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Genetic diversity of leafy kale genetic resources (*Brassica oleracea var. acephala* L.)

Sonja Umićević[1], Marina Antić[1,2], Dragana Rajković[3], Lovro Sinkovič[4], Nevena Nagl[3], Smiljana Goreta Ban[5], Vladimir Meglič[4], Vida Todorović[2], Ana Marjanović Jeromela[3], Barbara Pipan[4]

[1]*University of Banja Luka, Institute of Genetic Resources, Banja Luka, Republic of Srpska, Bosnia and Herzegovina*
[2]*University of Banja Luka, Faculty of Agriculture, Banja Luka, Republic of Srpska, Bosnia and Herzegovina*
[3] *Institute of field and vegetable crops, Novi Sad, Serbia*
[4] *Agricultural Institute of Slovenia, Ljubljana, Slovenia*
[5] *Institute of Agriculture and Tourism, Poreč, Croatia*

Abstract

Leafy kale (*Brassica oleracea var. acephala L.*) is one of the most important *Brassicaceae* species with health-promoting properties and great diversity. To investigate variability within and among accessions, 12 accessions (each accession comprised four individuals/genotypes) from the Balkan region were genotyped at 12 simple sequence repeats (SSRs) loci. The selected SSR markers originated from the genomes of *B. oleracea* (eight), *B. rapa* (two) and *B. napus* (two). DNA was extracted from seeds, cotyledons, shoots, and young leaves, depending on the germination energy of each seed, using magnetic extraction method. Genetic analysis included eight accessions from Serbia (24 genotypes), two accessions from Montenegro (eight genotypes), and one accession from Bosnia and Herzegovina and Croatia (with four genotypes each). Accession-specific polymorphism was found at the Ol12-FO2 and Ol10-F11a loci, where genotypes were qualitatively distinguished by horizontal agarose gel electrophoresis (binary detection). Among 12 loci, the average polymorphic information content (PIC) was 0.71, with the marker Ol13-C12 proving to be the

most informative (PIC = 0.89). However, the analysis of molecular variability (AMOVA) showed 11% diversity between accessions, 19% diversity between genotypes, and 70% diversity within genotypes, explaining the molecular diversity of native Balkan leafy kale germplasm. According to the genetic structure, four genetic groups were formed with an average expected heterozygosity of 0.70 between clusters. In order to exploit the genetic diversity, it would be advisable to evaluate these accessions at phenotypic level and use their potential in breeding programmes.

Key words: leafy kale, accession, genetic analysis, SSR, genotyping

Introduction

The *Brassicaceae* family, which includes about 350 genera and 3700 species, is one of the ten most economically important plant families with a wide range of agronomic traits (Love et al., 2005; Guo et al., 2017). The genus *Brassica L.* contains six economically important species cultivated worldwide: *B. oleracea, B. rapa, B. nigra, B. napus, B. juncea* and *B. carinata* (Sanchita et al., 2008). The wild type of *Brassica oleracea* generally grows along the Atlantic coast on limestone and chalk cliffs as a perennial plant with a life span of up to 10 years or longer (Christensen et al., 2011). Most similar to the wild form of *Brassica oleracea* is leafy kale (*Brassica oleracea* L. convar. *acephala* (DC.) Alef.) (Christensen et al., 2011). It is believed to have originated in the eastern Mediterranean and was used for human consumption as early as 2000 BC (Sarikamis et al., 2010). Landraces of leafy kale are still widely cultivated in some areas: along part of the Black Sea coast and in the central and northern parts of the Iberian Peninsula, where they are well adapted to local growing conditions (Dias, 1995; Okumus and Balkaya, 2007). These landraces are grown in gardens and small plots. The newly developed tender shoots are usually used for human consumption, while the older and coarser shoots are used as livestock feed (Balkaya and Yanmaz, 2005).

