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GENETIC CHARACTERISATION OF AUTOCHTHONOUS SWEET CHERRY GENOTYPES (*Prunus avium* L.) USING SSR MARKERS

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The autochthonous local genotypes of sweet cherries, which represent a very important genetic potential for future breeding programs. Understanding of the molecular basis biodiversity is one of the most important factors for the proper conservation, management and use of plant genetic resources.

This paper investigate the genetic variability of 14 genotypes of sweet cherries using 26 SSR markers. The study included eight autochthonous genotypes of sweet cherry taken from four different location and six virus-free reference varieties of sweet cherry. The average genetic distance between them was 0.43. The number of alleles per locus ranged from two to eight. The minimum number of two alleles of polymorphic loci showed EMPa003 and EMPa002, while the highest number of eight loci alleles had PceGA34 and UDP97-402**.** Results of genetic analysis that were done show that between autochthonous genotypes of sweet cherries 'Biljur-Bjelica', 'Barevka' and 'Ašlamka' (Kriškovci) there was no difference, it is to have the same genetic profil, which indicates that is the synonym, it is the same identity group. Also the autochthonous genotype 'Crveni Hrušt' and 'Nordwunder' cultivar had the same SSR profile on microsatellite loci tested. The remaining autochthonous genotypes of sweet cherry had a unique genetic profile.

Keywords: Prunus avium L., autochthonous genotype, varieties, genetic diversity, SSR

INTRODUCTION

Sweet cherry (*Prunus avium* L.) is a diploid species characterized by a haploid genome. Nowadays, it is grown on all continents and is one of the most important commercial fruits

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whose production on a global level has had a constant growth trend in the last few decades (DIRLEWANGER *et al.,* 2007). According to numerous authors, the Balkan Peninsula is one of the richest centers of genetic diversity of fruit species in Europe. The first written information on growing fruit trees in the territory of the Republic of Srpska and Bosnia and Herzegovina date back to the period of the Ottoman Empire (ĐURIĆ *et al*., 2009), but the first inventory and statistics in fruit growing were performed in the times of the Austro-Hungarian Empire, during the period 1882 - 1896 (ĐURIĆ *et al.,* 2009). Diversities within the population of the same species are reflected in the number of properties, i.e. genetic diversity enabling the species better adaptation to the environment and its survival in unfavorable environment conditions. In addition to wild species, the domestic, autochthonous varietes, made after longtime selection, are also important for biological diversity. The knowledge on autochthonous varietes has remained mostly traditional to this day and has usually been transferred through generations. In most of the cases, the varieties got their names based on a geographic area or a sort specificity that made them unique. OGNJANOV (2012) says that autochthonous cultivars are characterized with high tolerance capacity for biotic and abiotic types of stress. In addition to natural resistance to biotic and abiotic types of stress, autochthonous cultivars are sometimes also characterized by unusual morphological and pomological properties, and, very often, by excellent fruit quality. The fruits of these cultivars may not look perfect, i.e. not belonging to the first commercial class, but they have other properties like fullness of taste, sugars and acids ratio, and some varietes even have special aroma of their fruits (SKENDROVIĆ-BABOJELIĆ *et al.,* 2014).

In the territory of Banja Luka region, RADOŠ *et al*. (1996) identified and, based on pomological, economic and biological aspects, defined the genotypes of 'Banjalučka crnica', 'Cipov', 'Biljur-Bjelica', 'Crveni Hrušt', 'Ašlamka', 'Azijanka' and 'Barevka'. In addition to this research, which referred to morphological characteristics of the aforementioned sweet cherry genotypes, to this day, nobody has ever performed the molecular identification of autochthonous sweet cherry genotypes. Classical methods of describing morphological properties include certain deficiencies, such as: objectivity in identification, great similarities among the cultivars, the connection of the analysis for the fruiting period, etc. Unlike the morphological, the advantages of molecular identification have been proven through numerous research (GRANDO *et al*., 1996). Microsatellite markers are used not only for cultivar identification but also for the discrimination of clones and for the verification of synonyms and homonyms. Intensive application of microsatellite markers started after 2000, and they have been widely applied even within the *Prunus* genus. In order to evaluate the diversity and identify the cultivars and genotypes, the largest number of research with the application of microsatellite markers was implemented for sweet cherry (CANTINI *et al.,* 2001; WÜNSCH and HORMAZA, 2002; CLARKE and TOBUTT, 2003; VAUGHAN and RUSSELL, 2004; VAUGHAN *et al.,* 2004; PEDERSON, 2006; KACAR *et al*., 2005; LACIS *et al*., 2009; ZHONG *et al*., 2009; TANČEVA-CRMARIĆ *et al.,* 2011; ERCILSI *et al.,* 2011; BALLIAN, 2012; VOKURKA *et al.,* 2012; BARAĆ *et al.,* 2014; SHARMA *et al.,* 2015; FURSAD and ESNA-ASHARI, 2016). Based on results published by numerous authors, genotypes from natural populations showed a higher level of diversity within the cultivated varietes of sweet cherry (GANOPOULOS *et al*., 2010). The differences reflected on the level of diversity between the cultivated and wild varieties of *Prunus avium* L. originating directly from the fact that the genetic basis used in cultivating this plant species is very narrow (WUNSCH and

