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Genetic characterization of almond (*Prunus amygdalus* L) using microsatellite markers in the area of Adriatic Sea

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Abstract: The use of microsatellite (SSR) markers has successfully found its application in genetic characterization and examination of the origin of a large number of fruit species. Mediterranean germplasm is characterized by a great variety of almond genotypes. The study covered two geographically distant regions Montenegro (Bar) and Croatia (Sibenik) in a sample of 60 almond genotypes. Genetic analysis of almonds involved the use of ten microsatellite primers for genetic characterization of 60 examined genotypes, which successfully amplified PCR products and were highly polymorphic. Nine microsatellite markers used for the genetic characterization of almonds are derived from *Prunus persica* (UDP97-402, UDP98-411, UDP96-005, UDP98-407, BPPCT039, BPPCT014, BPPCT026, BPPCT034, BPPCT0kA) and one from *Prunus armeniaca* (PacA33). Statistical analyses (AMOVA and Fst) of the genetic characterization of the two almond populations revealed different levels of statistically significant genetic differentiation between the populations from the mentioned areas.

Key words: *Prunus amygdalus*, genetic diversity, microsatellite, genetic resources, molecular characterization

1. Introduction

Almond (*Prunus dulcis*) Miller, synonym *Prunus amygdalus*, originates from the family Rosaceae. The genus contains a large number of significant fruit species such as peach (*P. persica* L. Batsch), apricot (*P. armeniaca* L.), cherry *(P. avium* L.), sour cherry (*P. cerasus* L.) and plum (*P. domestica* L.). The number of chromosomes characteristic of *Prunus dulcis* is 2n = 16, which is identical with other species of the genus *Prunus* (Kester and Gradziel, 1996). A group of authors (Xu et al., 2004; Sánchez Pérez et al., 2006; Xie et al., 2006; Shiran et al., 2007; Zeinalabedini et al., 2007) studied the origin of cultivated genotypes (Zeinalabedini et al., 2009) as well as distinguishing of genetic base and characteristics of the extensive and mostly unused intraspecies genetic base of peaches and almonds in breeding programs (Martínez Gómez et al., 2003). The rich genetic diversity of fruit crops is present in Bosnia and Herzegovina (BiH), so many fruit species are a significant source of genetic variability and can serve as a highly valued starting material in breeding programs. Many studies in the field of plant genetic resources over the past 10 years have resulted in a large number of scientific papers on important fruit species using microsatellite markers in figs (Hadziabulic et al., 2005), pears (Gasi et al., 2013a), apples (Gasi et al., 2010, 2013b., 2013c.), chestnuts (Skender et al., 2010, 2012, 2017b) walnuts (Becirspahic et al., 2017a, 2017b), then buckwheat (Grahic et al., 2018). BiH is part of the Eastern Adriatic region, an area that stretches on more than 2000 km, from Italy in the north to Albania in the south. For a long time, many civilizations dominated this area, the Phoenicians, Greeks and Romans in ancient times, and later Venice, the Ottoman Empire and the Austro-Hungarian Empire, until the period of World War I. This area now includes four countries: Slovenia, Croatia, BiH and Montenegro, which belonged to the common state, Yugoslavia. During that long period of growing different fruit species (olives, figs, almonds, etc.) and exchanging materials, great genetic diversity has developed in this area (Lazovic et al., 2018). The research of indigenous populations, wild relatives, free populations and cultivated varieties of fruit species in recent years presents a challenge to a large number of researchers in B&H. Such interest

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indicates the existence of a large wealth of gene pool of fruit crops, still unexplored in order to preserve and exploit genetic resources in breeding programs (Aliman et al., 2010, 2013, 2016, 2020; Hadziabulic et al., 2011, 2017; Hasanbegovic et al., 2017, 2020; Skender et al., 2017a, 2017b, 2019). Today, molecular markers are routinely used to manage plant genetic resources and are particularly effective tools for identifying varieties and clones of cultivated plants. Among the available DNA markers, microsatellites combine several properties and represent the best markers due to their highly polymorphic nature and informative content, codominance, genome richness, availability, high reproducibility, and easy interlaboratory comparison (Kumar et al., 2009). In recent years, molecular markers have been used to study genetic diversity and varietal identification of peaches and almonds (Cipriani et al., 1999; Sosinski et al., 2000; Testolin et al., 2000; Dirlewanger et al., 2002; Testolin et al., 2004; Shiran et al., 2007; Dangl et al., 2009) set up the first set of almond SSR markers, which have been used successfully for molecular characterization and identification of almond cultivars (Martínez-Gómez et al., 2003; Testolin et al., 2004) and related *Prunus* species.

