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GENETIC DIVERSITY AND RELATIONSHIP ASSESSMENT OF ROMANIAN BLUEBERRY CULTIVARS USING SRAP MARKERS

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Abstract

Sequence-related amplified polymorphism (SRAP) molecular markers have proven to be an effective tool for assessing genetic diversity and cultivar identification in various plant species. Present study aimed to genotype nine Romanian blueberry (Vaccinium corymbosum) cultivars – 'Lax', 'Prod', 'Vital', 'Azur', 'Simultan', 'Delicia', 'Pastel', 'Safir', and 'Augusta' - using SRAP markers to evaluate their genetic variability and relationships. A set of polymorphic SRAP primers was selected to amplify DNA fragments, and the banding patterns were analysed to assess genetic similarity. Cluster analysis grouped the cultivars based on their genetic similarities, providing valuable insights into their genetic background and potential breeding applications. The study reveals the effectiveness of SRAP markers for the differentiation of Romanian blueberry cultivars, providing a dependable approach for cultivar identification, genetic conservation, and breeding initiatives. This research could be a valuable support for improved cultivars development.

Key words*: SRAP markers, genetic diversity, Vaccinium corymbosum, cultivar identification, molecular genotyping.*

INTRODUCTION

Blueberry (*Vaccinium corymbosum* L.) is known for its high nutritional value, antioxidant-rich fruit, and increasing consumer demand (Duan et al., 2022; Prior et al., 1998). Recently, blueberry cultivation has expanded significantly in Romania, due to favourable climatic conditions and growing market demand for fresh fruit consumption (Asănică et al., 2017). Recently, Hera (2024) published a study detailing the origin of the Romanian blueberry cultivars. However, the genetic background of many Romanian blueberry cultivars remains poorly characterized, which limits the effectiveness of breeding programs and germplasm conservation strategies.

Several molecular marker systems have been employed to study genetic variation within the genus *Vaccinium*, including Randomly Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR), and Simple Sequence Repeats (SSR) (Iordăchescu & Mihai, 2022). SRAP marker system is based on the amplification of open reading frames using primer pairs that contain the CCGG sequence in the forward primer and AATT sequence in the reverse primer, followed by 3 selective nucleotides at their 3' end (G. Li & Quiros, 2001). This simple system has been used for a variety of purposes, including mapping and gene tagging (G. Li & Quiros, 2001), assessment of genetic stability and somaclonal variation (Clapa et al., 2019; Ismail et al., 2022), phylogenetic studies (Ho et al., 2024), population structure analysis (Wang, 2020; Zhou et al., 2021), cultivar identification (Handa et al., 2015; Shamustakimova et al., 2021; Sun et al., 2016) and genetic diversity analysis (Y. Li et al., 2009; Szabo et al., 2021; Zagorcheva et al., 2024).

The use of SRAP markers in *Vaccinium* research is limited so far. Clapa et al. (2019) used RAPD and SRAP markers to check the genetic stability of micropropagated blackberry and blueberry (*V. corymbosum*) plants. In another study, SRAP markers and chloroplast *mat*K gene were used to determine the relationships and taxonomic status of intraspecific *Vaccinium uliginosum* L. germplasm resources located in the Changbai Mountain (Chen et al., 2022).

Present study we used SRAP markers to assess the relationships and genetic diversity among the nine Romanian blueberry cultivars. The goal of the study is to obtain data about the usefulness of SRAP markers in differentiating the cultivars and identifying genotypes that should be prioritized for conservation, as well as to

provide breeders with valuable information to be used in creating new, improved cultivars.

MATERIALS AND METHODS

Materials

The blueberry genotypes analysed in this study are the Romanian cultivars 'Lax', 'Prod', 'Vital', 'Azur', 'Simultan', 'Delicia', 'Pastel' and 'Augusta'.

Genomic DNA extraction

Genomic DNA was extracted from young leaves with the Innu PREP Plant DNA I KIT IPC 16 (Analytik Jena GmbH+Co, Jena, Germany). For the external lysis of the tissue approximately 100 mg fresh tissue was transferred to 1.5 ml tubes containing 600 µl lysis solution SLS and 20 µl proteinase K, and the tissue was disrupted with a micropestle. The samples were then incubated at 65°C for 1 hour, centrifuged at 14000 rpm for 5 min at room temperature to separate the lysed tissue, and supernatant transferred to prefilters fitted into collection tubes. The samples were centrifuges again for 2 min at 10000 x g, and 2 µl of RNase A (10 mg/ml) were added to the samples in the collection tubes, with a further 5 min incubation at room temperature. After external lysis, samples the transferred into the kit plaque and processed further in the InnuPure C16 instrument (Analitik Jena), using the Ext_Lysis_200_C16_04 program. DNA quality and quantity were evaluated with NanoDrop 1000 spectrophotometer (BioRad, California, USA). Genomic DNA integrity was checked electrophoresis on 1% agarose gel in 1xTAE buffer, and visualised with the Pharox FX system (BioRad, California, USA).