HORMAZA, 2002). The aims of these study were to determine molecular profile of eight local genotypes of sweet cherry grown in Bosnia and Herzegovina, Republic of Srpska by SSR markers, and to determine their genetic similarity or genetic distance.

MATERIALS AND METHODS

Genetic characterization has been performed on the following autochthonous genotypes of sweet cherry: 'Ašlamka' (the village of Maglajani), 'Banjalučka crnica', 'Biljur-Bjelica', 'Crveni Hrušt', 'Ašlamka' (the village of Kriškovci), 'Cipov' (the village of Donja Jurkovica) 'Azijanka' (Trapisti, Banja Luka) and 'Barevka' (Trapisti, Banja Luka) and six introduced virus-free varietes of sweet cherry: 'Nordwunder', 'Van', 'Burlat', 'Stella', 'Hedelfingen' and 'Giorgia'. The introduced sweet cherry varietes are referent and, in this research, they were used as a standard for determining the possible synonyms for the analyzed autochthonous genotypes. All analysis have been carried out at the Faculty of Agriculture in the Department of Plant Breeding, Genetics and Biometrics, University of Zagreb.

DNA extraction from plant tissue

The total DNA was isolated from young leaf tissue which previously dried in the process of lyophilisation. This procedure was performed on lyophilisator (Christ, model Beta 1-8 lyophilizator) where the plant tissue was grinded to fine powder. The isolation of the total genome DNA from a sweet cherry leaf was extracted using *DNeasy® Plant Mini Kit* according to the producer's manual (Qiagen, 2015). Quality of isolated DNA was checked in 0.8% agarose gel using horizontal electrophoresis (SEA 2000 Submerged Gel Electrophoresis System, Guest Elchrom Scientific AG, Switzerland). The gel was dyed by ethidium bromide, following by visualization under UV light, and digitally photographed using GelDoc (BioRad, USA). DNA concentration determination of the obtained DNA concentration was performed with fluorimeter (Biorad, China).

PCR amplification and SSR analysis

PCR-Polymerase Chain Reaction of multiplication of microsatellite region occurred by VeritiTM 96-well Thermal Cycler *(Applied Biosystems*, USA). It was prepared in the total volume of 10 μl with 20 ng DNA in a buffer system 10 mM Tris-HCl, pH 8.3 and 50 mM KCl, with 0.5 U Taq polymerase (Sigma), 2.0 mM $MgCl_2$, 0.2 mM of each dNTPa and 0.4 μM of each primer (*forward* and *reverse*). The first stage of PCR reaction is denaturation of DNA at 95°C for five minutes. The amplification of DNA molecule was performed in 11 cycles with denaturation temperatures of 94°C for 45 seconds and primers annealing at the temperature of 56°C for 30 seconds, and in each of the 11 cycles, the temperature was lower by 0.3°C compared to the previous one. After 11 cycles, the annealing temperature gradually lowered to 53°C, the same annealing temperature continued through the next 24 cycles. After all cycles were finished, the extended elongation followed and it lasted for 5 minutes at the temperature of 72°C.

Separation of PCR fragments was performed in the capillary sequencer, model ABI 3130 (*Applied Biosystems*, USA). The obtained information in the form of DNA fragments visible as

graphical peaks was analyzed by *GeneMapper 4.0* software module (*Applied Biosystems*, USA). The readings of every sample were additionally checked by manually, i.e. visual overview of amplification for each sample. Only the visually unquestionable fragments for each SSR fragment were used for further analysis**.**

Data analysis

All biometric analyses in this research were performed through software package SPSS 22 (IBM, 2013) and R (CRAN, 2016). Based on calculated values of similarity coefficient according to the aforementioned software packages, cluster analysis of grouping all possible pairs was also performed. The dendrogram presented in Chart 1 was also constructed with cluster analysis.

