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Original Article

Usefulness of a Locus LE21085 in the Genetic Differentiation of Tomato Varieties

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Abstract

The microsatellites are specific for each individual genome or species. In order to evaluate the genetic diversity and the relationships within the genus *Lycopersicon* microsatellites markers were used. The main objective of this study was to determine the usefulness of the locus LE21085 in the genetic differentiation among six morphologically different tomato varieties of *Lycopersicon esculentum* Mill. (var. *grandifolium* subsp. *cultum*; var. *cerasiforme* - red and yellow, var. *pruniforme,* var. *pyriforme* subsp. *subspontaneum*, and var. *racemigerum* subsp. *spontaneum*). For the microsatellites locus LE21085 were detected two alleles in all estimated tomato varieties, that differed by one base pair (122 and 123 bp). The biggest allele frequency was found for the allelic variant of 122 bp, and its values were: 0.8462 for *L. esculentum* subsp. *subspontaneum* var. *cerasiforme* (red), 0.6923 for *L. esculentum* subsp. *subspontaneum* var. *cerasiforme* (yellow), 0.5769 for *L. esculentum* subsp. *cultum* var. *grandifolium*, 0.6923 for *L. esculentum* subsp. *subspontaneum* var. *pruniforme*, 0.6154 for *L. esculentum* subsp. *subspontaneum* var. *pyriforme* and 0.8077 for *L. esculentum* subsp. *spontaneum* var. *racemigerum*. The average of observed heterozygosity for the locus LE21085 (Ho = 0.5641) was higher than average expected heterozygosity (He = 0.4158). The average PIC value for the locus LE21085 was 0.3294 and it was classified as a modest informative markers. From the obtained results it can be concluded that locus LE21085 could be used in genetic differentiation of tomato varieties, but in combination with other polymorphic microsatellite loci.

Keywords: DNA microsatellites, fragment analyses, locus LE21085, tomato

Introduction

Molecular markers have many advantages for plant variety identification over the more traditionally used morphological traits because of their independence from environmental influences, their generally high level of polymorphism, their almost unlimited availability, and their greater potential for automation (Bredemeijer *et al.*, 2002). Molecular techniques are also likely to be extremely discriminating and much more rapid (Vosman *et al.*, 2001). Genotypic differences detected by molecular markers can be used for cultivar identification and protection of the plant breeder's intellectual property rights (Garcia-Martinez *et al.*, 2006). According to Miller and Tanksley (1990) the molecular marker of choice must be very informative, especially in crop like tomato where genetic diversity is very limited (Bredemeijer *et al.*, 2002).

The microsatellites, also called simple sequence repeats (SSRs) appear as suitable molecular markers because of their highly polymorphic character. SSR markers have the advantages of being multiallelic, highly polymorphic, co-dominant and assayable by PCR. The first application of SSR markers in plants has been in cultivar identification. Around 1.4 million STRs were generated from tomato genome (Iquebal *et al.*, 2013). Microsatellites are useful for a number of analyses. The SSRs play significant role in mapping variety development, trait improvement and variety identification. According to He *et al.* (2003) microsatellite markers were demonstrated to be highly polymorphic and efficient for differentiating genetic materials, further suggesting their capacity for practical application in cultivar and seed purity identification and phylogenetic study. The enlightening, of microsatellites, as a genetic marker has already been shown with great success in several plant species (Alvarez *et al.*, 2001). A significant role of microsatellite markers in research of genetic diversity and variability of genus *Solanum*, as well as for tomato variety identification was confirmed in many studies (Smulders *et al.*, 1997; Bredemeijer *et al.*, 2002; He *et al.*, 2003; Villalta *et al.*, 2005; Garcia-Martinez *et al.*, 2006; Mazzucato *et al.*, 2008). SSR markers are becoming the preferred molecular

210

marker for variety identification, genetic mapping and marker-assisted selection in tomato (He *et al.*, 2003). According to Smulders *et al.* (1997), the level of polymorphism and the scorability of the banding patterns are important when choosing new microsatellite loci for identification purposes or for studies on genetic variation. The allelic variation may be correlated with the number of repeats within a particular microsatellite locus (He *et al.*, 2003).