In a study conducted by Cipriani et al. (1999), which identified a series of microsatellites in the genus of peach (*Prunus persica* L. Batsch), labeled UDP, the possibility of their application in related species of the genus *Prunus* was also investigated. The results were obtained, which indicate a high percentage of successful reproduction in these species (71% in sour cherries, 76% in cherries, apricots and Japanese plums, 82% in almonds and European plums and 94% in the nectarine genome) (Barac, 2016). The aim of this paper is to present the results of genetic characterization using SSR markers of almond genotypes from the free population from two areas along the eastern Adriatic coast, from Montenegro (Bar) and from Croatia (Sibenik). One of the aims was to determine the correlation between the genetic distances of the analyzed genotypes based on molecular data, using adequate statistical methods and analyses. Identification of the free population of almonds and explanation of the phylogenetic relationships among the genotypes of these areas is of great interest for continuous breeding programs to improve germplasm almonds.

2. Materials and methods

2.1. Plant material, experimental site

The analyzed genotypes of almonds were selected at the following locations in Montenegro (Bar) (latitude 42°09′80″N; longitude 19°09′49″E) and Croatia (Sibenik) (latitude 43°44′06″N; longitude 15°53′43″E). The sample included 30 genotypes of almonds in Bar and 30 genotypes in Sibenik. Sampling in 2018 included marking perspective trees and taking leaves in April from each marked tree. Healthy medium-sized sheets were taken with the aim of obtaining as much DNA as possible, better purity for isolation. The leaves were stored until lyophilization in the Gen Bank of the Faculty of Agriculture and Food in Sarajevo, in a freezer at –80 °C until the time of extraction. Cold drying of leaf's tissue by lyophilization was performed under vacuum, using a lyophilizer (Christ, model Alpha 1-2 LDplus). This method of drying plant material was used to prevent the degradation of DNA molecules. Dried samples of almond leaves were vacuumed in PVC bags and stored at –80 °C until DNA isolation. For DNA isolation, 10–20 mg of powdered leaf tissue was used. Isolation and genetic characterization were performed at the Institute of Genetic Engineering and Biotechnology, University of Sarajevo INGEB. DNA isolation was performed according to the principle of a modified CTAB protocol (Doyle and Doyle, 1987; Cullings, 1992) which is most commonly applied to plant samples. After successful DNA isolation from almond samples, the PCR protocol was established. Ten genomic microsatellite primers were used for DNA amplification, nine of which were developed in the species *Prunus persica* by (Cipriani et al., 1999; Testolini et al., 2000; Dirlewanger et al., 2002), which later found their application in the work of genetic analysis and identification of cultivars of *Prunus amygdalus* L. as well as a genetic microsatellite marker originating from *Prunus armeniaca*. Out of a total of 14 microsatellite markers used in the work of the mentioned authors, the ten with which the highest allelic polymorphism was registered were singled out and used in this paper (Table 1). The genetic microsatellite markers used in this study are a very reliable tool for studying genetic diversity, because they are adaptively neutral. Amplification of microsatellite sequences was performed in a PCR device ABI GeneAmp® PCR System 9700. Fluorescently labeled primers were used for amplification in order to be able to multiplex and analyze the PCR product on a DNA genetic analyzer. Amplification of selected loci was performed in two separate PCR reactions (mix 1 and mix 2) with five microsatellite loci each. The total volume in which the PCR reaction took place was 15 µL (Table 2). Taq DNA polymerase from Gdansk Company with an optimized protocol previously described by (Dangl et al., 2005) was used for amplification. The temperature regime for the amplification reaction was the same for both PCR reactions (Table 3). Allele sizes were determined by analysis of PCR products on an ABI 3500 genetic analyzer, by vertical capillary electrophoresis. LIZ 500 (Applied Biosystem) was used as an internal standard. The obtained data were processed using GeneMapper ID 5 software.

2.2. Biostatistical analyses of molecular data

The analysis of the informativeness of the examined microsatellite markers was made by calculating the

Table 1. Characteristics of 10 microsatellite markers originating from *Prunus persica* and 1 from *Prunus armeniaca* used for the study of almond genotypes.