Polymerase Chain Reaction

Five primer pairs were used as SRAP markers: ME1-EM1, ME2-EM2, ME3-EM3, ME4-EM4, and ME5-EM5 (Table 1).

Annealing temperature optimization was performed for each pair of SRAP primers and the results compared to those obtained by standard amplification (G. Li & Quiros, 2001) modified to fit with the Platinum II Hot Start kit (Table 3). Once it was decided the optimum annealing temperature for each pair of SRAP primers, the reactions were done in triplicate and only bands present in all reactions for each SRAP marker were scored.

Table 1. SRAP Markers

SRAP Marker	Primers	DNA Sequence (5'- 3')
ME1-EM1	ME1	TAGGTCCAAACCGGAAG
	EM1	GACTGCGTACGAATTAAT
ME2-EM2	ME2	TGAGTCCAAACCGGACA
	EM2	GACTGCGTACGAATTACA
ME3-EM3	ME3	TGAGTCCAAACCGGACT
	EM3	GACTGCGTAGGAATTACT
ME4-EM4	ME4	TGAGTCCAAACCGGAGA
	EM4	GACTGCGTACGAATTACA
ME5-EM5	ME5	TAGGTCCAAACCGGATC
	EM5	GACTGCGTACGAATTATC

PCR reactions were performed using the Platinum II Hot Start kit (Invitrogen) according to the manufacturer instructions. PCR setup was done according to Table 2.

Table 2. PCR reaction setup

Component	Volume	Final Concentration
Platinum™ II HotStart PCR Master Mix (2x)	5.0 µl	1x
10 µM forward primer	0.2 µl	0.2 µM
10 µM reverse primer	0.2 µl	0.2 µM
10 ng/µl template DNA	2.0 µl	2 ng/µl
Nuclease-free water	2.6 µl	-
Total	10.0 µl	-

Table 3. PCR cycling conditions

	Temperature	Time	Cycles
Standard SRAP amplification	94°C	2 min	1
	94°C	15 sec	5
	35°C	1 min	
	68°C	1 min	
	94°C	15 sec	30
	50°C	1 min	
	68°C	1 min	
	68°C	10 min	1
	4°C	HOLD	
Annealing temperature optimization	94°C	2 min	1
	94°C	15 sec	30
	47-57°C	1 min	
	68°C	1 min	
	68°C	1 min	1
	68°C	10 min	1
	4°C	HOLD	

Data Analysis

Polymorphism levels of SRAP markers were assayed based on the percentage of polymorphic loci, cumulative Shannon's information index, and polymorphism information content (PIC). Percentage of polymorphic loci was calculated using the formula:

$$Percentage\ of\ Polymorphic\ loci = \frac{Nr.\ of\ polymorphic\ loci}{Total\ nr.\ of\ loci} \times 100$$

Shannon information index, that quantifies the diversity in the presence/absence of a DNA fragment across genotypes was calculated for

each locus using the formula $H = -(p \cdot log_2(p) + (1 - p) \cdot log_2(1 - p)$, where p represents the frequency of band presence and 1-p represents the frequency of band absence at a certain locus.

Polymorphism information content (PIC) was calculated using the formula $PIC = 2pq = 2p(1-p)$ (Serrote et al., 2020), where p represents the frequency of band presence and $q=1-p$ represents the frequency of band absence at a certain locus. Amplified DNA was scored as present (1) or absent (0) in a *.csv file as a binary matrix. Data was then analysed with BIO-R software, Biodiversity Analysis with R for Windows, version 3.2, Centro Internacional de Mejoramiento de Maíz y Trigo (CIMMYT) and Microsoft® Excel® for Microsoft 365 MSO (Version 2406 Build 16.0.17726.20078) 64-bit.

RESULTS AND DISCUSSIONS

Annealing temperature optimization

Following the comparison of the results of the standard SRAP amplification and annealing temperature optimization reactions it was decided that for the ME1-EM1 and ME2-EM2 it will be used a marker specific annealing temperature (46°C and 48°C) and for the ME3-EM3, ME4-EM4, and ME5-EM5 markers will be used the standard SRAP amplification parameters.

SRAP markers assessment

The SRAP markers amplified a total of 59 loci, out of which 37 were polymorphic (Table 4).