The informative amount of polymorphic DNA markers can be quantitatively measured statistically by means of the polymorphism information content (PIC). Selected molecular markers must be very informative especially for crops like *Lycopersicon esculentum*, where genetic diversity seems very limited (Miller and Tanksley, 1990). Such microsatellites may show polymorphisms that can be useful for the analysis of genetic diversity, as well as determination of species relationships within the genus *Lycopersicon*. The interest for the microsatellite locus LE21085 is presented in the studies of Smulder *at al.* (1997), Bredemeijer *et al.* (1998), Alvarez *et al.* (2001), He *et al.* (2003), Villata *et al.* (2005), Garcia-Martinez *et al.* (2006), Mazzucato *et al.* (2008).

The main objective of this work was to examine the potential of the locus LE21085 in genetic differentiation among six morphologically different tomato varieties of *Lycopersicon esculentum* Mill.

Materials and Methods

Plant material

In this study, six tomato varieties of *Lycopersicon esculentum* Mill. (var. *grandifolium* subsp. *cultum*; var. *cerasiforme* – red and yellow, var. *pruniforme,* var. *pyriforme* subsp. *subspontaneum* and var. *racemigerum* subsp. *spontaneum*) were analysed. *Lycopersicon esculentum* var. *grandifolium* has large, red fruits with flattened shape. *Lycopersicon esculentum* var. *cerasiforme* produces small fruits with slightly flattened shape in red or yellow colour. *Lycopersicon esculentum* var. *pruniforme* forms small, red or yellow fruits with specific oval (plum) shape. *Lycopersicon esculentum* var. *pyriforme* forms pear-shaped small red fruits. *Lycopersicon esculentum* var. *racemigerum* is characterized by medium-large fruits with flattened shape in red or yellow colour. The plant material was obtained from the GeneBank of the Agricultural Institute in Skopje.

DNA isolation and PCR conditions

The DNA isolation and optimization of the PCR conditions were performed 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). The DNA was isolated from fresh leaves using Promega's Wizard ®Genomic DNA purification kit. The leaves were collected from ten individual plants per each variety. Also, pooled seeds were used for DNA isolation. The DNA isolation from seeds was done using modified CTAB method (Miskoska-Milevska *et al.*, 2011).

Namely, collected leaves were stored at -70 °C till the DNA isolation, even if the step was not part of the protocol. The amount of starting plant material for DNA isolation was 50 mg instead 10-20 mg. To remove RNA it was necessary to be added treatment with RNaze (10 µg/µL). Also, phenol/chloroform re-extraction was necessary for obtaining high quality DNA (Miskoska-Milevska *et al.*, 2011). The quality of the isolated DNA was checked by running it on 0.8% agarose gel. The optimization of the PCR conditions for amplification of the locus LE21085 was performed using appropriate primers, produced by Operon, Huntsville, AL. Some general data for the locus LE21085 and appropriate primer pair are presented in Table 1 (Miskoska-Milevska *et al.*, 2012). Visualization of the PCR products were done by running them on 2% agarose gel, stained with ethidium bromide and photographed under UV light by using G-Box system (Sygene).

Data analyses

The fragment analyses of the PCR products were performed on *Applied Biosystems* DNA analyzer (*ABI 3130*) using *GeneMapper®Software program*. The specific program *Power Marker Software* was used for data analyzing.

Results and Discussion

The analyzed microsatellite primers showed strong amplification within the estimated tomato varieties and were used for the fragment analysis (Fig. 1 and Fig. 2). The data from fragment analysis was presented in the form of electropherograms of heterozygous (Fig. 1) and homozygous samples (Fig. 2). The interpretation of the obtained electropherograms is significant because the further statistical analyses are based on these results. The peaks higher than 100 RFU and lower than 2,000 RFU were taken in consideration.

The fragment analyses of the locus LE21085 detected only two allelic variances (122-123 bp) and their frequencies are shown in Fig. 3. The detected alleles differed by one base pair, as well as in study of Alvarez *et al.* (2001). Alvarez *et al.* (2001) noticed 12 different alleles in the researched tomatoes (90-93-96-99-102-105-108-111-118-119-127-132 bp), and the number of specific alleles was six. According to these researchers, alleles of unexpected sizes occur across the genus. Thus, 31% of the microsatellites analysed appear to be concordant with the single-step mutation model and another 31% possibly follow this model with minor modification, although the presence of these series by itself does not prove that they proceeded by mutation with one repeat unit at a time (Alvarez *et al.*, 2001).