The evaluation of genetic diversity within collections of crop species is crucial for establishing efficient conservation and breeding practices to develop new and more productive varieties that are resistant to diseases and adapted to changing environmental conditions (El-Esawi et al., 2016). For example, resistance to clubroot disease caused by *Plasmodiophora brassicae* has been found in French landraces of *Brassica oleracea* (Manzanares-Dauleux et al., 2000), while resistance to downy mildew (*Peronospora parasitica*) has been reported for Portuguese landraces of *Brassica oleracea* (Dias et al., 1993). To

assess genetic diversity in crop species and their relationships, molecular markers have proven to be powerful tools (El-Esawi et al., 2016). Simple sequence repeats (SSRs) have been successfully used to assess genetic variability because they are codominant, have high polymorphism, and can reveal a large number of alleles for each locus, resulting in a high degree of variability and reproducibility (Mariette et al., 2001, as cited in El-Esawi et al., 2016)).

Many studies have been conducted on the genetic diversity of leafy kale worldwide (Abbas et al., 2009; Sarikamis et al., 2010; Christensen et al., 2011; El-Esawi et al., 2016), indicating its importance for both scientific research and breeding purposes. The aim of our current study was to evaluate genetic variability within and among leafy kale accessions from the Balkan region, which includes accessions from Serbia, Montenegro, Bosnia and Herzegovina, and Croatia.

Material and Methods

The leafy kale collection included 12 accessions originating from four Balkan countries: eight accessions from Serbia, two accessions from Montenegro, and one accession from Bosnia and Herzegovina and Croatia (Table 1).

Tab. 1. Leafy kale collection

Accession Number	*Country*	*Location*	*Habitat Type*	*GPS Coordinates*	*Origin*
1	Serbia	Ostojićevo	Private garden	45.889763, 20.160021	Prijedor, Bosnia and Herzegovina
2	Montenegro	Luštica (Herceg Novi)	Abandoned lot	42.397459, 18.595201	Unknown
3	Bosnia and Herzegovina	Trebinje	Private garden	42.710657, 18.333551	Bosnia and Herzegovina
4	Serbia	Ravno selo	Private garden	45.448647, 19.623525	Domesticated variety
5	Serbia	Ravno selo 1	Private garden	45.446249, 19.627071	Domesticated variety
6	Serbia	Lovćenac	Private garden	45.678675, 19.697395	Domesticated variety
7	Serbia	Lovćenac 1	Private garden	45.681600, 19.688885	Domesticated variety
8	Montenegro	Bečići	Private garden	42.283828, 18.881616	Unknown
9	Serbia	Banatski Despotovac	Private garden	45.366865, 20.661254	Unknown
10	Serbia	Kisač	Private garden	45.353780, 19.733362	Domesticated variety
11	Croatia	Dalmacija	Private garden	43.541935, 16.457828	Unknown
12	*Serbia*	*Klek*	*Private garden*	*45.420283, 20.484968*	*Bosnia and Herzegovina*

To detect intra-specific variability, each accession was represented by four genotypes. Plant material was grown in the greenhouse until reaching the stage of fourth true-leaf, and subsequently, DNA extraction was performed using one healthy leaf from each individual plant/genotype. Individual seed was used as a starting material for DNA extraction from genotypes the seeds of which did not germinate.

Homogenization of samples was performed using TissueLyser (Qiagen). DNA was extracted using the MagMax (Applied Biosystems) magnetic extraction method with the BioSprint DNA Plant Kit (Qiagen) according to Pipan and Meglič (2019). DNA quantification was performed using the Qubit dsDNA BroadRange Assay Kit and the Qubit 3.0 fluorometer. The DNA was visualized on agarose gel and Syngene GeneSnap software was used. Twelve microsatellite markers were used for genotyping the leafy kale collection (Table 2).

