UDC 575.633.11
https://doi.org/10.2298/GENSR1803791G
Original scientific paper

ASSESSMENT OF GENETIC RELATIONSHIPS AMONG COMMON BUCKWHEAT (*Fagopyrum esculentum* MOENCH) VARIETIES FROM WESTERN BALKANS USING MORPHOLOGICAL AND SSR MOLECULAR MARKERS

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Grahić J., M.Đikić, D. Gadžo, S. Šimon, M. Kurtović, I. Pejić, F. Gaši (2018): *Assessment of genetic relationships among common buckwheat (Fagopyrum esculentum Moench) varieties from Western Balkans using morphological and SSR molecular markers*.- Genetika, Vol 50, No., 791-802.

In order to assess the genetic relationships and diversity among common buckwheat from Western Balkans, three varieties produced under the names 'Čebelica', 'Darja' and 'Goluba' were evaluated with a set of 10 SSR and 32 morphological markers. Eight of ten primer pairs used managed to amplify SSR alleles, in average 7 alleles per locus. Analysis of molecular variance (AMOVA) revealed that only 2.6% of the total diversity was attributed to the differences among 'Darja' and 'Goluba'. The largest percentage of variance between varieties was detected among 'Goluba' and 'Čebelica' (f_{CT} = 0.136; $p < 0.001$). Factorial correspondence analysis also revealed a clear differentiation between these two varieties. Results of hierarchical clustering based on morphological data of the three analyzed common buckwheat varieties were not in a complete accordance with the

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results obtained through genetic analyses, as it displayed a much larger difference between 'Darja' and 'Čebelica', than between 'Goluba' and 'Čebelica'. However, the differentiation among the analyzed varieties based on SSRs and Euclidean distances, calculated using morphological data, was the same. Namely, both approaches identified 'Čebelica' as the most divergent material among the analyzed varieties. The results of the genetic characterization indicate that the purity of varieties of cross pollinated species produced in Western Balkans is questionable due to use of seed material of unverified origin, as well as the general use of farm saved seeds.

Key words: common buckwheat, SSR, morphological markers, factorial correspondence analysis, hierarchical clustering.

INTRODUCTION

Common buckwheat (*Fagopyrum esculentum* Moench), usually a diploid (2n = 16) annual crop plant, originating from southwestern China (OHNISHI, 1998), is widely cultivated in Asia, Europe and America. Its ability to grow under poor agronomic conditions and its short growing period, along with the fact that buckwheat seeds possess a well-balanced quantity of essential amino acids and excellent nutritional value (GAO *et al*., 2010), made it a very important crop in mountainous regions in countries of the northern hemisphere. Among the amino acids, rutin, quercetin, kaempferol-3-rutinoside and a trace quantity of flavonol triglycosides in buckwheat grains make them suitable even for special diets (HOLASOVA *et al*., 2002).

Common buckwheat 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-incompatible system (IWATA *et al*, 2005). The mentioned diversity has been studied using allozyme analysis (OHNISHI, 1988), RAPDs (MURAI and OHNISHI, 1996; SHARMA and JANA, 2002; IWATA *et al*., 2005), AFLPs (IWATA *et al*. 2005; KONISHI *et al*. 2005) and SSRs (IWATA *et al*., 2005; KONISHI *et al*., 2006; MA *et al*., 2009; SONG *et al*., 2011). All of these approaches proved to be effective in revealing genetic differences between samples of common buckwheat. The ability to easily detect co-dominant alleles, their high reproducibility and a very high level of polymorphisms (WEBER and MAY, 1989), makes SSR markers the preferred choice for a wide range of applications (GOLDSTEIN and SCHLÖTTERER, 1999), and of course, the ideal choice for detection of genetic variations within or among populations (TAUTZ, 1989).

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 (BAVEC *et al.*, 2002; NIKOLIĆ *et al*., 2010; GRAHIĆ *et al*., 2016a; GADŽO *et al*., 2016). Besides 'Darja', buckwheat producers from Southeastern Europe tend to use local buckwheat varieties, such us 'Čebelica' and 'Goluba' (NIKOLIĆ *et al*., 2010; GADŽO *et al*., 2016). It is important to mention that producers generally use farm-saved seeds as sowing material, not taking into account the disturbance of a varieties genetic integrity due to the cross-pollination of common buckwheat (GRAHIĆ *et al*., 2016b), nor the specific requirements of those populations regarding some agro technical measures, such as fertilization (POPOVIĆ *et al.*, 2013; GRAHIĆ *et al*., 2016a).