The Stepwise Mutation Model (SMM) implies that an allele mutates only by losing or gaining a single tandem repeat. According to this model, the allele with i repeats are assumed to mutate to an allele either in state $i+1$ or $i-1$ with an equal probability (Ohta and Kimura, 1973). In the present research, it is obviously appearance of alleles that differ in only one base pair (Fig. 1). It can be assumed that appearance of these alleles is in line with Stepwise Mutation Model (SMM) proposed by Ohta and Kimura (1973).

The knowledge of the mutation process is necessary for understanding the species relationships and for determination of genetic distance or population structure, respectively.

Usually, the assessment of species relationships is based on interpretation of allozyme variations, but IAM-model (Infinite Allele Model) shows as very successful in explanation of alozyme variations (Nei, 1987). The situation is much different for the microsatellite markers. As first, since more of the mutations include receiving or losing one repetitive unit (Weber and Wong, 1993), it is clear that there exists a high amount of homoplasy. This is important as homoplasy leads to the underestimation of the total amount of variation and genetic distance, and to the overestimation of the similarities among populations.

These two models, the IAM and SMM, represent the extremes of the situation. In the IAM no homoplasy exists, while in the SMM, a large amount is present. Microsatellites have been estimated to mutate at rate between 10^3 and 10^5 mutations per gamete (Edwards *et al.*, 1992; Bowcock *et al.*, 1994; Forbes *et al.*, 1995). Empirical data also suggests that the structure of the microsatellite repeat may be very important in determining the operating mutational process (Estoup *et al.,* 1995a, 1995b). Estoup *et al.* (1995a) examined the variation, found at two loci with irregular microsatellite repeats in bees in order to determinate the levels of homoplasy that exist between populations. Irregular repeats are core repeats that are interrupted by point mutations. The presence of these mutations allows alleles with similar sizes to be distinguished. Not surprisingly, as the taxonomic level of the comparisons increased (i.e. among species), the amount of homoplasy increased.

In this regard it is clear that evolution of microsatellites is a very complex process. More research work is needed in order to completely understand the mutations that are necessary for proper interpretation of microsatellites results.

For locus LE21085, Smulders *et al.* (1997) detected five different alleles in researched tomatoes, while Villata *et al.* (2005) noticed only one allele (130 bp). Four different alleles were detected for same locus in researched tomatoes by Bredemeijer *et al.* (2002) and Garcia-Martinez *et al.* (2006). Three alleles were noticed on this microsatellite locus by He *et al.* (2003) and Mazzucato *et al.* (2008), while Bredemeijer *et al.* (1998) found two alleles (103-116 bp).

The differences in allele number and size are evident, between this study and the studies mentioned before. This difference is probable due to the different plant material used in each research. Namely, Bredemeijer *et al.* (1998) and He *et al.* (2003) researched only cultivated tomato accessions, whereas in this study, tomato varieties from subsp. *cultum*, subsp. *subspontaneum* and subsp. *spontaneum* were included. Also, the difference in allele size could be due to the methodological approach. Different DNA analysers, as well and different working conditions on the same DNA analyser (for ex. different type of polymer, different capillary length) could be reason for receiving such differences in allele size. In this regard might be concluded that, working on same DNA analyser with the same working conditions (for ex. same capillary length, same type of polymer) is the best option.

The data presented in Fig. 3 showed that the allelic variances in size of 122 and 123 bp appeared on the locus LE21085 across all analysed varieties.

In the analysed varieties, the biggest allele frequency was found for the allelic variant of 122 bp, and its values were: (0.8462) for *Lycopersicon esculentum* subsp. *subspontaneum* var. *cerasiforme* (red), (0.6923) *Lycopersicon esculentum* subsp. *subspontaneum* var. *cerasiforme* (yellow), (0.5769) for *Lycopersicon esculentum* subsp. *cultum* var. *grandifolium*, (0.6923) for *Lycopersicon esculentum* subsp. *subspontaneum* var. *pruniforme*, (0.6154) for *Lycopersicon esculentum* subsp. *subspontaneum* var. *pyriforme* and (0.8077) for *Lycopersicon esculentum* subsp. *spontaneum* var. *racemigerum*.