Marker	Primer sequence (5′ → 3′)	A repetitive pattern	The origin of the marker	Reference	The size of base pairs
UDP97-402	F:TCCCATAACCAAAAAAAACACG:C R:TGGAGAAGGGTGGGTACTTG	(AG)17	*Prunus persica*	Testolini et al. (2000)	108–152
UDP98-411	F:AAGCCATCCACTCAGCACTC R:CCAAAAACCAAAACCAAAGG	CT and GT	*Prunus persica*	Testolini et al. (2000)	154–180
UDP96-005	F:GTAACGCTCGCTACCACAAA R:CCTGCATATCACCACCCAG	(AC)16TG(CT)2CA(CT)11	*Prunus persica*	Cipriani et al. (1999) Testolini et al. (2000)	155
UDP98-407	F:AGCGGCAGGCTAAATATCAA R:AATCGCCGATCAAAGCAAC	(GA)29	*Prunus persica*	Cipriani et al. (1999)	212
PacA33	F:TCAGTCTCATCCTGCATACG R:CATGTGGCTCAAGGATCAAA	(GA)16	*Prunus persica*	-	188–196
BPPCT039	F:ATTACGTACCCTAAAGCTTCTGC R:GATGTCATGAAGATTGGAGAGG	(GA)20	*Prunus persica*	Dirlewanger et al. (2002)	154
BPPCT014	F:TTGTCTGCCTCTCATCTTAACC R:CATCGCAGAGAACTGAGAGC	(AG)23	*Prunus persica*	Dirlewanger et al. (2002)	215
BPPCT026	F:ATACCTTTGCCACTTGCG R:TGAGTTGGAAGAAAACGTAACA	(AG)8GG(AG)6	*Prunus persica*	Dirlewanger et al. (2002)	134
BPPCT034	F:CTACCTGAAATAAGCAGAGCCAT R:CAATGGAGAATGGGGTGC	(GA)19	*Prunus persica*	Dirlewanger et al. (2002)	228
BPPCT040	F:ATGAGGACGTGTCTGAATGG R:AGCCAAACCCCTCTTATACG	(GA)14	*Prunus persica*	Dirlewanger et al. (2002)	135

Table 2. Proportion of components used in PCR reaction mix 1 and mix 2.

	mix 1	mix 2	
Components	Reaction concentrations	Components	Reaction concentrations
UDP97-402	0.50 μM	UDP96-005	0.50 μM
BPPCT026	0.50 μM	UDP98-411	0.50 μM
BPPCT034	0.50 μM	BPPCT039	0.50 μM
PacA33	0.50 μM	UDP98-407	0.50 μM
BPPCT040	0.50 μM	BPPCT014	0.50 μM
dNTP	0.3 mM	dNTP	0.3 mM
PCR pufer	1 X	PCR pufer	1 X
MgCl2	2 mM	MgCl2	2 mM
Taq pol.	0.5 U	Taq pol.	0.5 U
DNK	25 ng	DNK	25 ng
ddH2O	do 15 μl	ddH2O	do 15 μl

number of detected alleles (AN), the effective number of alleles (AE), the ratio between the effective and detected number of alleles (AE / AN), Shannon information index, and the observed (HO) and expected (HE) heterozygosity in the computer program Cervus. The genetic analyses processed in this study are deviations of microsatellite loci from Hardy-Weinberg equilibrium in the computer program GenAlEx. The coefficient for estimating

Table 3. PCR protocol temperature regime for two separate PCR reactions (mix 1 and mix 2).

Protocol			
	Temperature (°C)	Duration (min: s)	Number of cycles
Enzyme activation	94	1:00	
Denaturation	94	0:45	
Annealing	57	0:45	35
Elongation	72	2:00	
Final elongation	72	4:00	

genetic differentiation between the analyzed groups was presented by Wright's F_ST test. For the coefficient of genetic differentiation, the computer program SpaGedi v.1.2 was applied. Molecular variance analysis (AMOVA) was performed using the computer program GenoType. All analyses were performed with a bootstrap with 1000 permutations.