Until now, SSR molecular markers have not been used to assess the genetic relationships among common buckwheat varieties from Western Balkans, nor to characterize local populations of this crop present and grown in the countries of Southeastern Europe. In fact, diversity studies in this region are more frequently conducted through morphological evaluation of the analyzed crops (MLADENOVIĆ *et al*., 2012; GRAHIĆ *et al*., 2013; SAVIĆ *et al*., 2014; POPOVIĆ *et al*., 2013, 2014, 2017; JANKOVIĆ *et al*., 2018).

The specific objectives of this study are as following: (i) assess the genetic relationships among well-known diploid common buckwheat varieties from Wester Balkans using morphological and SSR molecular markers; (ii) examine the genetic diversity of the analyzed varieties.

MATERIALS AND METHODS

SSR analyses

Seeds from the three varieties of common buckwheat (*Fagopyrum esculentum* Moench), known and produced under the names 'Čebelica', 'Darja' and 'Goluba', analyzed in this study, are currently maintained at the Gene bank of the Faculty of Agriculture and Food Sciences in Sarajevo. The original seed material was obtained from *ex situ* seed collections from Slovenia ('Čebelica' and 'Darja') and Montenegro ('Goluba'). Analyzed buckwheat varieties were sown in pots, and 16 seedlings were taken from each variety for the purposes of further 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, previously published by IWATA *et al*. (2005), MA *et al*. (2009) and KISHORE *et al*. (2012) 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 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 (Applied Biosystems, Foster City, California, USA) was used to perform the PCR amplification of SSR sequences with the following temperature cycling program: initial denaturation at 94^{o}C for 3 min, 32 cycles of: 30 s at 94^{o}C, 45 s at 50^{o}C, 1 min at 72^{o}C, followed by 9 cycles of: 30 s at 94^{o}C, 45 s at 53^{o}C, 1 min at 72^{o}C, and by a final extension at 72^{o}C for 10 min. PCR products (1.5 μL) were diluted with ddH20 (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).

Morphological analyses

Each accession, analyzed in our study, was grown on 3 m long and 2 m wide plots. Plots were assigned according to a randomized complete block design with three replications. The field trial was conducted during 2014 and 2015 at the regeneration field of the Gene bank in Butmir, Sarajevo.

Seventeen quantitative and fifteen qualitative traits of 30 plant (per year) were analyzed on each accession, using descriptors established for Buckwheat (IPGRI, 1994): plant height (cm), number of internodes, number of branches, stem length (cm), stem diameter (cm),

thickness of stem tissue (cm), number of leafs, petiole length (cm), leaf blade length (cm), leaf blade width (cm), length of cymes (cm), number of flower clusters per cyme, number of cymes per plant, number of seeds per cyme, seed length (cm), seed width (cm), seed weight (g), cotyledon leaf color, growth and branch shoot habit, stem color, leaf color, leaf blade color, leaf vein color, petiole color, leaf blade shape, compactness of inflorescence, branched inflorescence, color of inflorescence stalk, flower color, seed shape, seed color and seed surface.

Biostatistical analyses

Population genetics software SPAGeDi 1.2 (HARDY and VEKEMANS, 2002) was used to examine the characteristics of microsatellite loci used, as well as for the calculation of fixation index statistics (Fst) (WEIR and COCKERHEIM, 1984). Analyses of molecular variance (EXCOFFIER *et al*., 1992), based on the stepwise mutation model (OHTA and KIMURA, 1973), was performed using GenoType software with 1000 permutations (MEIRMANS and 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).

Hierarchical clustering based on Euclidean distances between the 17 quantitative and 15 qualitative morphological traits was performed using the unweighted pair group method with arithmetic means (UPGMA). This analysis (hierarchical clustering) was performed in R software version 3.2.3 (R DEVELOPMENT CORE TEAM, 2016).