RESULTS

DNA amplification and subsequent analyses of relationship (genetic similarity and identity) among local sweet cherry varieties was performed on the basis of the DNA amplification of 24 microsatellite markers (Ex: Table 1). We therefore used a standardised set of internationally agreed SSR markers from the *Prunus* working group of the ECPGR-FN (European Collaborative Programme for Genetic Resources – Fruit Network; CLARKE and TOBUTT, 2009). Primers Ps05c03 and BPPCT-026 did not result in DNA amplification, so there are none of them in the matrix of final results. As a result of this research, with the aid of molecular markers, the total number of 116 alleles was identified on 14 varieties, out if which eight were autochthonous genotypes and six were referential varieties. Out of the total number of identified loci, 113 were polymorphic, and only three loci were monomorphic. The number of alleles per locus went from two to eight. The average number of locus which made the difference between varieties was 4.83.The lowest number of two polymorphic alleles appeared on loci EMPa003 and EMPa002, where as the highest number of eight alleles was provided by loci PceGA34 and UDP97-402. Seven polymorphic alleles were recorded with primer BPPCT-040, whereas primers UDP98-412, EMPaS02, BPPCT-37, EMPaS10, EMPaS06 and BPPCT-005 provided six amplified DNA fragments each. Primers CPPCT06, EMPaS01, EMPaS12, BPPCT-039 and UDP97-403 each amplified five alleles for each locus, whereas four polymorphic alleles for each of the following primers CPPCT-22, EMPaS14, BPPCT-026 and UDP98-411. Three loci were amplified with EMPa017 primers: two of them were monomorphic, and the remaining one was polymorphic. Three loci amplification was also recorded with UDP96-005 primer, where only one was monomorphic and two were polymorphic. The primers EMPa026 and BPPCT-034 also amplified three alleles and they were all polymorphic.

Additionally, the length of DNA fragments obtained through Polymerase Chain Reaction by applying the aforementioned primers of analyzed genotypes went from 101 to 258 of base pairs (bp). The average length of DNA fragments was 164 bp. The longest DNA fragment was recorded with EMPa017 primer on third amplified locus, and it had 258 bp, whereas the shortest fragment was provided by the first amplified locus of UDP98-412 primer, whose length was 101 bp. From the profile of vertical electrophoresis, numeric value (1) was awarded to amplified loci by applying 24 primers; and those that were not amplified, got the numeric values (0). Further calculating of coefficients of similarity (dissimilarity) according (Jaccard) and cluster analysis

was performed on the basis of binary matrix. The obtained results of coefficient of genetic similarity calculated according to Jaccard are presented in Table 2.