Tab. 2. Characteristics of microsatellite markers used for PCR amplification

Marker Name	*Forward Sequence*	*Reverse Sequence*	*Expected Length [bp]*	*Reference*
Ol12-FO2	GGCCCATTGAT ATGGAGATG	CATTTCTCAATG ATGAATAGT	200-250	Sarikamis et al., 2010
Ol10-A03a	CTGGTTTTCTC CTTCATCAG	CTGTGTAGCTTT TAGTCTTT	50–160	El-Esawi et al., 2016
Ol10-F11a	TTTGGAACGTC CGTAGAAGG	CAGCTGACTTCG AAAGGTCC	64–240	El-Esawi et al., 2016
Ol10-H02	AACAGGAAGA AACGACGAGG	AGAGAGCCATGA GAAGCACC	98–260	El-Esawi et al., 2016
Ol11-G11	GTTGCGGGCG AAACAGAGAA G	GAGTAGGCGATC AAACCGAG	70–210	El-Esawi et al., 2016
Ol11-H02	TCTTCAGGGTT TCCAACGAC	AGGCTCCTTCAT TTGATCCC	96–250	El-Esawi et al., 2016
Ol12-F11	AAGGACTCATC GTGCAATCC	GTGTCAGTGGCT ACAGAGAC	100–276	El-Esawi et al., 2016
Ol13-C12	AGAGGCCAAC AAAGAACACC	GAAGCAGCACC AGTGACAAG	84–196	El-Esawi et al., 2016
Ra2-E03	AGGTAGGCCC ATCTCTCTCC	CCAAAACTTGCT CAAAACCC	110–245	El-Esawi et al., 2016
Ra2-E11	GGAGCCAGGA GAGAAGAAGG	CCCAAAACTTCC AAGAAAAGC	74–208	El-Esawi et al., 2016
Na12-C08	GCAAACGATTT GTTTACCCG	CGTGTAGGGTGA TCTATGATGGG	68–190	El-Esawi et al., 2016
Na14-C12	*CACATTTTGGTT CAATTCGG*	*TACGACCTGGTTT CGATTC*	*92–198*	*El-Esawi et al., 2016*

Eight markers were designed based on their position in the genetic map of *B. oleracea var. acephala*, two markers were designed based on their position in

the genetic map of *B. rapa* and two markers based on their position in the genetic map of *B. napus*.

The optimal PCR mixtures and PCR reaction conditions for each specific marker were used as described by Pipan and Meglič (2019); Meglič and Pipan (2018); and Pipan et al. (2013) (Table 3). Amplification reactions were performed using the VerityTM 96-Well Thermal Cycler (Applied Biosystems) under the following conditions for the PCR programme 1: 94°C for 4 min, 15 cycles at a) 94°C for 1 min, b) 49.5°C + 0.7°C/cycle for 1 min, c) 72°C for 1 min; 23 cycles at a) 94°C for 30 s, b) 53°C for 30 s, c) 72°C for 1 min; 72°C for 5 min and for the PCR programme 2: 94°C for 5 min, 10 cycles at a) 94°C for 45 s, b) 56°C – 0.1°C/cycle for 45 s, c) 72°C for 1 min and 30 s; 30 cycles at a) 94°C for 45 s, b) 55°C for 45 s, c) 72°C for 1 min and 30 s; 72°C for 8 min. QuantaBio Mix (for one sample) was prepared as follows: 9.467 µl AccuStart II PCR ToughMix, 0.1 µl forward marker, 0.25 µl reverse marker, and 0.183 µl fluorescent dye. Biotools Mix (for one sample) was made by mixing: 1 µl 10× reaction buffer, 0.5 µl 50mM MgCl2 solution, 0.2 µl dNTP, 0.1 µl forward marker, 0.25 µl reverse marker, 0.183 µl fluorescent dye, 7.75 µl nuclease-free water, and 0.05 µl Biotools DNA Polymerase 500 Units 5U/µl.

Tab. 3. PCR conditions

Marker Name	*PCR Programme*	*PCR Chemistry*	*M13 Fluorescent Dye*
Ol12-FO2	1	QuantaBio	FAM
Ol10-A03a	1	QuantaBio	NED
Ol10-F11a	1	Biotools	HEX
Ol10-H02	2	Biotools	FAM
Ol11-G11	2	Biotools	NED
Ol11-H02	2	Biotools	HEX
Ol12-F11	1	Biotools	FAM
Ol13-C12	2	Biotools	NED
Ra2-E03	2	Biotools	HEX
Ra2-E11	2	Biotools	FAM
Na12-C08	1	QuantaBio	NED
Na14-C12	*1*	*QuantaBio*	*HEX*

Fragment analysis was performed using the Genetic Analyser (ABI 3500, Applied Biosystems). The fragment reaction was prepared using 7 µl formamide and 0.4 µl ROX-500 internal length standard in each sample along with pooled post-PCR products according to the different M13 fluorescent dyes as described by Pipan and Meglič (2019). Electropherograms were read in the GeneMappper 6.0 (Applied Biosystems) programme. Input matrices with allele lengths were evaluated in several programmes for population genetics data analysis: the GeneAlEx 6.1 software to calculate the average number of migrants, molecular variability, principal coordinate analysis, and deviations from Hardy-Weinberg equilibrium (Peakall and Smouse, 2006); the Microsatellite-Toolkit software to

calculate observed and expected heterozygosity and polymorphism information content (Park, 2001), the Structure 2.3.3 software to analyse genetic structure (Pritchard et al., 2009), and the Structure Harvester software to determine the number of genetic groups (Earl and von Holdt, 2012).

