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Does the geographic distance affect the genetic differentiation among bilberry populations sampled in Bosnia and Herzegovina?

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Abstract

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Isolation-by-distance (IBD) pattern among bilberry (*Vaccinium myrtillus* L.) populations has previously been reported for this species in northern Europe. However, the number of molecular studies conducted on bilberry, using everything from isoenzymes, RAPDs to microsatellite markers, are very few and far between. Considering that Bosnia and Herzegovina (B&H) is a country rich with diverse fruit genetic resources, conducting a genetic characterization of the naturally occurring *V. myrtillus* populations could yield valuable data for the conservation and utilization of this resource. This study entailed genotyping samples collected from three bilberry populations located in Fojnica, Kladanj, and Srebrenica municipalities using seven polymorphic microsatellite or SSR (simple sequence repeats) markers. The obtained molecular data was used to calculate the correlation between the physical distance of the individual B&H populations and a parameter of the genetic differentiation (pairwise *F_{st}*). The results of the correlation analyses revealed an absence of a significant isolation-by-distance pattern among the three examined B&H bilberry populations. In addition, the most pronounced genetic differentiation was detected between the Srebrenica and each of the two remaining B&H populations. At the same time, the values for *pF_{st}* were significant, albeit much lower, between the Fojnica and Kladanj populations. Bilberries from the sampled Srebrenica population appear to be distinct from the other B&H populations, possibly due to the different genetic origin of this population.

Introduction

The bilberry (*Vaccinium myrtillus* L.) plant is native to northern Europe but can also be found in parts of North America and Asia. It usually grows in meadows, heaths, or evergreen forests (Chu et al., 2011). In Bosnia and Herzegovina bilberry can be found on higher elevation of almost all mountains, as a part of the existing biocenosis (Kurtović et al., 2016). The assessment of genetic diversity in natural populations and the evaluation of the risk of genetic erosion often relies on factors such as population size and potential isolation (Ellstrand et al. 1993). In this context, populations of bilberry present in the Balkans are considerably smaller and fragmented compared to populations in Central and Western Europe, where the geographic distribution is continuous (Bjedov et al., 2015).

The first molecular research on *V. myrtillus* has been conducted in the 1990s, using biochemical markers, aka isoenzymes (Jacquemart, 1992; Jacquemart et al., 1994). The advancement in genetic studies on *V. myrtillus* involved the use of DNA markers that possess a series of advantages over isoenzymes. DNA markers are abundant within plant genome, and depending on the type, can be highly polymorphic. Most importantly, during the analysis of natural populations across multiple habitats, they are mostly unaffected by environmental factors.

Albert et al. (2003) were the first in this field to apply a combination of RAPD and AFLP markers to investigate clonal structure in wild blueberries. Later on, they exclusively utilized RAPD markers to assess the genetic diversity and structure across multiple populations of a given species (Albert et al., 2004), and the impact of clone presence on self-pollination (Albert et al., 2008). Even though RAPD markers have not been used in advanced genetic research on fruit tree germplasm for over 10 years, this marker system has continued to be employed in wild blueberries (Botau et al., 2014; Bjedov et al., 2015; Giordani et al., 2018; Nin et al., 2019). The potential reason for this may lie in

the challenges associated with implementing more advanced marker systems in this particular species (Carvalho et al., 2018; Gailite et al., 2020).

Molecular studies conducted exclusively using ISSR (Inter Simple Sequence Repeats) involved the analysis of wild blueberries propagated from seeds of *V. myrtillus* fruits collected in Iceland, Norway, Sweden, Finland, and Germany (Zoratti et al., 2015).

The first widely accepted codominant markers developed for the genetic characterization of blueberries were microsatellites or SSRs (Simple Sequence Repeats) isolated from the genome and complementary DNA (cDNA) of blueberry species in the *Cyanococcus* section (Boches et al., 2005). The first study that used SSR markers to analyze wild blueberry populations was published in 2020 by Gailite et al. (2020). They analyzed *V. myrtillus* populations from the Baltic states using microsatellites from complementary and chloroplast (cpDNA) deoxyribonucleic acid.

The only other two studies relying on microsatellites in the genetic characterization of wild blueberries had a very limited scope, as research for bachelor study level (Fahlgren, 2022) and in a master's thesis (Ytterdal, 2011). The additional characteristics of *V. myrtillus* populations, revealed through these molecular studies involve a restricted gene flow between populations (Bjedov et al., 2015; Zoratti et al., 2015; Gailite et al., 2020), that are manifested in variability within individual populations (Albert et al., 2004; Bjedov et al., 2015; Zoratti et al., 2015; Giordani et al., 2018) and a high degree of differentiation between populations (Bjedov et al., 2015). Furthermore, Zoratti et al. (2015) and Gailite et al. (2020) state that in North European populations, there is a high correlation between geographic and genetic distances.