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

Eight out of ten primer pairs used managed to amplify easy to score SSR alleles, the remaining two were discarded from further analyses. A total of 56 alleles were detected with the set of eight SSRs, resulting in an average of 7.0 alleles per locus (Table 1). Lower values (5.90) were reported by MA *et al*. (2009), who analyzed 41 common buckwheat population 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 loci (7.90). Much higher values (40.60) have been reported by IWATA *et al*. (2005) on buckwheat germplasm from Japan. Taking into consideration that the mentioned authors analyzed 19 indigenous varieties from Japan, one of the centers of origin of this species, and a large set of samples (570 samples in total), such a result is expected.

The Fem1303 primer pair produced the highest number (14) of alleles, whereas GB-FE-043 produced only two alleles. The observed heterozygosity (H_O) ranged from 0.152 for GB-FE-191 to 0.870 for Fes1497 (mean = 0.546), and expected heterozygosity (H_E) ranged from 0.433 for GB-FE-191 to 0.851 for Fes1638 (mean = 0.659). The mean expected heterozygosity detected in this study was somewhat higher than the one of 0.531 reported by SONG *et al*. (2011), but lower than that of the 19 varieties (0.819) used by IWATA *et al*. (2005). Fem1368, which detected 12 alleles, showed the highest PIC value (0.826). The PIC averaged over all markers was 0.607, and is higher than the average values (0.48) reported by MA *et al*. (2009) and SONG *et al*. (2011). Much higher values (0.84) for PIC were obtained by IWATA *et al*. (2005).

Table 1. Characterization of the 8 microsatellite loci used on three common buckwheat varieties from Western Balkans.

	Number of alleles	Size range (bp)	H_o	H_e	PIC
GB-FE-043	2	200/202	0.532	0.472	0.358
GB-FE-191	4	153/159	0.152	0.433	0.384
Fem1303	14	173/214	0.681	0.828	0.801
Fem1840	4	240/246	0.683	0.686	0.614
Fes1094	4	158/175	0.574	0.456	0.408
Fes1368	12	128/168	0.587	0.851	0.826
Fes1497	10	98/129	0.870	0.847	0.819
Fes1286	6	176/188	0.289	0.702	0.644
Mean	7		0.546	0.659	0.607

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

Genetic relationships

The genetic differentiation (WEIR and COCKERHAM, 1984) presented in Table 2 between the first pair of common buckwheat varieties from Western Balkans, 'Goluba'and 'Darja', for all 8 loci was very low (Fst = 0.008, *p* value not significant). This result was confirmed by AMOVA calculated for the same pair of varieties; most of the variance was retained within the groups (97.4%), whereas 2.6% of the total diversity was attributed to the differences among the two varieties of common buckwheat, indicating that they represent a very similar genetic material (Table 3). Even though IWATA *et al*. (2005) reported somewhat smaller values of variation accounted among varieties (2.34% of total variance), their published F_{CT} value (0.0235) was still significant ($p < 0.001$), indicating that it's very hard to find a high percentage of genetic purity in varieties of cross pollinated species.

Fst values calculated for the second ('Goluba' and 'Čebelica') and the third ('Darja' and 'Čebelica') pair of analyzed common buckwheat varieties were also low (0.080 and 0.055 respectively) but statistically significant ($p < 0.0001$). The largest variation between groups (F_{CT}) was detected among the second pair of varieties ('Goluba'and 'Čebelica') ($F_{CT} = 0.136$; $p < 0.001$). Somewhat smaller F_{CT} was calculated for the third analyzed pair (0.097; total variance among groups was 9.7%), but it was still significant ($p < 0.01$). F_{CT} values obtained for both pairs of varieties ('Goluba'and 'Čebelica', 'Darja' and 'Čebelica') are in concordance with their calculated Fst values, and they indicate the existence of genetic differences between these common buckwheat varieties. In contrast to the above mentioned results, genetic differentiation among fifteen population of *F. tataricum* 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 the populations, in that study, can be explained by the fact that *F. tataricum* is a self-pollinating species.