Results and Discussion

Informativeness and variability of microsatellite markers

Based on the genotyping results presented in Table 4, the average number of effective alleles was 2.87. The genetic variability defined by expected heterozygosity (He) had average values of 0.75, while the average value of the observed heterozygosity (Ho) was 0.73. Overall, the average polymorphic information content (PIC) was 0.71, which is higher than that of El-Esawi et al. (2016), where the average PIC was 0.571. The results show that the average Shannon information index (I), fixation index (F), and number of migrants were 1.06, -0.23, and 1.13, respectively. The average PIC value > 0.7 indicates that all observed microsatellite loci exhibit a high degree of polymorphism. The most informative microsatellite markers (i.e., with PIC > 0.8) were Ol13-C12 (PIC = 0.89; He = 0.91; I = 1.37), Ol10-A03a (PIC = 0.86; He = 0.88; I = 1.34), and Ol10-F11a (PIC = 0.84; He = 0.87; I = 1.06). The highest F was determined for Ol12-FO2 (0.21) and the lowest F for Na14-C12 (–0.61). In addition, the highest Nm was determined for Ra2-E11 (2.76) and the lowest Nm for Ol10-F11a (0.54).

Tab. 4. Genetic variability parameters among loci

Locus	N_e	He	H_o	*PIC*	*I*	*F*	F_{is}	F_{it}	F_{st}	N_m
Ol12-FO2	2.12	0.60	0.38	0.57	0.86	0.21	0.23	0.37	0.18	1.10
Ol10-A03a	3.58	0.88	0.75	0.86	1.34	-0.06	-0.06	0.14	0.19	1.05
Ol10-F11a	2.92	0.87	0.48	0.84	1.06	0.17	0.18	0.44	0.32	0.54
Ol10-H02	1.78	0.54	0.40	0.51	0.64	-0.08	-0.04	0.25	0.28	0.64
Ol11-G11	3.28	0.80	0.88	0.77	1.21	-0.36	-0.34	-0.11	0.18	1.16
Ol11-H02	2.89	0.76	0.75	0.73	1.07	-0.23	-0.27	0.01	0.22	0.88
Ol12-F11	2.94	0.79	0.94	0.74	1.11	-0.50	-0.47	-0.20	0.18	1.12
Ol13-C12	3.91	0.91	0.90	0.89	1.37	-0.29	-0.27	0.00	0.22	0.90
Ra2-E03	3.23	0.77	0.83	0.73	1.15	-0.35	-0.34	-0.09	0.19	1.09
Ra2-E11	2.62	0.67	0.92	0.60	1.00	-0.54	-0.51	-0.39	0.08	2.76
Na12-C08	2.67	0.70	0.63	0.65	1.03	-0.12	-0.10	0.10	0.18	1.14
Na14-C12	2.47	0.69	0.90	0.62	0.93	-0.61	-0.60	-0.32	0.18	1.16
Average	*2.87*	*0.75*	*0.73*	*0.71*	*1.06*	*-0.23*	*-0.22*	*0.02*	*0.20*	*1.13*

