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GENETIC VARIABILITY STUDY OF SEVERAL ROMANIAN BLUEBERRY CULTIVARS USING ISSR MOLECULAR MARKERS

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Abstract

High-bush blueberry is an important economic and nutraceutical species, due to its high content of anthocyanins and high antioxidant activity. Nowadays, climate changes are affecting plant growth and development, leading to changes in plants' adaptation to new environmental conditions. Consequently, the need to create new varieties and hybrids to cope with changes and meet the growing demands of consumers is imperative. One way to hasten plant breeding is to employ molecular methods, such as ISSR. In the present study, five ISSR molecular markers were used to study the genetic relationships between seven Romanian blueberry cultivars and one hybrid. The dendrogram obtained following ISSR analysis revealed the presence of two clusters, one cluster containing 'Lax', 'Prod', 'Vital', 'Azur', and the second one 'Simultan', 'Delicia', 'Compact', and the hybrid obtained through free pollination of variety 'Compact'. The shortest genetic distance was noted between the hybrid genotype and the 'Compact' cultivar. The longest genetic distance was noted between the varieties 'Compact' and 'Prod'. The study also revealed common markers for the hybrid studied and its maternal genitor, that could be used as markers in blueberry breeding.

***Key words**: Vaccinium corymbosum L., microsatellites, blueberry breeding, genetic variation.*

INTRODUCTION

Blueberries are very much appreciated fruits in today's culture, not only for their taste and flavour, but also for their nutraceutical qualities (Golovinskaia & Wang, 2021).
Consumed fresh or preserved by freezing, drying, or as gems and compotes, cooked in desserts or consumed for breakfast in yogurts, shakes and juices, they are part of people's diet, including those suffering of diseases such as diabetes or high blood pressure (Afrin et al., 2016; Bouyahya et al., 2022; Hameed et al., 2020; Wang et al., 2021). The demand for good looking and pleasant tasting fruits is linked with the growing demand for high fruit content of anthocyanins from the consumer side, and with the demand of resistance to biotic and abiotic stress from the growers' side (Asănică, 2018).
Consequently, the need for new cultivars answering all these demands is of high importance, and it translates into the plant breeders need for a wide range of genetically diverse genitors to be involved in obtaining novel varieties in shorten time.
Molecular biology techniques can supply at least partially the answers to these demands, as molecular markers can assess not only the genetic diversity across the range of genotypes available to plant breeders, but also label certain genes of interest for fruit quality or for cultivar resistance to stress, and hasten the time needed to check if the traits of interest have been transferred to the prospective new varieties (Debnath & An, 2019; Edger et al., 2022; Grover & Sharma, 2016; Iwata et al., 2016; Lobos & Hancock, 2015; Nadeem et al., 2018).
ISSR (Inter simple sequence repeat) technique takes advantage of the presence in the genomes of microsatellites or simple sequence repeats (SSRs). SSRs are regions that consist of short DNA sequences, two to five nucleotides in size, repeated in tandem multiple times (Gupta et al., 1996). The ISSR technique uses a single primer in the PCR reaction, its sequence based on the microsatellite sequence, to amplify the region

between two identical microsatellites. Since the sequences amplified may or may not be conserved, the technique is useful in studies such as genetic diversity assessment and gene mapping (Pradeep Reddy et al., 2002).
Microsatellite molecular markers have already been mined and developed in *V. macrocarpon* Ait. (Schlautman et al., 2015; Xu et al., 2021; Zhu et al., 2012), and have been used to study genetic diversity in various blueberry populations (Bhatt & Debnath, 2021; Rodriguez-Bonilla et al., 2020; Vega-Polo et al., 2020).
Present study analyses the genetic diversity of eight Romanian blueberry genotypes using ISSR markers, as well as the inheritance of genetic material from the maternal genitor 'Compact' to a hybrid obtained through free pollination, demonstrating the utility of the technique to plant breeders.

MATERIALS AND METHODS

Materials

Eight blueberry (*Vaccinium corymbosum* L.) genotypes, seven Romanian cultivars ('Lax', 'Prod', 'Vital', 'Azur', 'Simultan', 'Delicia', and 'Compact') and a hybrid resulted by free pollination of the cultivar 'Compact', were analyzed in the present study.

Genomic DNA extraction

Extraction of genomic DNA from young leaves was performed using the Innu PREP Plant DNA I KIT IPC 16 (Analytik Jena GmbH+Co, Jena, Germany) according to the manufacturer instructions. Frozen tissue was grounded to powder with liquid nitrogen.
Briefly, for each sample, approximately 100 mg of powder was transferred to 1.5 ml tubes, and then 600 µl lysis solution SLS and 20 µl proteinase K were added to the sample. Thereafter, the samples were incubated for 1 hour at 65°C, centrifuged for 5 min at 10000 x g, and then the supernatant was transferred to prefilters fitted to collection tubes. After an additional centrifugation for 2 min at 10000 x g, 2 µl of RNase A (10 mg/ml) were added and samples were incubated for 5 min at room temperature. After external lysis, samples were further processed in the InnuPure C16 (Analitik Jena), using the Ext_Lysis_200_C16_04 program. DNA quality and quantity were checked with Nanodrop 1000 (Biorad).