3. Results and discussion

3.1. Genetic analysis of almonds

Genetic analysis of almonds involved the use of ten microsatellite primers for genetic characterization of 60 examined genotypes, which successfully amplified PCR products and were highly polymorphic. Microsatellite primers, which were used in the development of this paper, showed high polymorphism in previous studies by a group of authors who analyzed almonds (Hongmei et al., 2009; Distefano et al., 2013; Halász et al., 2019), where in most cases significantly fewer genotypes were analyzed. The SSR profiles of all almond samples for all ten microsatellite primers from the area of Sibenik and Bar (Tables 4 and 5). The total number of detected alleles in the Sibenik group at ten SSR loci was 93, i.e. 9.3 alleles on average per locus, and ranged from 5 per locus (BPPCT014) to 14 per locus (BPPCT034) (Table 5). The lowest effective number of alleles, within the Sibenik group, was 1.648, for the locus (BPPCT014), while the highest was 9.091 for the locus (BPPCT034). The average effective number of alleles for this group was 4.74. The average values of the ratio between the effective and detected number of alleles (AE / AN), in the Sibenik group, ranged from 0.269 (UDP98-402) to 0.660 (BPPCT026 and BPPCT039). Shannon information index (I) of diversity, for ten SSR loci, in the analyzed group Sibenik, was high and ranged from 0.822 to 2.359. The expected heterozygosity (Ho), in the Sibenik group, for the analyzed 10 SSR loci, ranged from 0.233 (UDP97-402, Paca33, BPPCT040 and BPPCT014) to 0.867 (BPPCT034), with an average value of 0.427. The observed heterozygosity (He) ranged from 0.393 (BPPCT014) to 0.890 (BPPCT034), averaging 0.709. The results presented in Table 5 for the total number of detected alleles, in the Bar group, on ten SSR markers, were 74, and the average was 7.40. Detected alleles ranged from 4 for loci (UDP97-402 and BPPCT014) to 11 for loci (BPPCT034). The lowest effective number of alleles within the Bar group was 1.449 for the locus (BPPCT014), while the highest was 6.143 for the locus (BPPCT034). The average effective number of alleles for this group was 3.869. Mean ratios between the effective and detected allele numbers (AE / AN) in the Bar group ranged from 0.362 (BPPCT014) to 0.594 (PacA33). The largest number of private alleles was detected in the Sibenik group (8), while a smaller number of private alleles were detected in the Bar group (4). The highest number of rare alleles was detected in the Sibenik group and was (46), and the lowest number of rare alleles was detected in the Bar group (32). The highest average number of detected alleles was recorded in the Sibenik group (9.300), while a slightly lower number of detected alleles was recorded in the Bar group (7.400). The expected heterozygosity found in the Bar group was (0.690), which is lower in comparison with the Sibenik group (0.709). Analyzing the observed heterozygosity, it can be stated that the Bar group recorded a lower Bar (0.397) compared to the Sibenik group (0.427) (Table 5). Based on the presented results, it can be concluded that with the increase of heterogeneity within populations, due to uncontrolled exchange of genetic material, the differences between them decrease. The high value of the average number of alleles per locus is a consequence of the analysis of an extremely large number of individuals, as well as the more important fact that it is a material collected in one of the groups of origin of this culture. Differences in these values can be attributed to differences in germplasm diversity used in this study. However, given the number of individuals included in this study, the values for genetic diversity compared to other papers can be considered high. In a study by Sosinski et al. (2000), a high level of heterogeneity was observed for all loci (0.697), which can be attributed to cross-pollination and incompatibility of almonds. The high values of polymorphic loci (71%), the average number of alleles per

Table 4. Allele frequency calculated for all analyzed almond genotypes from Sibenik and Bar at 10 SSR loci.