The average observed heterozygosity for the locus LE21085 (Ho = 0.5641) was higher than average expected heterozygosity (He=0.4158), meaning increased level of heterogeneity in the researched tomato varieties (Table 2).

The polymorphism information content (PIC) is used to quantitatively measure the informativeness of polymorphic DNA markers. In the analysed tomato varieties, average PIC value for the locus LE21085 was 0.3294 (Table 2). According to the classification of Botstein *et al.* (1980), this locus belongs to the group of modest informative markers.

The genetic differentiation test (θ) in the analysed tomato varieties presented minor genetic differentiation for the locus LE21085 (0.0297). Also, this test showed modest differentiation for the locus LE21085 on subspecies level (0.0551) (Miskoska-Milevska *et al.*, 2015).

Table 1. General data for microsatellite locus LE21085 and primers used in this study

Locus	Repeat motif	Primer sequences (5'-3')
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

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

Table 2. Genetic variability and polymorphism of locus LE21085 in the researched tomato varieties

Locus	Number of genotypes	Number of alleles	*He*	*Ho*	PIC
LE21085	3.0000	2.0000	0.4158	0.5641	0.3294

He – expected heterozygosity
Ho – observed heterozygosity
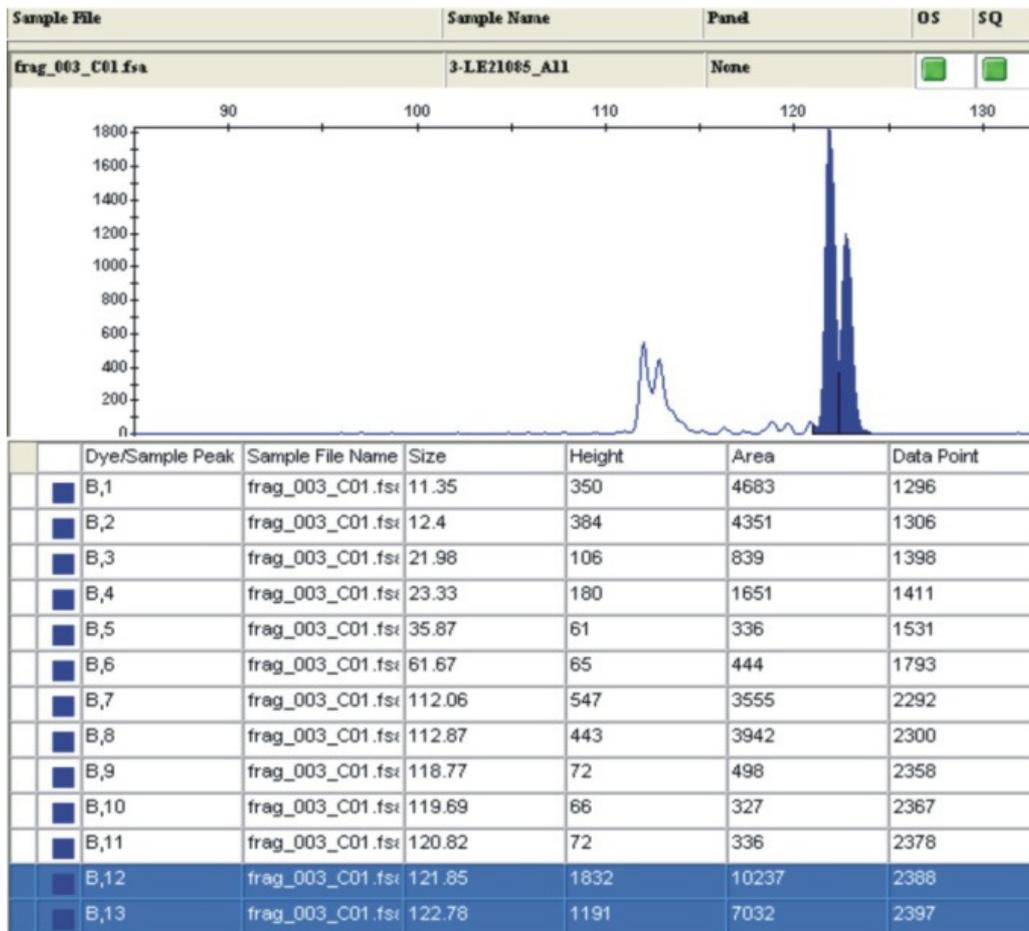PIC – test for determination of informativeness for analysed DNA microsatellite locus