Table 1. List of SSR primers names, sequences and references

Primers name	Marking primers in research	Fluorescent color	Sequences (Forward and Reverse 5' → 3')	References
UDP98-412	P1	6Fam	AGGGAAAGTTTCTGCTGCAC GCTGAAGACGACGATGATGA	Testolin et al., 2000.
CPPCT-22	P2	Vic	CAATTAGCTAGAGAGAATTATTG GACAAGAAGCAAGTAGTTTG	Aranzana et al., 2002.
EMPaS02	P3	6Fam	CTACTTCCATGATTGCCTCAC AACATCCAGAACATCAACACAC	Vaughan and Russell, 2004.
Ps05c03	P4	Ned	AGATCTCAAAGAAGCTGA AGCTTATGCATATACCTG	Sosinski et al., 2000.
CPPCT06	P5	Vic	AATTAACTCCAACAGCTCCA ATGGTTGCTTAATTCAATGG	Aranzana et al., 2002.
BPPCT-37	P6	6Fam	CATGGAAGAGGATCAAGTGC CTTGAAGGTAGTGCCAAAGC	Dirlewanger et al., 2002.
EMPaS01	P7	6Fam	CAAAATCAACAAAATCTAAACC CAAGAATCTTCTAGCTCAAACC	Vaughan and Russell, 2004.
PceGA34	P8	Ned	GAACATGTGGTGTGCTGGTT TCCACTAGGAGGTGCAAATG	Downey and Iezzoni, 2000.
EMPaS14	P9	Ned	TCCGCCATATCACAATCA AC TTCCACACAAAAACCAAT CC	Vaughan and Russell, 2004.
EMPaS10	P10	Vic	GCTAATATCAAATCCCAG CTCTC TGAAGAAGTATGGCTTCT GTGG	Vaughan and Russell, 2004.
EMPa002	P11	6Fam	TGACAGGTCATCATACCA TTTG CAGGATTAAGCATTGCAA ATTA	Clarke and Tobutt, 2003.
EMPa017	P12	6Fam	ATTTCAATGTGGGGATGA GC TGAAGTGAGGGAAATGGA GC	Clarke and Tobutt, 2003.
EMPa003	P13	Vic	AGCCATTCTGAAAAGGTGGA GCATTCAGCCAACAAAATCA	Clarke and Tobutt, 2003.
EMPa026	P14	Ned	ATTGAAAAAGCCAAAGAGCG TTCACGGTTTGAAGCAAGTG	Clarke and Tobutt, 2009.
EMPaS06	P15	6Fam	AAGCGGAAAGCACAGGTAG TTGCTAGCATAGAAAAGAATTGTAG	Vaughan and Russell, 2004.
EMPaS12	P16	6Fam	TGTGCTAATGCCAAAAATACC ACATGCATTTCAACCCACTC	Vaughan and Russell, 2004.
BPPCT-034	P17	Vic	CTACCTGAAATAAGCAGAGCCAT CTACCTGAAATAAGCAGAGCCAT	Dirlewanger et al., 2002.
BPPCT-038	P18	Pet	TATATTGTTGGCTTCTTGCATG TGAAAGTGAAACAATGGAAGC	Dirlewanger et al., 2002.
BPPCT-039	P19	Vic	ATTACGTACCTAAAGCTTCTGC GATGTCATGAAGATTGGAGAGG	Dirlewanger et al., 2002.
BPPCT-040	P20	Ned	ATGAGGACGTGTCTGAATGG AGCCAAACCCCTCTTATACG	Dirlewanger et al., 2002.
UDP96-005	P21	6Fam	GTAACGCTCGCTACCACAAA CCTGCATATCACCACCCAG	Cipriani et al., 1999.
UDP97-403	P22	Pet	CTGGCTTACAACTCGCAAGC CGTCGACCAACTGAGACTCA	Cipriani et al., 1999.
UDP98-411	P23	Ned	AAGCCATCCACTCAGCACTC CCAAAAACCAAAACCAAAGG	Testolin et al., 2000.
BPPCT-026	P24	6Fam	ATACCTTTGCCACTTGCG TGAGTTGGAAGAAAACGTAACA	Dirlewanger et al., 2002.
UDP97-402	P25	Vic	TCCCATAACCAAAAAAAACACC TGGAGAAGGGTGGGTACTTG	Cipriani et al., 1999.
BPPCT-005	P26	Ned	GCTAGCAGGGCACTTGATC ACGCGTGTACGGTGGAT	Dirlewanger et al., 2002.

The coefficient of genetic similarity of 14 examined genotypes of sweet cherry ranged from 0.14 to 1.00. The lowest similarity, 0.14, was between local genotype 'Cipov' and 'Stella', while the highest similarity (1.0) was determined between 'Crveni Hrušt' and 'Nordwunder', and also among 'Biljur-Bjelica', 'Barevka', and 'Ašlamka' (Kriškovci), revealing all the genotypes with the coefficient of similarity equaling 1.00 are in fact genetic synonyms, or the same variety with different local names.

Table 2. The analysis of similarity coefficient according to Jaccard

Genotypes of sweet cherry	Crveni Hrušt	Ašlamk (Kriškovci)	Banjalučka crnica	Biljur-Bjelica	Cipov	Ašlamka (Maglajani)	Barevka	Azijanka	Nordwunder	Burlat	Stella	Van	Hedelfingen	Giorgia
Crveni Hrušt	1.00	0.32	0.37	0.32	0.23	0.35	0.32	0.49	1.00	0.30	0.41	0.33	0.36	0.36
Ašlamka (Kriškovci)		1.00	0.44	1.00	0.44	0.24	1.00	0.34	0.32	0.20	0.19	0.21	0.31	0.23
Banjalučka crnica			1.00	0.44	0.34	0.31	0.44	0.32	0.37	0.26	0.24	0.23	0.33	0.23
Biljur-Bjelica				1.00	0.44	0.24	1.00	0.34	0.32	0.20	0.19	0.21	0.31	0.23
Cipov					1.00	0.21	0.44	0.30	0.23	0.17	0.14	0.18	0.29	0.24
Ašlamka (Maglajani)						1.00	0.24	0.45	0.35	0.33	0.37	0.29	0.27	0.34
Barevka							1.00	0.34	0.32	0.20	0.19	0.21	0.31	0.23
Azijanka								1.00	0.49	0.28	0.43	0.38	0.40	0.33
Nordwunder									1.00	0.30	0.41	0.33	0.36	0.36
Burlat										1.00	0.34	0.26	0.33	0.38
Stella											1.00	0.45	0.49	0.37
Van												1.00	0.32	0.42
Hedelfingen													1.00	0.39
Giorgia														1.00