Table 4. SRAP markers efficiency

SRAP Marker	Total loci	Polymorphic loci	Percentage of polymorphism	Cumulative Shannon Index	Mean PIC across all loci	Mean PIC across polymorphic loci only
ME1-EM1	7	4	57.14	2.07	0.20	0.35
ME2-EM2	10	8	80.00	4.43	0.30	0.37
ME3-EM3	17	10	58.82	5.39	0.21	0.36
ME4-EM4	11	6	54.55	3.73	0.24	0.44
ME5-EM5	14	9	64.29	4.60	0.22	0.33

The number of total loci ranged from 7 (ME1-EM1) to 17 (ME3-EM3), whereas the number of polymorphic loci varied from 4 (ME1-EM1) to 10 (ME3-EM3). The polymorphism was above 50% for all markers, with the highest percentage of polymorphism (80%) detected for the marker ME2-EM2. ME1-EM1 marker has the lowest percentage of polymorphism (57.14%) and the lowest value of the cumulative Shannon index (2.07), which indicates that it has a limited power to detect polymorphism. On the other hand, ME3-EM3 marker displayed the highest degree of polymorphism, the highest cumulative Shannon index (5.39), and it amplified DNA on the highest number of total loci.

PIC values for dominant markers, such as SRAPs, cannot be higher than 0.5 when using the $PIC = 2pq = 2p(1-p)$ formula, when the allele frequencies are equal (p=q=0.5). In this case, Serrote et al. (2020) proposed for dominant markers informativeness the following ranges: low (0.00-0.10), medium (0.10-0.25), high (0.30-0.40) and very high (0.40-0.50). For the markers studied here, mean PIC values across all loci ranged between 0.20 (ME1-EM1) and 0.30 (ME2-EM2), indicating a medium to high informativeness for all markers. However, if only polymorphic loci are taken into consideration, the PIC values increase, ranging from 0.33 (ME5-EM5) to 0.44 (ME4-EM4), high to very high informativeness.

When looking at the total polymorphism, Shannon diversity index, and PIC values, ME2-EM2 appeared to be the best informative marker, with the polymorphism (80.00%), the highest mean PIC across all loci (0.30), and a strong cumulative Shannon Index (4.43), making it ideal for broad diversity analysis and germplasm differentiation. The ME3-EM3 marker, has slightly lower polymorphism (58.82%) and moderate PIC values, but the highest cumulative Shannon Index (5.39), and amplified the greatest number of loci (17). Therefore, this marker can be used successfully for overall genetic diversity assessment. The ME4-EM4 marker produced fewer polymorphic loci (6) and had a moderate Shannon Index (3.73), but showed the highest PIC value across polymorphic loci (0.44). This indicates that this marker can be used for studies requiring precise identification of cultivars.

Genetic diversity analysis

One of the ways to estimate the genetic difference between two genotypes is to calculate Rogers' genetic distances (Mohammadi & Prasanna, 2003). Analysis of the binary data with the Bio-R software generated the Rogers' distances between the blueberry genotypes (Table 5).

Table 5. Rogers distances between blueberry genotypes

NAME	gLax	gProd	gVital	gAzur	gSimultan	gDelicia	gPastel	gSafir	gAugusta
1. gLax	0	0.69749	0.69749	0.69749	0.36761	0.73521	0.56949	0.67783	0.56949
2. gProd	0.69749	0	0.51988	0.69749	0.67783	0.69749	0.73521	0.54525	0.56949
3. gVital	0.69749	0.51988	0	0.61512	0.67783	0.6576	0.61512	0.63671	0.61512
4. gAzur	0.69749	0.69749	0.61512	0	0.63671	0.6576	0.6576	0.59275	0.61512
5. gSimultan	0.36761	0.67783	0.67783	0.63671	0	0.63671	0.59275	0.61512	0.59275
6. gDelicia	0.73521	0.69749	0.6576	0.6576	0.63671	0	0.80539	0.59275	0.61512
7. gPastel	0.56949	0.73521	0.61512	0.6576	0.59275	0.80539	0	0.78843	0.6576
8. gSafir	0.67783	0.54525	0.63671	0.59275	0.61512	0.59275	0.78843	0	0.63671
9. gAugusta	0.56949	0.56949	0.61512	0.61512	0.59275	0.61512	0.6576	0.63671	0

Colour scale ranges from blue (low distance, genetically similar genotypes) to red (high distance, genetically distant genotypes).

Rogers distance values among the cultivars ranged from 0.3676 (between 'Simultan' and 'Lax') to 0.8054 (between 'Pastel' and 'Delicia'), identifying closely related as well as distantly related genotypes.