Genotype	Population	UDP97-402		PacA33		BPPCT026		BPPCT034		BPPCT040		UDP96-005		BPPCT014		UDP98-411		UDP98-407		BPPCT039	
SG1	Sibenik	112	112	176	176	142	150	226	234	134	134	132	132	178	178	160	164	190	190	134	144
SG2	Sibenik	112	112	176	176	146	150	246	246	138	138	132	132	178	178	160	160	184	184	138	138
SG3	Sibenik	112	112	176	176	142	142	242	246	134	134	132	154	178	178	164	164	184	184	150	150
SG4	Sibenik	112	112	176	176	146	146	246	246	134	134	132	156	178	178	160	160	184	184	138	138
SG5	Sibenik	112	112	178	178	142	146	244	244	138	138	128	140	178	178	166	166	184	184	138	148
SG6	Sibenik	112	112	176	176	142	146	208	234	142	142	132	132	178	178	162	164	178	178	134	134
SG7	Sibenik	112	112	176	176	146	150	208	246	136	136	132	132	178	178	162	162	184	184	134	134
SG8	Sibenik	112	112	188	188	138	146	220	234	128	128	132	154	194	194	162	162	172	190	156	156
SG9	Sibenik	124	124	176	176	140	146	208	226	134	134	132	132	192	192	160	160	172	174	134	138
SG10	Sibenik	112	118	188	188	148	158	216	242	134	142	124	158	178	178	160	160	182	182	148	148
SG11	Sibenik	112	112	176	176	138	138	220	242	130	130	132	132	178	178	160	160	186	190	134	150
SG12	Sibenik	124	132	176	180	142	150	236	240	130	130	132	132	178	178	160	168	186	200	134	134
SG13	Sibenik	118	118	176	176	144	150	208	226	142	150	138	140	186	186	160	168	182	182	150	150
SG14	Sibenik	114	124	170	178	142	146	220	234	134	134	142	154	178	194	160	160	172	172	154	154
SG15	Sibenik	114	128	178	178	148	148	208	208	136	136	126	154	178	194	164	166	186	186	148	148
SG16	Sibenik	112	112	176	176	146	150	220	234	134	134	140	154	178	194	164	166	172	182	150	158
SG17	Sibenik	112	112	178	178	138	138	220	234	134	134	124	154	194	198	160	160	172	172	150	150
SG18	Sibenik	124	136	176	176	148	148	226	246	134	134	140	148	178	178	160	160	184	184	140	150
SG19	Sibenik	114	114	186	186	148	148	242	248	134	142	142	142	178	178	162	164	186	186	126	150
SG20	Sibenik	112	112	176	176	140	148	242	248	142	142	154	154	178	178	164	164	186	186	150	150
SG21	Sibenik	112	112	176	176	138	150	224	242	138	146	132	132	178	178	166	166	172	182	148	148
SG22	Sibenik	112	112	176	188	142	158	226	242	132	132	132	140	178	194	160	160	180	180	126	140
SG23	Sibenik	112	112	176	188	144	148	220	242	142	142	140	154	178	178	160	164	186	186	134	150
SG24	Sibenik	112	112	176	188	142	150	242	250	142	142	122	140	178	178	170	170	180	180	126	154
SG25	Sibenik	112	112	176	184	138	142	220	252	126	132	122	154	178	178	164	168	186	186	138	156
SG26	Sibenik	112	112	176	176	148	148	216	226	134	134	126	154	178	186	160	160	186	186	140	148
SG27	Sibenik	112	118	176	188	148	148	234	246	134	136	132	154	178	192	160	160	180	188	140	150
SG28	Sibenik	112	112	176	176	134	148	216	242	136	146	140	140	178	178	160	164	188	188	154	154
SG29	Sibenik	112	112	176	176	134	148	226	246	144	144	132	154	178	178	160	162	180	186	126	138
SG30	Sibenik	112	128	176	176	142	148	216	236	132	132	132	154	178	178	160	164	198	198	160	160
BRG1	Bar	132	132	180	180	142	142	220	250	140	140	126	126	178	178	150	150	196	200	154	154
BRG2	Bar	132	132	184	184	140	142	216	252	142	146	134	134	178	194	160	164	196	196	134	154
BRG3	Bar	112	132	184	184	140	140	216	250	144	146	154	154	178	194	160	164	212	212	134	154
BRG4	Bar	112	112	178	178	142	146	226	250	142	142	154	154	178	178	164	166	212	212	148	154
BRG5	Bar	112	112	178	178	146	146	226	226	138	138	142	154	178	178	166	166	178	196	148	154
BRG6	Bar	112	112	178	178	142	146	226	250	142	142	154	154	178	178	164	166	212	212	148	154
BRG7	Bar	112	112	178	178	142	146	250	250	142	146	154	154	178	178	164	166	186	186	148	154
BRG8	Bar	112	112	178	178	142	146	226	226	142	146	142	154	178	178	166	166	186	186	148	154
BRG9	Bar	112	112	184	184	144	158	220	244	144	156	124	154	178	178	170	170	182	182	148	148
BRG10	Bar	112	112	170	178	144	158	222	226	144	156	124	124	178	178	164	164	182	188	140	140
BRG11	Bar	112	112	170	178	146	146	226	226	146	146	154	154	178	178	166	166	186	186	148	154
BRG12	Bar	112	112	178	178	142	146	226	250	142	146	142	154	178	178	164	166	186	186	148	154
BRG13	Bar	112	112	166	178	146	148	232	232	142	142	140	140	178	192	164	164	186	186	154	154
BRG14	Bar	132	132	180	180	142	142	220	248	132	140	126	126	178	178	164	164	196	200	150	162
BRG15	Bar	112	132	180	180	142	142	220	250	132	132	126	158	178	178	164	164	196	196	150	150
BRG16	Bar	112	132	180	180	142	142	220	250	132	132	126	158	178	178	164	164	196	196	150	150

Table 4. (Continued).