ISSR reactions

PCR reactions were performed using the Platinum II Hot Start kit (Invitrogen) according to the manufacturer instructions. PCR setup was done according to Table 1.

Table 1. ISSR reaction setup

Component	Volume	Final concentration
Nuclease-free water	0.2 µl	-
Platinum™ II Hot-Start PCR Master Mix (2x)	5 µl	1x
10 µM Primer P59	0.3 µl	0.3 µM
10 ng/µl Template DNA	2.5 µl	2.5 ng/µl
Platinum GC Enhancer	2 µl	-
Total	10 µl	-

Annealing temperature optimization (between 30°C and 35°C) was done for all primers. PCR program consisted of an initial denaturation step of 2 min at 94°C, followed by 35 cycles of denaturation 15 sec at 94°C, annealing 15 sec at 48/51°C (depending on the primer), and extension 1 min at 68°C, and a final extension step of 2 min at 68°C. The nucleotide sequences of the primers used are presented in Table 2.

Table 2. ISSR primers nucleotide sequences

Primers	Nucleotide sequence
ISSR 2	5'- ACACACACACACACACG-3'
ISSR 3	5'- CTCTCTCTCTCTCTCTRC-3'
ISSR 5	5'- CACACACACACACACARG-3'
ISSR 8	5'-GAAGAAGAAGAAGAAGAA-3'
ISSR 9	5'-ATGATGATGATGATGATG-3'

DNA fragments amplified in the ISSR reactions were separated on 1.5% agarose gel, visualized with the Pharox FX system (BioRad, California, USA), and band lengths were measured using the Quantity One software (Version 4.6.9., BioRad, California, USA).

Data analysis

Data were analysed with BIO-R software, Biodiversity Analysis with R for Windows, (version 3.0, BioRad, California, USA). Amplicons were scored as present (1) or absent (0) as a binary matrix in a *.csv file.

The Resolving Power of a primer (Rp) was calculated with the formula $\sum I_b$, $I_b = 1 - [2(0.5 - p_i)]$, where I_b = band informativeness, and p_i is the proportion of the genotypes contain-ning the band (Prevost & Wilkinson, 1999).

RESULTS AND DISCUSSIONS

All primers used proved to be polymorphic, demonstrating on one hand their suitability for this type of analysis, and on the other hand the variability of the genotypes under study. Out of the five ISSR primers used, three are based on dinucleotide repeats (ISSR2, ISSR3, and ISSR5) and two are based on trinucleotide repeats (ISSR 8 and ISSR 9) (Table 2).

Primer ISSR 2 amplified the 1370 bp band only in the 'Compact' variety, the 740 bp band only in the 'Vital' variety, the 650 bp band only in the 'Delicia' variety, and the 460 bp band only in the 'Azur' variety. With the primer ISSR 3, the 1490 bp and 430 bp bands were specific for the 'Lax' variety, the 1630 bp band was specific for the 'Prod' variety, the 1600 bp band was specific for the 'Vital' variety, the 340 bp band was specific for the 'Azur' variety,

Since one of the genotypes studied is a hybrid obtained by free pollination of 'Compact' variety, the amplified fragments with the same sizes for both hybrid and its maternal genitor were noted, as these markers could be inherited from the parent genotype. A total of 14 bands were common between the hybrid studied and its maternal genitor, 4 for ISSR 2 (940 bp, 790 bp, 600 bp, and 520 bp), 3 for ISSR 3 (960 bp, 800 bp, and 540 bp), 1 for ISSR 5 (700 bp), 4 for ISSR 8 (2250 bp, 1300 bp, 680 bp, and 570 bp), and 2 for ISSR 9 (2300 bp and 1700 bp) (Table 3). However, two of the amplified fragments, with lengths of 790 bp amplified with ISSR 2, and 680 bp amplified with ISSR 8, should not be taken into consideration, as they are monomorphic among all genotypes studied. The rest of the common amplified fragments are potentially inherited from the maternal genitor and should be sequenced, and their location identified within the genome, to check if they are within regions that contain genes of interest for resistance to biotic/abiotic stress or genes of interest related to fruit quality and could be used as potential markers to select for/against traits of interest by the plant breeders.

