GENETIC PURITY ASSESSMENT OF COMMON BUCKWHEAT VARIETY 'DARJA' WITH THE USE OF SSR MOLECULAR MARKERS

Jasmin Grahić[1], Mirsad Kurtović[1], Silvio Šimon[2], Mirha Đikić[1], Fuad Gaši[1]

[1]*Faculty of Agriculture and Food Sciences, University of Sarajevo, Sarajevo, Bosnia and Herzegovina*
[2]*Institute of Viticulture and Enology, Croatian Center for Agriculture, Food and Rural Affairs, Zagreb, Croatia*

***Correspondence**
E-mail:
grahic.jasmin@live.com

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Abstract

In order to assess the genetic purity of common buckwheat variety 'Darja' which is the most commonly produced variety of this crop in Bosnia and Herzegovina, 10 SSR markers have been used. Five samples have been collected from different production regions in B&H (Breza, Nisici Plateau, Ustikolina, Bihac and Bosanska Krupa) and compared to the reference 'Darja' sample obtained from an *ex situ* seed collection from Slovenia. Seven out of ten primer pairs used managed to amplify SSR alleles. Analyses of molecular variance (AMOVA) showed a significant differentiation between the reference and all analyzed 'Darja' samples. Furthermore, the factorial correspondence analysis revealed a clear differentiation between the reference and 'Darja' samples from the most known production regions of common buckwheat in B&H clustering four out of five analyzed samples very close together. The most divergent one among the analyzed samples was the one from Ustikolina. Genetic purity of varieties of all of cross pollinated species produced in Bosnia and Herzegovina is questionable due to the general use of farm-saved seeds.

Research article

Key words: *common buckwheat, Darja, SSR, genetic integrity, factorial correspondence analysis*

Introduction

Common buckwheat (*Fagopyrum esculentum* Moench), usually a diploid (2n = 16) annual crop plant, originating from southwestern China (Ohnishi, 1998a), is widely cultivated in Asia, Europe and America. It has a narrow gene pool (Ma et al., 2009) and its limited distribution contributes to the fact that most of the varieties grown are local populations adapted to their environmental conditions through cultivation (Song et al., 2011). High genetic diversity among and within common buckwheat varieties is a result of complete allogamy due to the heterostylous self-incompatibility system (Iwata et al., 2005). The mentioned diversity has been studied using allozyme analysis (Ohnishi, 1988b), RAPDs (Iwata et al., 2005; Sharma & Jana, 2002; Murai & Ohnishi, 1996), AFLPs (Iwata et al., 2005; Konishi et al., 2005) and SSRs (Song et al., 2011; Ma et al., 2009; Konishi et al., 2006; Iwata et al., 2005). All of these approaches proved to be effective in revealing genetic differences between samples of common buckwheat.

In terms of field crop production, alternative cereals, such as common buckwheat, are of an immense importance for European agriculture (Đikić et al., 2013). The common buckwheat variety 'Darja' is presumed to be the one of the most cultivated varieties in the countries of Southeastern Europe (Gadžo et al., 2016; Grahić et al., 2016a; Nikolić et al., 2010; Bavec et al., 2002). Besides 'Darja', buckwheat producers from Southeastern Europe tend to use local common buckwheat varieties, such us 'Čebelica' and 'Goluba' (Gadžo et al., 2016; Nikolić et al., 2010), and even local varieties of another species from the *Fagopyrum* genus - *Fagopyrum tataricum* (L.) Gaertn (Tartary buckwheat). It is important to mention that producers generally use

farm-saved seeds as sowing material, not taking into account the disturbance of varieties' genetic integrity due to cross-pollination of common buckwheat (Grahić et al., 2016b), nor the specific requirements of those populations regarding some agro technical measures, such as fertilization (Grahić et al., 2016a).

Until now, SSR molecular markers have not been used to assess genetic purity of common buckwheat varieties that are produced in Bosnia and Herzegovina (B&H). In fact, diversity studies of field crops in Western Balkan countries are more frequently conducted through morphological evaluations (Savić et al., 2014; Grahić et al., 2013; Mladenović et al., 2012).

The specific objective of this study is to assess the genetic purity of five different samples of 'Darja' which were collected from the most known production regions of this crop in B&H.