BRG17	Bar	112	132	180	180	142	142	220	250	132	132	126	158	178	178	164	164	196	196	150	150
BRG18	Bar	112	132	180	180	142	142	220	250	132	132	126	158	178	178	164	164	196	196	150	150
BRG19	Bar	132	132	180	180	142	142	220	248	132	140	126	126	178	178	164	164	196	200	150	150
BRG20	Bar	132	132	180	180	142	142	220	248	132	140	126	126	178	178	164	164	196	200	150	150
BRG21	Bar	132	132	180	180	142	142	220	248	132	140	126	126	178	178	164	164	196	200	150	150
BRG22	Bar	132	132	180	180	142	142	220	248	132	140	126	126	178	178	164	164	196	200	150	150
BRG23	Bar	112	112	178	178	142	146	226	250	142	142	142	154	178	178	164	166	196	196	148	154
BRG24	Bar	112	112	180	180	146	148	220	226	146	146	124	140	186	194	168	168	180	180	146	150
BRG25	Bar	112	136	178	178	142	148	220	232	126	132	124	138	178	194	168	168	186	186	140	140
BRG26	Bar	112	118	176	178	140	142	226	242	134	134	124	126	194	194	160	162	196	196	150	150
BRG27	Bar	112	136	166	166	148	148	208	232	142	142	124	134	178	194	160	162	178	178	142	142
BRG28	Bar	112	112	176	178	142	148	208	232	136	136	124	162	194	194	160	162	186	186	150	150
BRG29	Bar	112	112	176	176	134	158	208	226	132	132	124	124	178	178	160	162	186	186	140	140
BRG30	Bar	112	112	176	176	148	158	208	242	132	132	124	124	178	178	160	160	180	180	142	150

Table 5. Number of detected alleles (AN), effective number of alleles (AE), ratio between effective and detected number of alleles (AE / AN), Shannon information index (I), observed (HO) and expected (HE) heterozygosity for ten SSR markers on 30 almond samples from the Sibenik area and 30 almond samples from the Bar area.

	Sibenik						Bar					
Locus	A_N	A_E	A_E/A_N	I	Ho	He	A_N	A_E	A_E/A_N	I	Ho	He
UDP97-402	7.000	1.883	0.269	1.057	0.233	0.469	4.000	1.989	0.497	0.835	0.267	0.497
PacA33	7.000	2.093	0.299	1.108	0.233	0.522	6.000	3.564	0.594	1.459	0.167	0.719
BPPCT026	9.000	5.941	0.660	1.935	0.700	0.832	7.000	3.186	0.455	1.462	0.533	0.686
BPPCT034	14.000	9.091	0.649	2.359	0.867	0.890	11.000	6.143	0.558	2.019	0.833	0.837
BPPCT040	11.000	5.310	0.483	1.980	0.233	0.812	10.000	5.325	0.533	1.907	0.433	0.812
UDP96-005	12.000	4.327	0.361	1.823	0.633	0.769	9.000	5.070	0.563	1.823	0.467	0.803
BPPCT014	5.000	1.648	0.330	0.822	0.233	0.393	4.000	1.449	0.362	0.586	0.200	0.310
UDP98-411	6.000	3.249	0.542	1.427	0.400	0.692	7.000	3.377	0.482	1.519	0.367	0.704
UDP98-407	11.000	6.667	0.606	2.091	0.267	0.850	8.000	4.478	0.560	1.729	0.267	0.777
BPPCT039	11.000	7.258	0.660	2.143	0.467	0.862	8.000	4.110	0.514	1.636	0.433	0.757
Average	9.300	4.747	0.486	1.674	0.427	0.709	7.400	3.869	0.512	1.497	0.397	0.690