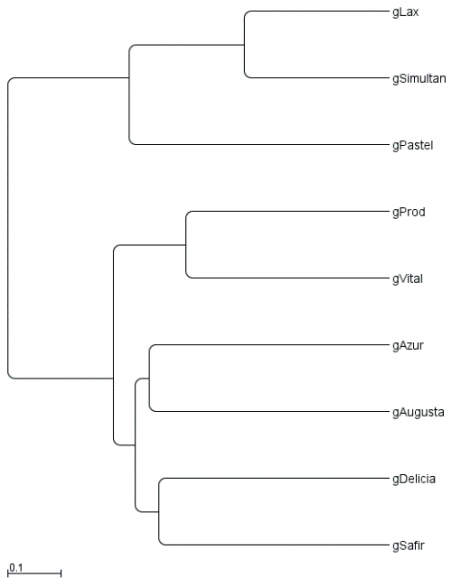

Figure 1. Hierarchical clustering dendrogram based on Rogers' distances, generated by the Bio-R software

A dendrogram based on the Rogers' distances is presented in Figure 1. The genotypes are grouped into two main clusters, the first cluster with the 'Lax', 'Simultan', and 'Pastel' cultivars, and the second cluster with the rest of the cultivars. The low genetic distance between 'Simultan' and 'Lax' indicates that the two genotypes are closely related. Besides this pair, in the second cluster, 'Prod' is grouped with 'Vital', 'Azur' with 'Augusta', and 'Delicia' with 'Safir', indicating close relationships between the pairs. Indeed, 'Lax', 'Simultan', and 'Pastel' were obtained by open pollination of 'Spartan' cultivar, and 'Azur' and 'Augusta' from a cross between 'Berkeley' and 'Bluecrop' cultivars (Hera, 2024).

2D principal component analysis (PCA) results are presented in Figure 2, illustrating the relationships between the blueberry cultivars as well. CP1 component explains 27.6% of the genetic variation and separates the 2 clusters from the dendrogram – on the right side the first cluster, and on the left side the second cluster. CP2 component explains 19.4% of the genetic variation, further grouping the blueberry cultivars.

'Pastel' cultivar, is the most genetically dissimilar, when compared to the other cultivars.

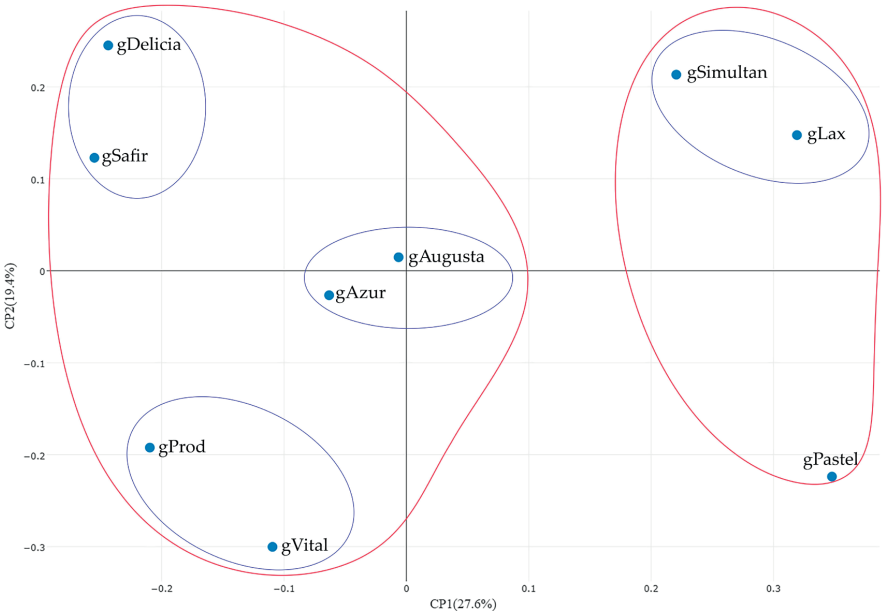

Figure 2. 2D PCA plot based on Rogers' distances, generated by the Bio-R software. Pairs of genotypes from the dendrogram are circled with blue, and the 2 clusters are circles with red.

This cultivar has some alleles that are present in few other genotypes, and should be conserved to preserve the genetic diversity, since such diversity is essential for the development of new improved cultivars that need to cope with the changing environment and answer the customers everchanging demands (Salgotra & Chauhan, 2023).

CONCLUSIONS

The five SRAP markers used were effective in differentiating the nine Romanian blueberry cultivars and revealed high levels of polymorphism (54-80%), and high genetic variation among cultivars. Among them, ME2-EM2 and ME4-EM4 markers are the most suitable for cultivar identification, due to their high degree of polymorphism and high PIC values. In terms of breeding, the same markers should be used to select genitors for crosses to introduce new traits. As the marker ME3-EM3 has the highest cumulative Shannon index, hence capturing the most diversity, it should be used for genetic conservation studies by monitoring diversity over time, and safeguarding against genetic loss.

The nine Romanian cultivars were grouped into 2 main clusters, and further into pairs, except for the cultivar 'Pastel', that has the most genetically diverse genotype, making it a good candidate for breeding programs as a genitor, and a priority for conservation.

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