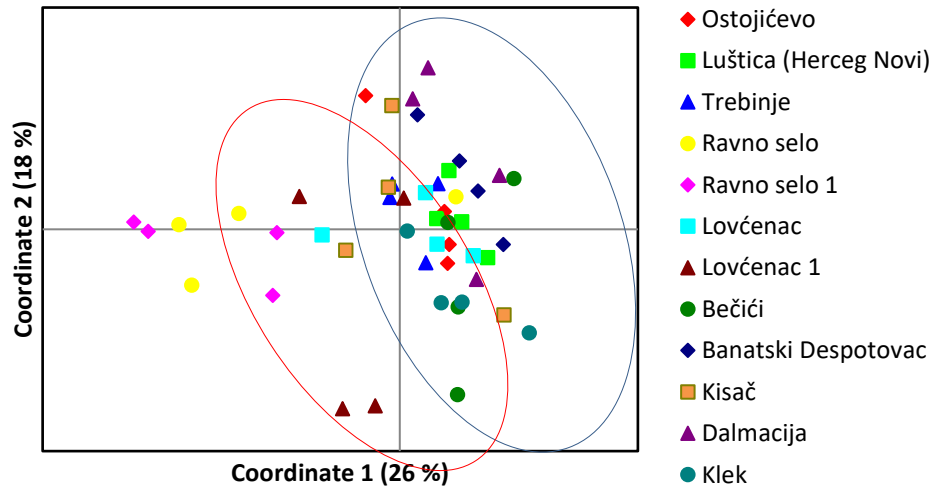
* N_e – Number of effective alleles; H_e – Expected heterozygosity; H_o – Observed heterozygosity; *PIC* – Polymorphism information content; *I* – Shannon's information index; *F* – Fixation index; F_{is} – Genotype genetic differentiation relative to accession; F_{it} – Genotype genetic differentiation relative to the total populations; F_{st} – Genetic differentiation of the accessions compared to the total population; N_m – Average number of migrants

Statistically significant deviations ($P < 0.05$) from Hardy-Weinberg equilibrium occurred at 7 loci. First, there were statistically significant deviations from Hardy-Weinberg equilibrium at locus Ol10-F11a in the Ostojićevo, Luštica (Herceg Novi), and Lovćenac 1 accessions. The same was shown at locus Ol11-H02 in the Ravno selo, Lovćenac 1, and Klek, and also at locus Ra2-E11 in the Lovćenac, Banatski Despotovac, and Klek accessions. In addition, there were statistically significant deviations from Hardy-Weinberg equilibrium at locus Ol12-F11 in the Trebinje and Ravno selo 1 accessions, the Ra2-E03 locus in the Trebinje and Klek accessions, the Na12-C08 locus in the Trebinje and Ravno selo 1, and at the Ol10-H02 locus in Ravno selo. The highest genetic differentiation of the accessions compared to the total population (F_{ST}) was found for the Ol12-FO2 locus (0.318) and the lowest for the Na12-C08 locus (0.083). The average F_{ST} in this study was 0.2 and similar results for *B. oleracea* germplasm were obtained by El-Esawi et al. (2016), where the F_{ST} was 0.271. The highest genetic differentiation of genotype relative to accession (F_{IS}) was found for the Ol10-F11a locus (0.23) and the lowest for the Ra2-E11 locus (–0.6). The average F_{IS} in this study was –0.217 and similar results were obtained by El-Esawi et al. (2016) where the F_{IS} was –0.374. The highest genotype genetic differentiation relative to the total populations (F_{IT}) was found for the Ol12-FO2 locus (0.442), while the lowest was for the Na12-C08 locus (–0.385). The aerage F_{IT} in this study was 0.018 and similar results were obtained by El-Esawi et al. (2016), where F_{IT} was 0.1.

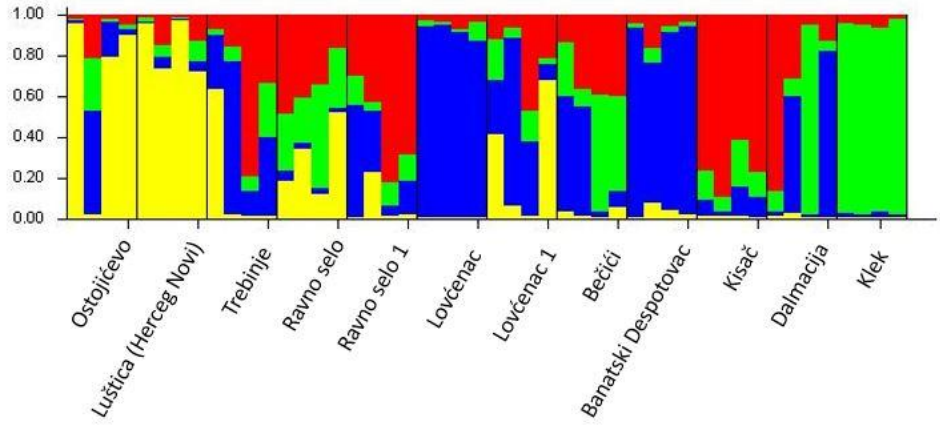
Genetic diversity of the leafy kale collection