In order to get a clearer picture of the relationships between the analyzed varieties, a multivariate approach based on FCA analysis was used. The three-dimensional plot (Fig. 1) revealed a clear differentiation between the varieties 'Goluba'and 'Čebelica', whereas 'Darja' samples generally clustered closer to 'Čebelica' samples, showing some overlapping between those two, but still showing a clear distinction. Both of these observations are in concordance with the results of AMOVA (Table 3). In contrast, 'Goluba'and 'Darja' samples partially

overlapped, which could indicate that those two varieties originate from the same base material. Considering the nonsignificant values for Fst and F_{CT}, this was completely expected. One of the reason for the obtained results could lie in the fact that the genetic integrity of buckwheat varieties from Western Balkan region is highly disrupted due to the usage of farm-saved seeds as sowing material.

Table 2. F-statistics for each of the pairs of analyzed buckwheat varieties ('Goluba', 'Darja' and 'Čebelica') based on 8 simple sequence repeat loci.

'Goluba'and 'Darja'		
SSRs	Fst	*P-value*
GB-FE-043	-0.011	0.924
GB-FE-191	-0.009	0.879
Fem1303	0.026	0.243
Fem1840	0.017	0.464
Fes1094	-0.019	0.375
Fes1368	0.014	0.425
Fes1497	0.021	0.260
Fes1286	-0.011	0.933
All loci	0.008	0.441
'Goluba'and 'Čebelica'		
SSRs	Fst	*P-value*
GB-FE-043	-0.006	0.611
GB-FE-191	0.146	0.064
Fem1303	0.041	0.073
Fem1840	-0.009	0.938
Fes1094	0.028	0.238
Fes1368	0.047	0.056
Fes1497	0.038	0.088
Fes1286	0.322	<0.0001
All loci	0.080	<0.0001
'Darja' and 'Čebelica'		
SSRs	Fst	*P-value*
GB-FE-043	0.045	0.230
GB-FE-191	0.055	0.277
Fem1303	0.052	0.053
Fem1840	-0.021	0.670
Fes1094	0.025	0.187
Fes1368	0.055	0.053
Fes1497	-0.011	0.631
Fes1286	0.228	0.003
All loci	0.055	0.003

Table 3. Analysis of molecular variance (AMOVA) based on the 8 simple sequence repeat loci for each of the pairs of analyzed buckwheat varieties ('Goluba', 'Darja' and 'Čebelica').

Source of variation	df	Variance components	Total variance (%)	f_{CT}	*P*
'Goluba'and 'Darja'					
Among groups	1	0.81	2.6	0.026	0.129
Within groups	30	30.93	97.4		
'Goluba'and 'Čebelica'					
Among groups	1	5.58	13.6	0.136	<0.001
Within groups	30	35.58	86.4		
'Darja' and 'Čebelica'					
Among groups	1	3.35	9.7	0.097	<0.01
Within groups	30	31.31	90.3		

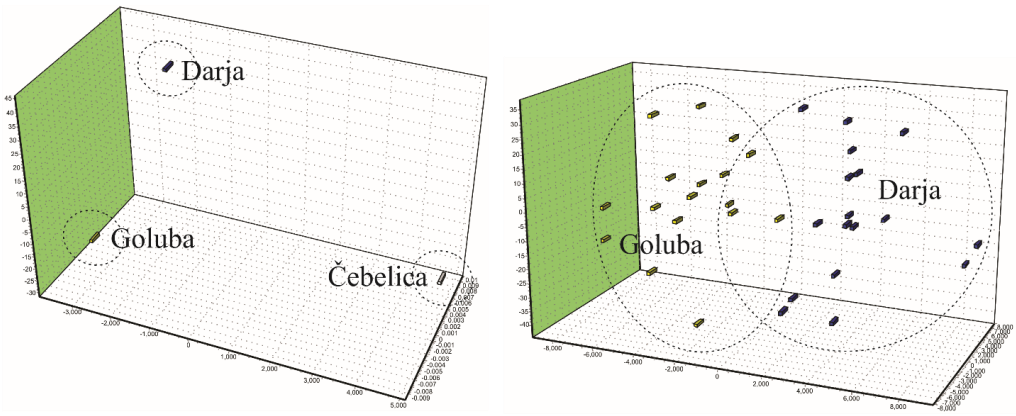

Fig. 1. Multivariate analysis (Factorial Correspondence Analysis - FCA) of simple sequence repeat data for three common buckwheat varieties from Western Balkans - variety centroids shown (left) and for two common buckwheat varieties from Western Balkans (Goluba and Darja) - individuals shown (right).