Materials and methods

SSR analyses

Seeds from six samples of 'Darja', analyzed in this study, are currently maintained at the Gene bank of the Faculty of Agriculture and Food Sciences in Sarajevo. The first sample, that served as 'Darja' reference, was obtained from an *ex situ* seed collection from Slovenia. Remaining five samples were collected from the best known production regions of common buckwheat in B&H (Breza, Nisici Plateau, Ustikolina, Bihac and Bosanska Krupa); all of them were produced under the name 'Darja'. Seeds were sown in pots, and 16 seedlings were taken from each sample for the purposes of genetic analyses.

Genomic DNA was extracted from green leaves of the collected buckwheat seedlings using peqGOLD plant DNA kit-a (Peqlab) according to the manufacturer's instructions. Ten primer pairs (Table 1), previously published by Kishore et al. (2012), Ma et al. (2009) and Iwata et al. (2005) were used for SSR amplifications.

The M13F-tail PCR method (Schuelke, 2000) was used to measure the size of PCR products. PCR amplification was carried out in the total volume of

Table 1. Microsatellite (simple sequence repeats – SSR) code and DNA sequences of 10 primer pairs used in the assessment of genetic purity of 'Darja', a common buckwheat variety

SSR name	Forward and reverse primers
GB-FE-043 [1]	F:TGTAAAACGACGGCCAGTTTCAGCACCTGGATGGAC
	R: TGTCCCCAATGTGAAAGG
GB-FE-191 [1]	F:TGTAAAACGACGGCCAGTAATCAATGACCAGCACGC
	R:CTGATGGAGGATGCCAAA
Fem1303 [2]	F:TGTAAAACGACGGCCAGTAGGAGACGGGAGAGAAGCAG
	R:GGATGTTTGGGTGATTTCAG
Fem1322 [2]	F:TGTAAAACGACGGCCAGTAAGCATTCATTCATTCATTC
	R:GAGTTTGTTGTGTTTGGAGG
Fem1582 [2]	F:TGTAAAACGACGGCCAGTTCTGGAGAAGCTAAACCCAC
	R:CCGCAGTTTGTAGGGAGGGA
Fem1840 [2]	F:TGTAAAACGACGGCCAGTACGACGAAGACAAATGAGGA
	R:ATATGGACGGCCTGGATTAT
Fes1368 [3]	F:TGTAAAACGACGGCCAGTCAACCACTCAAAGCCTCATC
	R:CTTTCATATCCCTAACACAC
Fes1497 [3]	F:TGTAAAACGACGGCCAGTGTTGGCTGACGAAGACCGAC
	R:AAAGAGAGCGAGAGGCACTG
Fes1094 [3]	F:TGTAAAACGACGGCCAGTGAAGCCTTGGAAGAAGTGAAAT
	R:TAAAGCTCATCCCAATATGCAA
Fes1286 [3]	F:CCATATTCTACTTTCCGACC
	R:GAAGCACAAGGAAATGAGGG

[1]Ma et al. (2009); [2]Iwata et al. (2005); [3]Kishore et al. (2012)

11 µl, containing 2.5 µl of genomic DNA (1 ng/µl), 0.065 µl of the specific forward primer (5 µM), 0.32 µl of M13 universal primer (5 µM), 0.32 µl of normal reverse primer (5 µM), 1 µl of 10 x PCR buffer, 1 µl of dNTP (2.0 mM), 0.5 µl of Betaine (1M) and 0.05 µl of Taq polymerase (5 U/µl). A Veriti TM Thermal Cycler was used to perform the PCR amplification of SSR sequences (Applied Biosystems, Foster City, California, USA) with the following temperature cycling program: initial denaturation at 94°C for 3 min, 32 cycles of: 30 s at 94°C, 45 s at 50°C, 1 min at 72°C, followed by 9 cycles of: 30 s at 94°C, 45 s at 53°C, 1 min at 72°C,

Table 2. Characterization of the 7 microsatellite loci used on six common buckwheat samples (16 samples per sample)