DISCUSSION

After cluster analysis of grouping and the analysis of similarity coefficient according to Jaccard (Table 2.) of the examined genotypes (cultivars) of sweet cherry, heterogeneous grouping in two separate groups was clearly visible. The following genotypes belong to the first group: autochthonous genotype 'Crveni Hrušt' from Kriškovci village, 'Azijanka' from Trapisti, 'Ašlamka' from Maglajani village, as well as all six referential varieties of sweet cherry: 'Nordwunder', 'Van', 'Burlat', 'Stella', 'Hedelfingen' and 'Giorgia'. The following genotypes belong to the second group: 'Biljur-Bjelica', 'Ašlamka' and 'Banjalučka crnica' taken at the

location of Kriškovci village, as well as 'Barevka' (Trapisti) and 'Cipov' from the village of Donja Jurkovica.

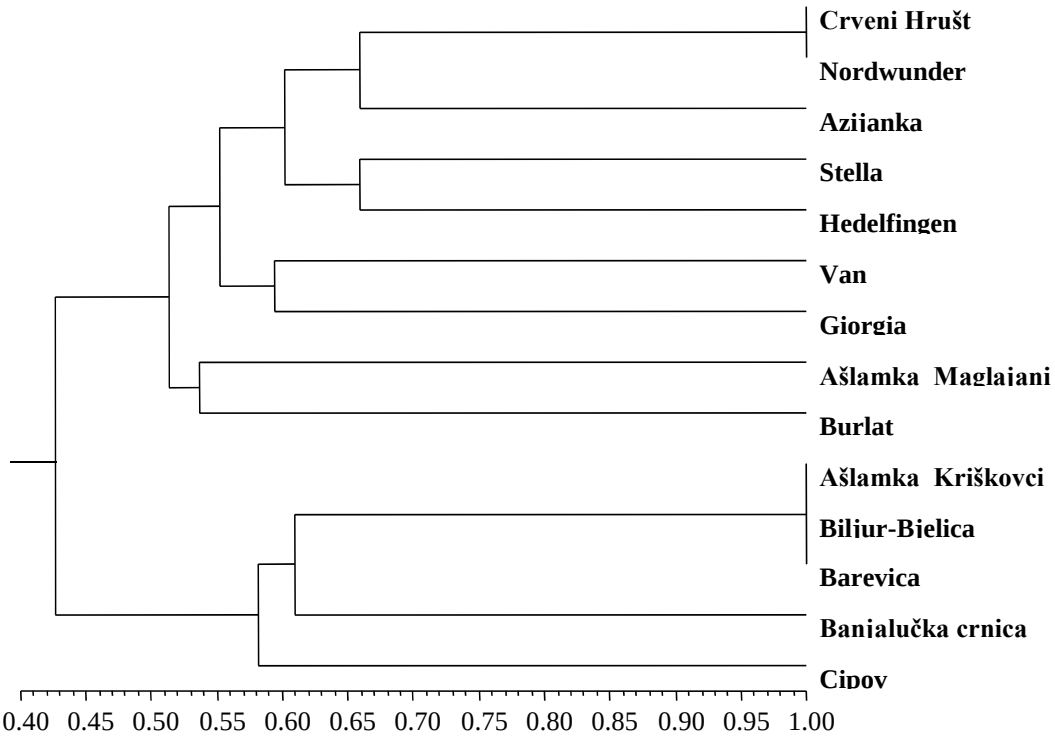

Chart 1. The dendrogram grouping the genotypes in accordance with the coefficients of similarity