locus (8.76), He (0.775), the average content of polymorphism information (0.475) and PI (0.258) observed in this study indicate that SSR markers can recognize genetic variation between examined almond genotypes. In the study of (Martínez-Gómez et al., 2003), the average number of alleles per locus was 4.7, which is significantly lower than in this study, while in the study of Martí i AF et al. (2015), the average number allele per locus was significantly higher at 14.6. Xie et al. (2006) concluded an average number of alleles per locus of 7.8, and the observed heterozygosity was 0.678 in the genetic characterization of 23 Chinese and 15 international almond cultivars using 16 microsatellite markers. Chalak et al., (2006) in a study on 36 almond genotypes represented in Lebanon using 6 microsatellite markers, came to the following results: the average number of alleles per locus was 12.5, the expected heterozygosity ranged from 0.78 to 0.88, averaging 0.83. The observed heterozygosity was 0.8. In a study by Fathi et al., (2008) where the sample consisted of 56 almond genotypes, using 35 SSR markers, it was concluded that the total number of alleles was 215, and the average number of alleles per locus was 8.76. The average value of the Shannon index was 1.79, and the average He ranged from 0.92 to 0.17, averaging 0.775, which is very similar to the results obtained in this study. In a study by Gouta et al. (2010), where 10 microsatellite markers were

Table 6. Deviation of ten examined SSR loci from Hardy-Weinberg (HW) equilibrium in the total set of samples, as well as within individual groups (ns = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Locus	Sibenik	Bar
UDP97-402	***	ns
PacA33	***	***
BPPCT026	ns	***
BPPCT034	**	**
BPPCT040	***	***
UDP96-005	ns	***
BPPCT014	***	ns
UDP98-411	***	***
UDP98-407	***	***
BPPCT039	***	***

Table 7. Pairwise genetic differentiation - Fst value calculated between the groups of almonds Sibenik and Bar.

	Sibenik	Bar
Sibenik	0.000	
Bar	0.061	0.000

used in a population of 82 almond cultivars, it was concluded that the total number of alleles was 159, which is an average of 15.9 per locus. The average number of effective alleles was 7.5. The mean expected heterozygosity was 0.86, while the mean observed heterozygosity was 0.68. In the study by Kadkhodaei et al. (2011) conducted in Iran, which included the study of 53 genotypes/cultivars of almonds, with 9 microsatellite markers, the average number of alleles per locus ranged from 8 on UDA022 to 17 on UDA002, with an average of 12.86. Higher average values of the number of effective alleles were recorded in the mentioned study 5.59. Moreover, higher values of the average Shannon information index (I) were recorded in this study and amounted to 1.97, expected heterozygosity of 0.80 and average PIC of 0.89, which can be related to a large geographical distance, since the genotypes examined are originally from Spain, Iran, and America. Higher average values of the observed heterozygosity than those obtained in this paper were published by El Hamzaoui et al. (2012), where 16 microsatellite markers were used in a sample of 127 almond genotypes native to Morocco. The value of the observed heterozygosity was 0.596, while the average expected heterozygosity in it was 0.699 and was slightly lower compared to this study. It can be stated that the total number of alleles was 238, i.e. it ranged from 4 to 24 alleles per locus. The average number of alleles per locus was 14.88. The Fst value in the same study ranged from 0.00726 to 0.04354, with no statistical significance. In the research of Rahemi et al. (2012), which included 89 genotypes of almonds and other species of the genus *Prunus* from Iran, was that the observed heterozygosity (Ho) was 0.581, while the expected heterozygosity (He) was 0.885. In the same study, the average number of alleles per locus was 34. Analyzing the results of this study in relation to the study of El Hamzaoui et al. (2012), it can be concluded that they are approximately the same sample size. A higher average number of alleles per locus was recorded in studies conducted by Distefano et al. (2013), where the sample included 300 almond cultivars, on 9 SSR markers, the average number of alleles per locus was 18. In the study by Dicenta et al. (2015), three local populations of almonds from Apulia and Saradinia were investigated in a total sample of 96 almond genotypes, where 11 microsatellite markers were used, and the results were obtained that emphasize the average number of alleles per locus for samples from Sardinia, 14.3, and for samples from the Apulia group 11.9. Analyzing the number of private alleles obtained in this study, it can be concluded that it ranged from 48, in groups originating from Sardinia, to 24 in the group from Apulia, which is a total of 62 private alleles. The average number of effective alleles originating from the two groups of Sardinian and Apulian ranged from 8.5 to 7.4, and the average observed heterozygosity in the groups of almonds from Sardinia and Apulian ranged from 0.71 to 0.66. The average expected heterozygosity in the two mentioned groups of almonds ranged from 0.88 to 0.81. Similar results were obtained by a group of scientists (Martí i AF et al., 2015) where the average number of alleles per locus was 18.66 per locus. The study by Forcada i CF et al. (2015) used 98 almond samples from five continents located at the Centro de Investigacióny Tecnología Agroalimentariade Aragón (CITA; Spain), where 40 microsatellite markers were used, the average number of alleles per locus was 13.9. The observed heterozygosity ranged from 0.24 (BPPCT030) and 0.94 (CPPCTO40), averaging 0.66 at 40 SSR loci. Expected and observed heterozygosity was compared with the fixation index (F) where the mean was 0.11. Significantly higher values of all parameters were obtained in the above study because the initial sample was very diverse from five different continents and because 40 microsatellite markers were used. Halász et al. (2019), in a study that included 86 genotypes of almonds originating from Central Asia to America, using 15 SSR markers, for the purpose of genetic characterization, found an average number of alleles of 18.86 per locus. In the research of Rahemi et al. (2012), which included 89 genotypes of