Of all studied accessions, 10 accessions amplified 100% polymorphic loci, while only the Luštica (Herceg Novi) and Bečići accessions had 91.67% polymorphic loci. The analysis of molecular variability (AMOVA) showed 11% diversity among accessions, 19% diversity among genotypes, and 70% diversity within genotypes. Similar results were presented in the study by Christensen et al. (2011), where the variation within individuals was 62%. From the AMOVA results listed, it is clear that the leafy kale collection analyzed has high diversity indicating a common origin. According to Christensen et al. (2011), accessions from Portugal, Turkey, Croatia, and Bosnia and Herzegovina consisted of mixed genotypes and share parts of their genome with other accessions because of common ancestry or gene flow. Principal coordinate analysis (PCoA) was performed based on the covariance matrix of genetic distances. Graph 1 shows that the leafy kale accessions are clustered into two groups. Moreover, the Ravno selo, Lovćenac, Lovćenac 1, and Kisač accessions occurred in both groups, suggesting a common origin of the studied accessions. The first coordinate

explained 26% of the variability, the second coordinate 18%; cumulatively the first three coordinates explained 62% of the variability. The analysis of genetic structure, performed using the Bayesian clustering approach (Structure Harvester, Graph 2), revealed four genetic clusters. Lovćenac, Banatski Despotovac, and Klek had similar genetic variability within accessions.



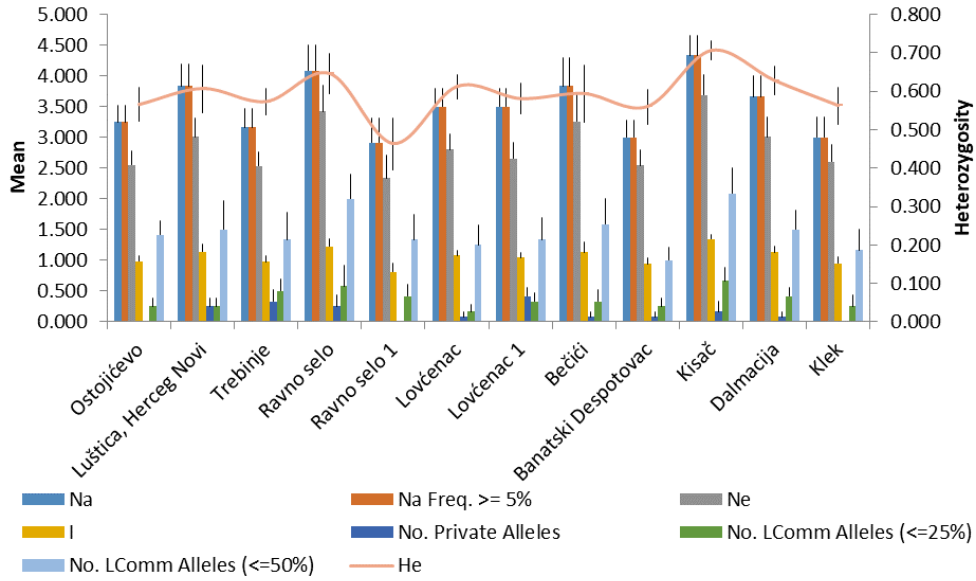
Graph 1. Distribution of leafy kale accessions in Principal Coordinate Analysis (PCoA)



* Each colour represents one genetic cluster, while vertical bars stand for each individual
Graph 2. Genetic structure of leafy kale accessions

The results of the comparison of the allelic patterns of the codominant data of the studied leafy kale accessions are shown in Graph 3. Among the studied accessions, the most genetically diverse accession was Kisač with the

highest value of He (0.71), I (1.34), the number of effective alleles (Ne) (3.69), the number of locally common alleles found in 25% or fewer populations (0.67), and the number of locally common alleles found in 50% or fewer populations (2.08). The Ravno selo accession had the second highest genetic diversity, with values of He (0.65), I (1.23), Ne (3.43), the number of locally common alleles found in 25% or fewer populations (0.58), the number of locally common alleles found in 50% or fewer populations (2.00), and the number of private alleles (0.25). The highest value for the number of private alleles was calculated for the following accessions: Lovćenac (0.42), Trebinje (0.33), Luštica (Herceg Novi) (0.25), and Ravno selo (0.25), indicating a conserved diversity within these genetic resources that is higher than the diversity in commercial varieties where targeted selection takes place.