In this study, samples from three bilberry populations in Bosnia and Herzegovina were genotyped using microsatellites in order to calculate the correlation between the physical distance of the individual populations and a parameter of the genetic differentiation (pairwise

Fst). The aim of this research was to investigate the effect of geographic distance on the genetic differentiation among examined bilberry populations in Bosnia and Herzegovina.

Material and methods

Leaf samples of *V. myrtillus* were collected in the spring of 2016 from five randomly selected plants from three plots within each of the three examined populations. The sampled plant material included sampling the youngest leaves considering they contain the least compounds that hinder the extraction of high-quality genomic DNA. The leaves were placed in paper envelopes and stored in a refrigerator at +4°C before lyophilization. Lyophilization of plant tissue was conducted using the Alpha 1-2 LSCbasic device (Martin Christ, Osterode am Harz, Germany). Samples were then stored at -80°C until DNA isolation. The populations are located in three locations (Fojnica, Kladanj, and Srebrenica) in Bosnia and Herzegovina.

Fojnica, Kladanj, and Srebrenica represent distinct municipalities in Bosnia and Herzegovina. "Kladanj" population is identified in municipality of Kladanj (44°22'01"N 18°54'65"E) Konjuh locality, at an elevation of 1005 m, on terrain with a slope of 37°. "Srebrenica" population is in municipality of Srebrenica, (43°99'27"N 19°41'67"E), Šljivice locality at an elevation of 824 m, on terrain with a slope of 27°; and "Fojnica" population is identified in municipality Fojnica (43°58'520"N 17°54'20"E), at the Gradina locality, at an elevation of 775 m, on terrain with a slope of 30°. The shortest geographical distance between mentioned locations is between Srebrenica and Kladanj (86,5 km). Fojnica is located 197 km away from Srebrenica, and 112 km from Kladanj.

DNA extraction from plant tissue was carried out using the commercial kit "Qiagen DNeasy Plant Mini Kit" (Qiagen, Valencia, California, USA), following the manufacturer's provided protocol. Genomic DNA quality and quantity were assessed

using 0.8% agarose gel electrophoresis in 0.5x TBE buffer. Gel imaging and analysis were performed with GelDoc XR (Bio-Rad Laboratories, Richmond, California, USA). Evaluation was based on fragment migration, dispersion, and light intensity compared to a known λ DNA sample. Seven microsatellite loci, analyzed in this study, were adopted from Boches et al. (2005) (CA483, CA421, CA112, CA344, CA794, CA94, CA855). Genetic differentiation among individual populations (pairwise fixation index Fst) (Weir and Cockerham, 1984) and the analysis of molecular variance (AMOVA) was conducted using the GenoType computer program (Meirmans and Van Tienderen, 2004). Correlations between the physical distance of the sampled individuals were investigated using Mantel test. The test was conducted on matrices of genetic differentiation values (Fst) and geographical distances and computed based on 1,000 permutations using the computational software PASSaGE v. 2.0 (Rosenberg and Anderson, 2011).

Results and Discussion

In order to determine the differences among populations, genetic differentiation was calculated using the pairwise fixation index Fst (Weir and Cockerham, 1984) and analysis of molecular variance (AMOVA).

Average values of pFst obtained during pairwise comparison of Bilberry populations in Bosnia and Herzegovina ranged from 0.0485 to 0.1002, with minimal value of genetic differentiation being among the populations of Kladanj and Fojnica (0,0485), and maximum among Srebrenica and Fojnica populations (0,1002). Fst value for Srebrenica and Kladanj populations was 0,0882 (Table 1). All Fst values were statistically significant ($P < 0,05$) indicating significant genetic differentiation among all three populations of *V. myrtillus*. The most pronounced genetic differentiation was detected between the Srebrenica population and the other two analyzed

Table 1. pFst (Weir i Cockerham, 1984) for every analysed SSR locus and analysed pair of bilberry populations in B&H.