almonds and other species of the genus *Prunus* from Iran, was that the observed heterozygosity (Ho) was 0.581, while the expected heterozygosity (He) was 0.885. In the same study, the average number of alleles per locus was 34.

3.2. Hardy-Weinberg (H-W) equilibrium and pairwise genetic differentiation

Deviation from Hardy-Weinberg equilibrium in the total set of ten examined SSR loci is shown in Table 6. In the analyzed groups Sibenik and Bar for 80% of analyzed SSR loci, a significant deviation from Hardy-Weinberg equilibrium (H-W) was detected. Analyzing the loci PacA33, BPPCT040, UDP98-411, UDP98-407 and BPPCT039, it can be concluded that they deviated the most from the (H-W) equilibrium in the examined groups of almonds. In a study by Gouta et al. (2013), the average fixation index was (F = 0.13), indicating a heterozygosity deficit and a significant deviation from Hardy-Weinberg expectation (p 0.01) for nine of the 10 markers examined.

The results of AMOVA and Fst parameters show the existence of genetic differentiation of 0.061 between the groups of Sibenik and Bar is shown in Table 7. In general, genetic differentiation between groups is relatively small, but statistically significant, which leads to the conclusion that much of the germplasm of all groups was introduced and originated from the same source, but that additional factors influenced the creation of genetic differentiation between groups. A study by Gouta et al. (2013) states that F values at different levels were significant (FCT = 0.06484, FSC = 0.03187, FST = 0.09464, P 0.001) for a similar percentage of genetic variation in the population (88.7%). The dendrogram based on the (FST) values between population pairs showed the distribution of genetic diversity for all associated, and two main groups (A and B) were distinguished. Group A includes foreign populations and cultivars from the north of Tunisia (Bizerte), while group B includes the rest of the population of Tunisia from the central (Sidi Bouzid) and southern (Sfax, Tozeur and Nefta) part of Tunisia. In the research of Rahemi et al. (2012) that included 89 genotypes of almonds and other species of the genus *Prunus* from the area of Iran and the result was that the (PIC) was 0.874, while the average (Fst) was 0.271 and the fixation index Fis) was 0.151.

3. Conclusion

By the genetic characterization of almond populations from the area of Croatia-Sibenik and Montenegro-Bar using 10 microsatellite markers, they showed a high degree of genetic variability. The results of AMOVA, Fst and fCT values were statistically significant, indicating a certain degree of differentiation between the compared groups of almonds. The value of the calculated Fct for the two examined populations is 0.061. Large physical distance provides quality sampling when it comes to genetic diversity research. In general, the genetic differentiation between the groups is relatively small but statistically significant, leading to the conclusion that much of the germplasm of groups is introduced and originates from the same source, but that additional factors influenced the creation of genetic differentiation between given groups. This study represents a contribution to the conservation and management of almond germplasm, revealing the free population of Croatian and Montenegrin almond genotypes as a valuable source of genetic diversity. Identification of the free population of almonds and explanation of the phylogenetic relationships among the genotypes of these areas is of great interest for continuous breeding programs to improve germplasm almonds.

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