Table 3. The number of polymorphic and monomorphic loci corresponding to each ISSR primer used

Primer	Number of polymorphic loci	Number of monomorphic loci	Amplified fragments sizes (bp)*	Resolving power
ISSR 2	16	1	1370, 1360, 1320, 1080, 1050, 1020, 940, 890, **790**, 740, 670, 650, 620, 600, 520, 500, 460	8.50
ISSR 3	22	0	1630, 1600, 1490, 1440, 1200, 1170, 1150, 1000, 960, 880, 860, 800, 750, 740, 710, 605, 540, 430, 410, 380, 340	11.75
ISSR 5	21	0	2160, 2110, 2060, 1860, 1520, 1570, 1400, 1370, 1340, 1240, 1200, 1180, 1080, 1040, 1000, 750, 720, 700, 600, 570, 550	12.25
ISSR 8	28	1	2570, 2500, 2350, 2250, 2100, 2000, 1840, 1780, 1670, 1590, 1560, 1470, 1300, 1250, 1110, 950, 860, 800, **680**, 640, 580, 570, 560, 420, 410, 300, 290	14.50
ISSR 9	15	0	2440, 2400, 2300, 2180, 1800, 1700, 1610, 1500, 1340, 1050, 800, 770, 540, 530, 500	7.25

*Fragments written with bold letters represent monomorphic loci. Fragments underlined represent common markers for the hybrid studied and its maternal genitor, the 'Compact' cultivar.

The genetic variability of the genotypes under study is reflected into the calculated Rogers' genetic distances visible in Table 4. As expected, the shortest genetic distance was noted between the hybrid genotype and its maternal genitor, the 'Compact' cultivar (0.52).

The longest genetic distance was noted between the cultivars ‘Compact’ and ‘Prod’ (0.72). These results are also apparent in the dendrogram presented in Figure 1.
The UPGMA dendrogram is grouping the eight genotypes studied into two clusters, and in each cluster, the genotypes are grouped two by two. In the first cluster, ‘Lax’ is coupled with ‘Prod’, and ‘Vital’ with ‘Azur’, whereas in the second cluster, ‘Simultan’ is coupled with ‘Delicia’ and the hybrid with its maternal genitor, ‘Compact’.

Table 4. Rogers’ genetic distances calculated with the Bio-R software

Cultivar	Lax	Prod	Vital	Azur	Simultan	Delicia	Compact	Hybrid
Lax	0.00	0.64	0.67	0.69	0.65	0.63	0.66	0.57
Prod	0.64	0.00	0.63	0.66	0.69	0.68	0.72	0.62
Vital	0.67	0.63	0.00	0.60	0.65	0.71	0.66	0.68
Azur	0.69	0.66	0.60	0.00	0.61	0.64	0.70	0.61
Simultan	0.65	0.69	0.65	0.61	0.00	0.58	0.59	0.59
Delicia	0.63	0.68	0.71	0.64	0.58	0.00	0.59	0.54
Compact	0.66	0.72	0.66	0.70	0.59	0.59	0.00	0.52
Hybrid	0.57	0.62	0.68	0.61	0.59	0.54	0.52	0.00

The shortest Roger’s genetic distance between varieties is marked with green, and the longest genetic distance is marked with blue.

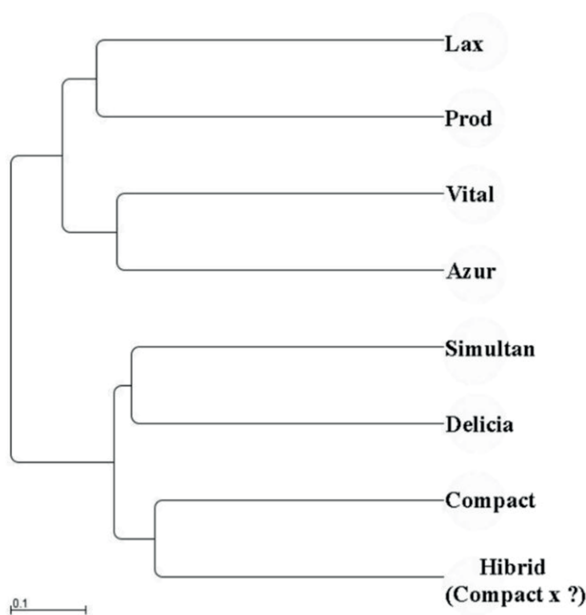

Figure 1. Dendrogram based on the ISSR data generated with the Bio-R software

CONCLUSIONS

Five ISSR molecular markers were used to assess the genetic variability among eight blueberry genotypes, the shortest genetic distance being observed between the hybrid genotype and its maternal genitor, the 'Compact' variety, and the longest genetic distance, observed between the cultivars 'Prod' and 'Compact'.

Twelve amplified DNA fragments were identified in the hybrid under study as regions potentially inherited from the maternal genitor, 'Compact', fragments that should be assessed in a future project for becoming putative markers for traits of interest.

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