Graph 3. Comparison of allelic patterns of codominant data between the leafy kale

Conclusion

This study demonstrated that 12 proposed SSR markers can be used to study diversification of leafy kale accessions. The most informative SSR markers (i.e., with PIC > 0.8) were Ol13-C12, Ol10-A03a, and Ol10-F11a. In general, wild populations and landraces are thought to be more diverse compared to commercially bred cultivars. The analyzed leafy kale accessions from the Balkan region had average polymorphic information content 0.71, with 70% diversity

detected within genotypes. The present study provides a new perspective on genetic diversity of leafy kale landraces originating from the Balkan region, which is important for further promotion and sustainable use of germplasm not only for scientific research purposes but also for breeding practice. In order to utilize the genetic diversity, it would be practical to evaluate these accessions at phenotypic level and use their potential in breeding programmes.

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Генетички диверзитет генетичких ресурса раштана (*Brassica oleracea var. acephala L.*)

Соња Умићевић[1], Марина Антић[1,2], Драгана Рајковић[3], Ловро Синкович[4], Невена Нагл[3], Смиљана Горета Бан[5], Владимир Меглич[4], Вида Тодоровић[2], Ана Марјановић Јеромела[3], Барбара Пипан[4]

[1] *Универзитет у Бањој Луци, Институт за генетичке ресурсе, Бања Лука, Република Српска, Босна и Херцеговина*
[2] *Универзитет у Бањој Луци, Пољопривредни факултет, Бања Лука, Република Српска, Босна и Херцеговина*
[3] *Институт за ратарство и повртарство, Нови Сад, Србија*
[4] *Кметијски инштитут Словеније, Љубљана, Словенија*
[5] *Институт за пољопривреду и туризам, Пореч, Хрватска*

Сажетак

Раштан (*Brassica oleracea var. acephala L.*) је једна од најважнијих врста из породице купусњача која повољно утиче на здравље људи и одликује се великом разноврсношћу. Да би се истражила варијабилност унутар и између принова, 12 принова (свака принова се састојала од четири индивидуе/генотипа) са територије Балканског полуострва је генотипизовано користећи 12 ССР маркера. Одабрани ССР маркери потичу из генома *B. oleracea* (осам), *B. rapa* (два) и *B. napus* (два). ДНК је изолована из сјемена, котиледона, изданака и младих листова, у зависности од енергије клијања сваког сјемена, методом магнетне екстракције. Генетичком анализом обухваћено је осам принова из Србије (24 генотипа), двије принове из Црне Горе (осам генотипова) и једна принова из Босне и Херцеговине и Хрватске (са по четири генотипа). Полиморфизам специфичан за принове пронађен је на локусима Ol12-FO2 и Ol10-F11a, гдје су генотипови квалитативно разликовани хоризонталном електрофорезом у агарозном гелу (бинарна детекција). Код укупно 12 локуса, просјечан садржај полиморфне информације (PIC) био је 0,71, при чему се као најинформативнији показао маркер Ol13-C12 (PIC = 0,89). Међутим, анализа молекуларне варијабилности (AMOVA) показала је 11% разноврсности између принова, 19% разноврсности између генотипова и 70% разноврсности унутар генотипова, објашњавајући молекуларну разноврсност изворне гермплазме раштана са територије Балканског полуострва. Према генетичкој структури формиране су четири генетичке групе са просјечном очекиваном хетерозиготношћу од 0,70 између кластера. Да би се искористио генетички диверзитет, било би препоручљиво да се ове принове карактеришу

на фенотипском нивоу и да се њихов потенцијал искористи у програмима оплемењивања.

Кључне ријечи: раштан, принова, генетичка анализа, ССР, генотипизација

Corresponding author: Sonja Umićević
E–mail: sonja.raseta@igr.unibl.org

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