	Referent sample				Analyzed samples				All			
	Number of alleles	H_o	H_e	PIC	Number of alleles	H_o	H_e	PIC	Number of alleles	H_o	H_e	PIC
GB-FE-043	2.0	0.438	0.417	0.323	3.0	0.744	0.520	0.402	3.0	0.691	0.511	0.394
GB-FE-191	4.0	0.143	0.429	0.386	3.0	0.304	0.393	0.352	4.0	0.280	0.398	0.362
Fem1303	8.0	0.625	0.708	0.652	15.0	0.455	0.617	0.597	16.0	0.484	0.635	0.614
Fem1840	3.0	0.700	0.679	0.572	3.0	0.581	0.631	0.552	3.0	0.595	0.641	0.562
Fes1094	4.0	0.500	0.427	0.387	5.0	0.304	0.276	0.264	5.0	0.337	0.301	0.288
Fes1368	6.0	0.643	0.762	0.692	10.0	0.474	0.565	0.539	11.0	0.500	0.620	0.592
Fes1497	8.0	0.786	0.870	0.820	11.0	0.883	0.673	0.624	11.0	0.868	0.713	0.671
Mean	5.0	0.548	0.613	0.547	7.1	0.535	0.525	0.476	7.6	0.536	0.546	0.498

H_o – observed heterozygosity; H_e – expected heterozygosity; PIC – polymorphism information content.

and by a final extension at 72°C for 10 min. PCR products (1,5 µl) were diluted with ddH_20 (1:50), then added to 8.75 µl HiDi and 0.25 µl Genescan 500 LIZ size standard. Detection of PCR products was conducted using an ABI 3130 Genetic Analyzer (Applied Biosystems) and the obtained data were analyzed using the software package GeneMapper 4.0 (Applied Biosystems).

Biostatistical analyses

Population genetics software SPAGeDI 1.2 (Hardy & Vekemans, 2002) was used to examine the characteristics of microsatellite loci. Analyses of molecular variance (Excoffier et al., 1992), based on the stepwise mutation model (Ohta & Kimura, 1973), was performed using GenoType software with 1000 permutations (Meirmans & Van Tienderen, 2004). Cervus 3.0.7 was used to calculate the polymorphism information content (Kalinowski et al., 2007). A multivariate analyses, FCA (factorial correspondence analysis) based on allele frequencies was performed using Genetix 4.02 (Belkhir et al., 2001). Input data for all statistical software's used was prepared using MADC v. 2.0 computer program (Grahić and Grahić, 2017).

Results and Discussion

SSR polymorphism

Of ten primer pairs used, seven managed to generate scorable SSR alleles. The remaining three primer pairs were discarded from further analyses. The total of 35 and 50 alleles were detected with the set of seven SSRs for the reference and five analyzed 'Darja' samples respectively, resulting in an average of 5.0 and 7.1 alleles per locus (Table 2). Lower values were reported by Ma et al. (2009) (5.90), who analyzed 41 common buckwheat populations from the National Agrobiodiversity Center in Korea. Song et al. (2011), who analyzed 179 accessions of common buckwheat obtained somewhat higher number of alleles per locus (7.90). Much higher values have been reported by Iwata et al. (2005) (40.60) for buckwheat germplasm from Japan. All calculated parameters (observed and expected heterozygosity and the PIC value) had higher average values in 'Darja' referent sample.

Seven SSRs identified 3 specific alleles in the referent sample and 18 specific alleles in the analyzed samples which indicates that the uncontrolled production of common buckwheat in B&H, mostly in regards to the use of farm-saved seeds as sowing material, led to the loss of genetic purity of the most commonly used common buckwheat variety in Western Balkans (Table 3).

Genetic relationships

Results of the analyses of molecular variance (AMOVA) show significant differentiation between the reference and all analyzed 'Darja' samples, with relatively high percentage of variation attributed to the differences among groups, proving that none of the analyzed samples have kept their genetic integrity (Table 4). The largest percentage of variance between groups (25.1) was detected between 'Darja' and 'Darja (Bosanska Krupa)' (fCT = 0.251; $p < 0.001$). Somewhat smaller fCT was calculated for the fourth analyzed pair (0.174; total variance among groups was 17.4%), but it was still highly significant ($p < 0.001$). In contrast to the

Table 3. Specific alleles found in the referent and the analyzed buckwheat samples

	Specific alleles	
	Darja	**Analyzed samples**
GB-FE-043		198
GB-FE-191	153	
Fem1303	204	173, 181, 185, 193, 197, 199, 214, 218
Fem1840		
Fes1094		185
Fes1368	164	143, 149, 155, 157, 159
Fes1497		108, 118, 127

In contrast to the above mentioned results, genetic differentiation among fifteen populations of F. tataricum (L.) Gaertn, analyzed by Kishore et al. (2012) showed that 83.49% of the total genetic variation was attributed to genetic diversity among populations and 16.51% occurred within the populations. The high percentage of variance between populations, in that study, can be explained by the fact that Tartary buckwheat is a self-pollinating species.