Kladanj and Srebrenica		
SSR-ovi	Fst	P-value
CA483	0,2436	<0,0001
CA421	0,0161	0,4340
CA112	0,1462	<0,0001
CA344	0,3004	0,0020
CA794	-0,0170	0,5800
CA94	-0,0554	0,5940
CA855	-0,0104	0,9190
All loci	0,0882	0,0030
Kladanj and Fojnica		
SSR-ovi	Fst	P-value
CA483	0,0185	0,2180
CA421	0,0965	<0,0001
CA112	0,0111	0,4410
CA344	0,3571	<0,0001
CA794	0,0059	0,5980
CA94	-0,0189	0,7910
CA855	-0,0037	0,7110
All loci	0,0485	0,0180
Srebrenica and Fojnica		
SSR-ovi	Fst	P-value
CA483	0,3395	<0,0001
CA421	0,1020	0,0090
CA112	0,1261	0,0030
CA344	-0,0070	0,8720
CA794	0,0174	0,3080
CA94	-0,0544	0,6940
CA855	-0,0299	0,2890
All loci	0,1002	<0,0001

populations (Kladanj and Fojnica). Simultaneously, pFst values were statistically significant between the Fojnica and Kladanj populations, although considerably lower. AMOVA analysis revealed that the majority of the total variance was contained within the populations (93%), with a smaller portion attributed to differences between populations (7%) (Table 2). Furthermore, AMOVA conducted between individual pairs of examined populations

also indicated that the majority of the total variance resided within the populations (80.6% for Kladanj and Srebrenica, 91.8% for Kladanj and Fojnica, and 87.6% for Srebrenica and Fojnica), while a smaller portion was attributed to differences between populations (19.4% for Kladanj and Srebrenica, 8.2% for Kladanj and Fojnica, and 12.4% for Srebrenica and Fojnica) (Table 2). Mantel test results showed the absence of statistically significant correlation between the

Table 2. AMOVA result for all examined Bosnia Herzegovina populations, as well as for each analysed pair of Bilberry populations.

Source of variations	SS	Variance Components	Total Variance (%)	fCT	P
All populations					
Between populations	2	3,03	7,0	0.070	0.001
Within population	39	40,23	93,0		
Kladanj and Srebrenica					
Between populations	1	9,27	19,4	0,194	0,002
Within population	25	38,52	80,6		
Kladanj and Fojnica					
Between populations	1	3,60	8,2	0,082	0,012
Within population	28	40,26	91,8		
Srebrenica and Fojnica					
Between populations	1	5,94	12,4	0,124	0,003
Within population	25	41,90	87,6		

matrices of genetic differentiation values (F_{st}) and geographical distances ($P > 0,05$, $R=0,418$), as well as the absence of statistically significant correlation between the matrices of genetic differentiation values (f_{CT}) and geographic distances ($P > 0,05$, $R=-0,443$) (Table 3). The absence of statistically significant correlation between spatial and genetic distances has also been detected in bilberry populations from Serbia in a study by Bjedov et al. (2015). Additionally, McCallum et al (2023) found significant genetic flow throughout the region, not directly correlated with the geographic distances among locations in a study on *V. myrtillus* in Scotland. This study reports that certain geographically distant populations are genetically more related than those closer in proximity, and vice versa. Furthermore, the mentioned study notes that by analyzing genealogical data, it can be concluded that ancient

populations of *V. myrtillus* are present in Western Balkans, whereas the populations present in the Eastern Balkans are relatively young. Different origins of B&H bilberry populations could explain the expressed genetic differentiation, especially between northern (Srebrenica) and western populations (Kladanj and Fojnica).

On the other hand, Zoratti et al. (2015) and Gailite et al. (2020) report the presence of statistically significant correlations between genetic and geographical distances of bilberry populations in northern Europe (Iceland, Norway, Finland, Sweden and Germany), and Baltic countries (Estonia, Latvia and Lithuania). It is however important to note that geographical distances among sampled populations in these studies, are far greater than in studies conducted in Serbia and Bosnia and Herzegovina.

Table 3. Mantel test results between matrices of genetic differentiation values (pF_{st} and f_{CT}) and geographic distances (1.000 permutations)

	Distance matrices	P value	R value
SSR	pF_{st}	$> 0,05$	0,418
SSR	AMOVA (f_{CT})	$> 0,05$	-0,443
Geographical data	Spatial distance		

Conclusion

The results of the correlation analyses revealed an absence of a significant isolation-by-distance pattern among the three examined B&H bilberry populations. The most pronounced genetic differentiation was detected between the Srebrenica population and the other two analyzed B&H populations of *V. myrtillus*. This is possibly due to the diverse genetic origin of Srebrenica population. Simultaneously, pFst values were statistically significant between the Fojnica and Kladanj populations, although considerably lower. Genetic characterization, conducted using microsatellite markers, indicated the presence of pronounced genetic variability in the studied populations of bilberry (*Vaccinium myrtillus*) in the continental part of Bosnia and Herzegovina.

Conflict of interest

The authors declare no conflict of interest.

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