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ANALIZA DIVERSITĂȚII GENETICE LA UNELE SOIURI DE MĂR FOLOSIND MARKERI ISSR

GENETIC DIVERSITY ANALYSIS AMONG SEVERAL APPLE GENOTYPES USING ISSR MARKERS

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Abstract

Apple *(Malus domestica* Borkh.) is one of the most cultivated fruit trees species worldwide, its fruits being consumed not only for their organoleptic attributes, but also for their nutraceutical properties. Therefore, the genetic variability of the species is extremely important to insure a large enough pool of cultivars to accommodate consumer demands for various fruit traits, such as taste, flavor, color, shape, etc., as well as to preserve cultivars that have less desirable organoleptic properties but are resistant/tolerant to biotic and/or abiotic stress and could be good genitors for these traits. The current study presents the use of ISSR method to assess the genetic variability among seven Romanian apple cultivars from the orchard collection of University of Agronomic Sciences and Veterinary Medicine of Bucharest, Romania: 'Florina', 'Remar', 'Ciprian', 'Iris', 'Rebra', 'Generos', and 'Redix'. In addition, the method proved to be useful in identifying closely related individual genotypes, allowing the identification and elimination of duplicates from collections, without compromising the collection's genetic variability.

Cuvinte cheie: *Malus domestica* Borkh., variabilitate genetică, markeri moleculari, microsateliți.
Key words: *Malus domestica* Borkh., genetic variability, molecular markers, microsatellites.

1. Introduction

Apple (*Malus domestica* Borkh.) is a fruit trees species important for human diet, its fruits being consumed fresh but also processed (Gardiner et al., 2007, Badulescu et.al., 2019). Consumer preferences for fruit traits such as color, shape, taste, and flavor are changing overtime, so new cultivars are needed to accommodate the consumers' demands (Volz et al., 2007; Yue and Tong, 2011; Butac and Militaru, 2017). Moreover, such organoleptic traits and genes coding for resistance to various biotic and abiotic stresses are often linked in the genomes to other less desirable traits (Gianfranceschi et al., 1996). Therefore, a wide pool of genotypes with good genetic variability for all existing traits is very important to ensure enough diverse genitors for plant breeders to create novel cultivars with desirable qualities for both consumers and farmers.

Even though apple is extensively cultivated in Romania, the genetic variability of Romanian genotypes is low, creating problems especially with respect to apple scab resistance (Militaru et al., 2019). Accurate genotype identification methods are needed to select genotypes that will be used in breeding programs to create new apple varieties. The classical methods of characterization and identification of genotypes present in collections are mainly based on the observation of morphological characters, which can be influenced by environmental factors or location (Harris et al., 2002).

The characteristics of a good and very useful DNA marker are that the marker is ubiquitous and evenly distributed throughout the genome, highly polymorphic, co-dominant in expression to enable effective discrimination between homozygotes and heterozygotes, ideally genome-specific in nature, and multi-functional (Amiteye, 2021). Inter simple sequence repeats (ISSR) are genomic sections flanked by simple sequence repeat or microsatellite sequences. Microsatellite sequences are genomic regions that consist of simple sequence motifs of short DNA sequences - usually di-, tri- and tetra- or penta-nucleotides in size - repeated multiple times in tandem.

Molecular markers, such as those based on microsatellites, can be used not only to identify genotypes at the molecular level, but also to determine genetic variability at the DNA level (Udriște & Bădulescu, 2019). For instance, SSR markers have been used to determine genetic variability in Swedish heirloom cultivars (Garkava-Gustavsson et al., 2013), local cultivars in Northeastern Spain (Pina et al., 2014), Morocco (Khachtib et al., 2022), Turkey – Anatolia (Öz et al., 2020), and apple heirlooms in Italy (Testolin et al., 2019). ISSR markers have been used as well for determining the genetic diversity in Brazilian apple collection with low chilling requirements (Mariano et al., 2019), in Turkey and foreign

cultivars (Udun et al., 2016), in some western Romanian cultivars (Giancarla et al., 2021), and in Portuguese local cultivars (Ferreira et al., 2012).

