight estimated microsatellites loci showed major differentiation in the analysed tomato subspecies and varieties. In the investigated tomatoes, four microsatellites loci (locus LEEF1Aa, locus LE20592, locus TMS9 and locus LE2A11) showed major differentiation on varieties and subspecies level. Based on microsatellites loci research, analysis of molecular variance (AMOVA) showed that 25,3 % of the total variance in investigated varieties was among varieties and 74,7 % of the total variance in investigated varieties was within the varieties.

Obtained data was important for evaluation of the genetic distance among the analyzed tomatoes and creation of the precise dendrogram according to Neighbor-Joining method (MISKOSKA-MILEVSKA *et al*., 2011b). Namely, estimated θ and Nei's standard genetic distance allowed investigated tomato varieties to be divided into three main clusters. Also, investigated tomato species were clearly separated in three clusters which is in agreement with the classification of Brezhnev (MISKOSKA-MILEVSKA *et al*., 2011b).

In conclusion, the eight DNA microsatellite loci showed to be adequate option for tomato genetic differentiation. Based on the gained data, it can be recommended that these microsatellite loci could be used in further population genetic studies of tomatoes. Namely, those microsatellites markers could be used for studies of genetic diversity, mapping, and variety identification in different tomato varieties.

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## ANALIZA DNA MICROSATELLITA ZA GENETIČKU DIFFERENCIJACIJU PARADAJZA

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Izvod

Najčešće korišćen metod za određivanje genetičke raznovrsnosti među populacijama je test genetičke diferencijacije. Cilj ovog istraživanja je bio ispitivanje primenljivosti osam DNK mikrosatelitskih lokusa (LECH13, LE21085, LEMDDNa, LEEF1Aa, LELEUZIP, LE20592, TMS9 i LE2A11) za genetičke diferencijacije šest morfološki različitih varieteta paradajza (var. *grandifolium* iz subsp. *cultum*; var. *cerasiforme* - crven i žut, var. *pruniforme* i var. *pyriforme* iz subsp. *subspontaneum*; i var. *racemigerum* iz subsp. *spontaneum*). Fragment analiza je izvedena korišćenjem *Applied Biosystems* DNA analyzer *(ABI 3130*) i *GeneMapper*® softverskog programa. Podaci su analizirani korišćenjem specifičnog programa *Power Marker Software*. Prosečan broj utvrđenih alela bio je 3.625. Takođe, prosečna PIC vrednost za svih osam mikrosatelitskih lokusa bila je 0,3571. Dobijeni podaci iz testa za genetičku diferencijaciju ispitivanih varieteta paradajza pokazali su malu diferencijaciju za lokus LELEUZIP (- 0,0009), umerenu diferencijaciju za lokus LECH13 (0.0896), lokus LEMDDNa (0,0896) i lokus LE21085 (0,0551) i veliku diferencijaciju za lokus LE2A11 (0.7633), lokus LEEF1Aa (0.6167), lokus TMS9 (0,4967) i lokus LE20592 (0,4263). Kod ispitivanih varijeteta paradajza, lokus LE21085 (0,0297), lokus LECH13 (0,0256) i lokus LELEUZIP (0,0005) su pokazali malu diferencijaciju, lokus LEMDDNa (0,1333) je pokazao umerenu diferencijaciju, dok lokus TMS9 (0,5929), lokus LEEF1Aa (0,5006), lokus LE2A11 (0,4013) i lokus LE20592 (0.2606) su pokazali veliku diferencijaciju. Na osnovu dobijenih rezultata, ovi mikrosatelitski lokusi mogu se koristiti u daljim proučavanjima populacione genetike kod paradajza.

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