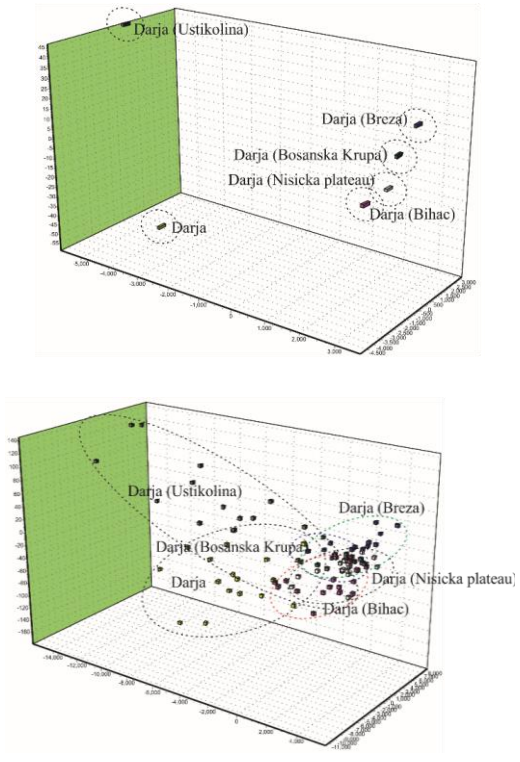

Figure 1. Multivariate analysis (Factorial Correspondence Analysis - FCA) of simple sequence repeat data for the reference and analyzed samples of 'Darja'- sample centroids (above) and individuals (beneath) shown

The three-dimensional plot (Fig. 1) of the factorial correspondence analysis revealed a clear differentiation between the reference and 'Darja' samples from the best known production regions of common buckwheat in B&H, confirming the results gained by AMOVA. Four out of five analyzed samples (Breza, Nisici Plateau, Bosanska Krupa and Bihac) clustered very close together, indicating a history of exchange of sowing material between those regions. Another explanation for this scenario could be the exchange of genetic material between 'Darja' and the limited number of local buckwheat populations during the last two decades, since 'Darja' was imported to B&H. Furthermore, other locally used buckwheat varieties, of known and unknown origin, could have played a significant role, leaving traces of introgression into the genetic material of what is today perceived as 'Darja' variety in BiH.

Table 4. Analysis of molecular variance (AMOVA) based on the 7 simple sequence repeat loci for each combination of reference and analyzed samples

Source of group variation	**df**	**Variance components**	**Total variance (%)**	**f_{CT}**	***P***
'Darja' and 'Darja (Breza)'					
Between	1	3.41	13.1	0.131	0.001
Within	30	22.67	86.9		
'Darja' and 'Darja (Nisici Plateau)'					
Between	1	3.41	13.4	0.134	0.001
Within	30	22.11	86.6		
'Darja' and 'Darja (Ustikolina)'					
Between	1	3.01	9.1	0.091	0.009
Within	30	30.1	90.9		
'Darja' and 'Darja (Bihac)'					
Between	1	4.85	17.4	0.174	0.001
Within	30	23.06	82.6		
'Darja' and 'Darja (Bosanska Krupa)'					
Between	1	7.32	25.1	0.251	0.001
Within	30	21.79	74.9		

There are still many uncertainties regarding the history of buckwheat production in B&H, leaving enough room for the assumption that the same local populations are present in the mentioned regions. The most divergent one among the analyzed samples was the one from Ustikolina, but the reasons for that are still unclear.

Conclusions

Molecular markers proved very effective when it comes to the assessment of genetic purity of common buckwheat samples from B&H. None of the analyzed samples showed sufficient similarity with the referent 'Darja' sample, indicating that irregularities regarding the production of the crop led to the loss of genetic purity of this variety. The only way to avoid this scenario in the future lies in the use of certified sowing material.

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