In the first group, the autochthonous genotype 'Crveni Hrušt' and 'Nordwunder' varieties had the same coefficient of genetic similarity - 1.00. Therefore, we can say that, between these cultivars, at this level of research, no differences in the examined microsatellite markers were identified. Additionally, in the second group, the autochthonous genotypes of 'Biljur-Bjelica', 'Barevka' and 'Ašlamka' (Kriškovci) stood out and showed there was no difference between them, i.e., they had identical genetic profile. Genetic analysis of autochthonous genotypes of sweet cherry included in this research implemented using microsatellite markers points to the possibility of the existence of synonyms. Basically, genotypes with identical genetic profiles could be the same identity group. At this level of research, other autochthonous genotypes showed they had unique genetic profiles and there is clear difference among them. The varieties description based on molecular markers only is not possible just like the identification of varieties only based on morphological and phenotype appearance is often unreliable and does not

have to be accurate. Genetic profile of the variety regardless of the molecular technique used, cannot provide the description of the variety the way it really is according to all its properties for which it is grown in the first place. On the other hand, nowadays, thanks to the intensive development of biotechnology, the molecular markers are unavoidable, very accurate and informative tool used in sort identification. SSR markers have been recognized as useful genetic markers in plants due to its high degree of polymorphism, abundance in genomes, codominance, and suitability for automation (GUPTA *et al*., 1996).

In this research, the most informed primers were PceGA34 and UDP97-402. BALLIAN (2004), as well as STANYS *et al*. (2012) in own researches used SSR markers for the identification of sweet cherry varieties. As the most informed primer in BALLIAN (2004) research, is showed the UDP97-402 primer (developed for genome of peach), while by STANYS *et al*. (2012) was PceGA34 primer (developed for genome of charry), which is the case in this research paper. Primers EMPa003 and EMPa002 were showed the lowest degree of polymorphism for analzsed genotipes. Vaughan et al. (2007) are in their research determined total number of 100 alels, where by EMPA002 primer showed only two polimorphy alels in compare to 100 alels. The same results are determined in this research paper. In order to identify genetic profile of a variety with certainty, it is necessary to support the molecular analysis with the morphological analysis and vice versa. Despite the fact that morphological characteristics of these autochthonous sweet cherry genotypes were not included in this research, since they were performed before this molecular identification. Based on everything mentioned before, it is necessary to state that phenotype appearance of the analyzed genotypes was supported and confirmed by this genetic characterization.

CONCLUSIONS

Microsatellite (SSR) primer pairs PceGA34 and UDP97-402 are informative and have a high genotype differentiation power. Usage of these markers enables identification of autochthonous genotypes of sweet cherry. The autochthonous genotypes of ‘Biljur-Bjelica’, ‘Barevka’ and ‘Ašlamka’ (Kriškovci) stood out and showed there was no difference between them, i.e., they had identical genetic profile, while autochthonous genotypes of ‘Cipov’, ‘Azijanka’, ‘Ašlamka’ (Maglajani) and ‘Banjalučka crnica’ had a unique genetic profile**.** All results obtained in this research should be used as a basis for future additional research and necessary detection of *S*-alleles of these autochthonous genotypes for their identity to be definitely confirmed.

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GENETIČKA KARAKTERIZACIJA AUTOHTONIH GENOTIPOVA TREŠNJE (*Prunus avium* L.) UZ POMOĆ SSR MARKERA

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Izvod

Autohtoni genotipovi trešnje, predstavljaju veoma važan genetički potencijal za buduće oplemenjivačke programe. Upravo je poznavanje molekularne osnove biodiverziteta jedna od najvažnijih faktora za pravilno očuvanje, upravljanje i primjenu biljnih genetičkih resursa. U ovom radu istražena je genetička varijabilnost 14 genotipova trešnje uz pomoć 26 mikrosatelitskih markera (SSR). Istraživanjem je obuhvaćeno osam autohtonih genotipova trešnje uzetih sa četiri različita lokaliteta i šest referentnih bezvirusnih sorti. Prosječna genetička udaljenost između njih iznosila 0.43. Broj alela po lokusu se kretao od dva do osam. Najmanji broj od dva polimorfna alela pokazali su lokusi EMPa003 i EMPa002, dok su najveći broj od osam alela dali lokusi PceGA34 i UDP97-402. Rezultati urađene genetičke analize pokazuju da između autohtonih genotipova 'Biljur-Bjelica', 'Barevka' i 'Ašlamka' (Kriškovci) ne postoji razlika, tj. da imaju isti genetički profil, što upućuje na to da riječ o sinonimu, tj. o istoj identitetnoj grupi. Takođe su i autohtoni genotip 'Crveni Hrušt' i sorta 'Nordwunder' imale isti SSR profil na ispitivanim mikrosatelitskim lokusima. Preostali autohtoni genotipovi imali su unikatne genetičke profile.

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