Factorial correspondence analysis also revealed that the variety 'Čebelica' is the most divergent among the analyzed common buckwheat varieties from Wester Balkans.

Morphological analyses

Summaries of the 17 measured quantitative and 15 qualitative morphological traits which were observed and analyzed are presented in Table 4 and Table 5.

Table 4. Average values with the standard error for the 17 quantitative traits measured on 30 plants (per year) from each of the analyzed varieties: 'Goluba', 'Darja' and 'Čebelica'.

Variety	'Goluba'	'Darja'	'Čebelica'
plant height	97.83 ± 6.201	83.61 ± 3.086	129.24 ± 2.822
number of internodes	11.45 ± 0.587	9.43 ± 0.558	12.88 ± 0.369
number of branches	3.10 ± 0.233	3.41 ± 0.257	2.35 ± 0.162
stem length	97.70 ± 6.228	80.51 ± 3.063	127.80 ± 2.878
stem diameter	0.40 ± 0.046	0.40 ± 0.019	0.53 ± 0.035
thickness of stem tissue	0.10 ± 0.002	0.16 ± 0.006	0.12 ± 0.005
number of leafs	28.55 ± 2.756	18.37 ± 5.209	35.78 ± 3.446
petiole length	2.99 ± 0.29	2.45 ± 0.39	5.48 ± 0.352
leaf blade length	4.41 ± 0.284	3.37 ± 0.363	6.11 ± 0.312
leaf blade width	3.78 ± 0.266	2.80 ± 0.288	5.47 ± 0.321
length of cymes	5.85 ± 0.195	2.81 ± 0.259	7.52 ± 0.548
number of flower clusters per cyme	2.88 ± 0.563	1.60 ± 0.195	1.98 ± 0.289
number of cymes per plant	23.85 ± 2.122	22.83 ± 2.749	32.98 ± 3.311
number of seeds per cyme	4.40 ± 0.587	8.77 ± 0.735	4.91 ± 0.444
seed length	0.580 ± 0.0133	0.461 ± 0.0157	0.650 ± 0.0143
seed width	0.300 ± 0.0000	0.222 ± 0.0131	0.353 ± 0.0080
seed weight	0.026 ± 0.0008	0.019 ± 0.0013	0.023 ± 0.0011

Table 5. Summary of the 15 qualitative traits measured on 30 plants (per year) from each of the analyzed varieties: 'Goluba', 'Darja' and 'Čebelica'.

Variety	'Goluba'	'Darja'	'Čebelica'
cotyledon leaf color	green	green	green
growth and branch shoot habit	erect shorter	erect longer	erect longer
stem color	pink	green	red
leaf color	green	green	green
leaf blade color	green	green	green
leaf vein color	green	green	red
petiole color	pink	pink	red
leaf blade shape	sagittate	sagittate	sagittate
compactness of inflorescence	semi-compact	semi-compact	loose
branched inflorescence	yes	yes	yes
color of inflorescence stalk	pink	green	green
flower color	white	white	white
seed shape	ovate	triangular	triangular
seed color	brown	brown	brown
seed surface	smooth	smooth	smooth

The three analyzed common buckwheat varieties formed two clusters based on the Euclidean distances utilizing the UPGMA method (Fig. 2). 'Goluba' and 'Darja' showed higher similarity with each other than with the variety 'Čebelica', causing them to group in the same cluster. However, differentiation based on morphological traits is not in a perfect accordance

with the results obtained through genetic analyses. Namely, the table of Euclidean distances shows that there is a much bigger difference between 'Darja' and 'Čebelica', than between 'Goluba' and 'Čebelica', despite the fact that all analyses based on SSR data showed a different scenario (Table 6), possibly proving that the assessment of genetic relationships solely with the help of morphological markers unreliable. DOLNIČAR *et al.* (2016) reported similar results when analyzing lightsprouts from different potato varieties, concluding that molecular markers showed more strength in resolving relationships between genotypes then morphological markers.

Table 6. Euclidean distances between three analyzed populations.