Fig. 1. The electropherograms of locus LE21085 in the researched tomato varieties
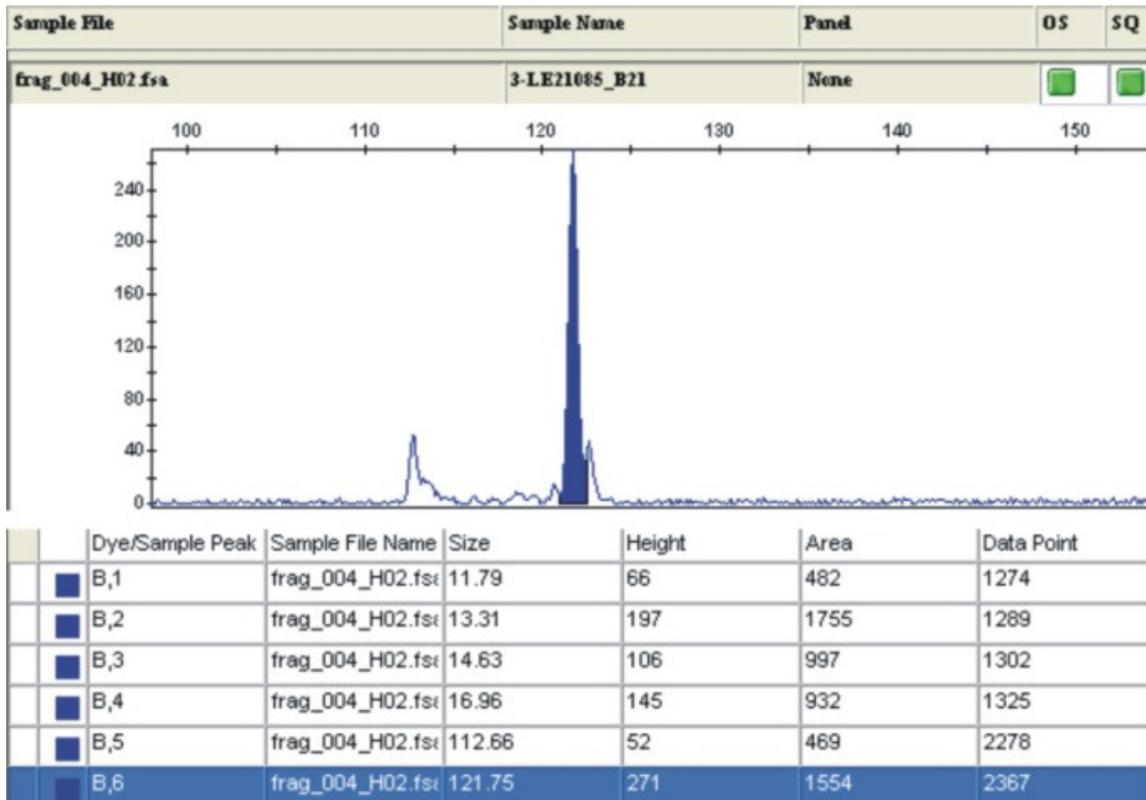

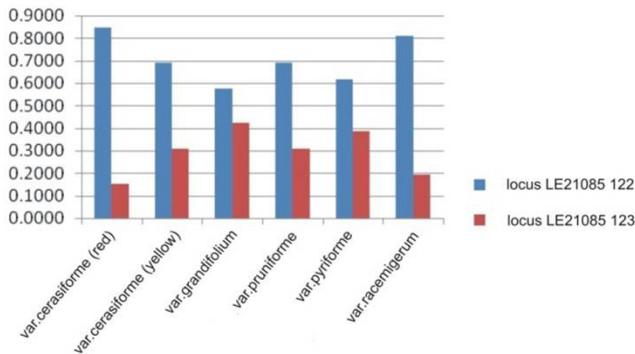Fig. 2. The electropherograms of locus LE21085 in the researched tomato varieties

Fig. 3. The allelic variances and their frequencies for locus LE21085 in the researched tomato varieties

Conclusions

The microsatellite locus achieved amplification across all the six tomato varieties. The detected allelic variances were different by one base pair. For distinguishing the tomato varieties it is necessary, that the locus LE21085 to be combined with other more polymorphic microsatellite loci.

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