	'Goluba'	'Darja'	'Čebelica'
'Goluba'	0.0	35.2	63.0
'Darja'	35.2	0.0	95.5
'Čebelica'	63.0	95.5	0.0

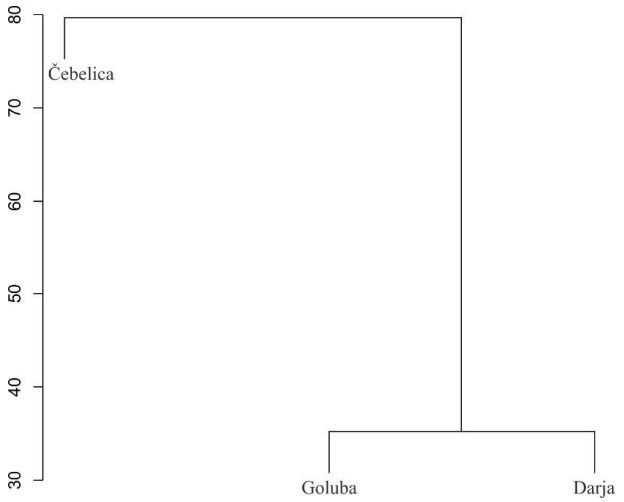

Fig. 2. Hierarchical clustering based on the Euclidean distances between 3 analyzed common buckwheat varieties utilizing the UPGMA method.

CONCLUSION

Two ('Goluba'and 'Darja') out of three analyzed common buckwheat varieties originating from Western Balkans represent a very similar genetic material. Slovenia's indigenous variety 'Čebelica' is the most divergent material among the analyzed varieties. Accordingly, both molecular and morphological markers proved very effective when it comes to assessment of genetic relations of common buckwheat, differentiating the analyzed varieties in the same manner. In order to fully utilize the production potential of a cross pollinated species like common buckwheat, producers from Western Balkans need to base their production on commercially sold seed material with guaranteed genetic purity.

Received, December 12th, 2017
Accepted April 18th, 2018

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ISPITIVANJE GENETIČKIH ODNOSA IZMEĐU SORTI OBIČNE HELJDE (*Fagopyrum esculentum* MOENCH) IZ ZAPADNOG BALKANA UPOTREBOM MORFOLOŠKIH I SSR MARKERA

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Izvod

Kako bi se analizirali genetički odnosi i diverzitet kod obične heljde iz Zapadnog Balkana, ispitane su tri sorte koje se proizvode pod imenima ‘Čebelica’, ‘Darja’ i ‘Goluba’, i to korišćenjem 10 SSR i 32 morfološka markera. Osam od deset korišćenih markera uspelo je da amplificira u proseku 7 SSR alela po lokusu. Analizom molekularne varijanse (AMOVA) je otkriveno da se samo 2,6% ukupnog diverziteta odnosilo na razlike između ‘Darja-e’ i ‘Goluba-e’. Najveća razlika je utvrđena između sorti ‘Goluba’ i ‘Čebelica’ (f_{CT} = 0,136; $p < 0,001$). Faktorijska korespodentna analiza je takođe pokazala jasnu diferencijaciju između prethodno navedenih sorti. Rezultati hijerarhijskog klasterisanja, dobijeni korišćenjem morfoloških podataka izmerenih kod tri analizirane sorte heljde, nisu u potpunosti u skladu sa rezultatima dobijenim genetičkim analizima. Naime, hijerarhijsko klasterisanje je pokazalo da postoji mnogo veća razlika između ‘Darja-e’ i ‘Čebelica-e’ nego između ‘Goluba-e’ i ‘Čebelica-e’, ali je grupisanje sorti zasnovano na Euklidskoj distanci i ono zasnovano na SSR podacima dalo jednake rezultate. Oba pristupa, tj. primena SSR i morfoloških markera, identifikovala su ‘Čebelica-u’ kao najdivergentiji materijal od analiziranih sorti. Sortna čistoća stranoopodnih vrsta koje se proizvode na području Zapadnog Balkana je veoma upitna zbog činjenice da proizvođači kao setveni materijal uglavnom koriste seme neproverenog porekla, te ono dobijeno žetvom date kulture prethodne sezone.

Primljeno 12.XII.2017.
Odobreno 18. IV. 2018.