In the present study, four ISSR markers were used to observe the genetic variability among seven apple genotypes present in the orchard collection of the USAMV in Bucharest and to analyze the possibility of using the method for the identification of genotypes by molecular methods.

2. Material and methods

2.1. Materials

In the current study were analyzed seven cultivars present in the collection of University of Agronomic Sciences and Veterinary Medicine of Bucharest, Romania: 'Florina', 'Remar', 'Ciprian', 'Iris', 'Rebra', 'Generos', and 'Redix'.

2.2. Methods

Genomic DNA extraction

Young leaves were used for the extraction of genomic DNA. Tissue frozen previously at -70°C was grounded to a fine powder with liquid nitrogen. The extraction was performed with InnuPREP Plant DNA I KIT IPC 16 (Analytik Jena), according to the manufacturer's instructions. Briefly, for each sample, approximately 150 mg of powder was transferred to 1.5 ml tubes, and 600 µl lysis solution SLS and 20 µl Proteinase K. The tubes were incubated for 1 hour at 65°C, then centrifuged for 5 min at 10000 x g, and the supernatant transferred to prefilters. After another 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 this step of external lysis, samples were transferred to the reagent plates and into the InnuPure C16 (Analitik Jena) apparatus for automatic genomic DNA extraction, using the Ext_Lysis_200_C16_04 program.

Genomic DNA quantity and quality were determined with NanoDrop 1000 spectrophotometer.

Genetic diversity analysis using ISSR molecular markers

PCR reactions were done using the Invitrogen™ Platinum™ II Hot-Start Master Mix, in a total volume of 10 µl. The reaction contained 5 µl of Invitrogen Platinum II Hot-Start PCR Master Mix (2X), 2 µl Platinum™ GC Enhancer, 0.3 µl 10 ng/µl ISSR primer, and 2.5 µl 10 ng/µl genomic ADN. 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 30 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 (Fig. 1)..

PCR products amplified in the ISSR reactions were separated on 1.5% agarose gel (in 1 x TAE buffer), visualized using the Pharox FX system (BioRad). DNA amplicons were measured using the Quantity One software (Version 4.6.9., BioRad) (Figure 1). Four ISSR primers (Table 1) were used for the genetic diversity analysis. Amplicons were marked as present (1) or absent (0) as a binary matrix in a *.csv file. Data were analyzed with BIO-R software (Biodiversity Analysis with R for Windows), version 3.0.

3. Results and discussions

All ISSR primers used were polymorphic. The DNA amplicons had lengths between 310 and 3300 bp, and a total number of 62 loci, each amplicon with a different length being considered a locus. Only 11 loci were monomorphic. The electrophoretic profiles for each genotype were different and could be used as a fingerprint for genotype identification.

In the dendrogram generated by the Bio R software (Fig. 2), the genotypes were split into two clusters. One cluster included cultivars 'Remar' and 'Iris', and the second cluster included cultivars 'Florina', 'Ciprian', 'Rebra', 'Generos', and 'Redix'.

These data confirm the fact the genotypes are genetically different, the closest being 'Remar' and 'Iris' which were obtained by seed irradiation from 'Prima' cultivar. Except for these two cultivars, the rest of the genotypes have various genitors (Table 2). In the second cluster the closest cultivars are 'Ciprian' and 'Generos'.

Genetic similarity or dissimilarity is apparent also from the Roger's Genetic Distance Matrix (Table 3), where the lowest value (0.46442), highlighted with yellow, depicts the most similar pair of cultivars, 'Iris' and 'Remar', and the highest value (0.76696), highlighted with blue, indicates the most dissimilar pair, 'Rebra' and 'Florina'.

Molecular markers have already been used to detect the presence of genes linked to presence of various genes of interest. For instance, SCAR markers were used to screen for the genes linked to resistance to *Venturia inequalis* in multiple apple cultivars (Patrascu et al., 2006; Patochi et al., 2009; Militaru et al., 2020). In future studies remains to be seen after the sequencing of DNA fragments from loci specific to certain cultivars, if some markers could be linked to useful traits that may be used in

marker assisted plant breeding to create novel cultivars with improved organoleptic qualities and better biotic and abiotic stress resistance.

4. Conclusions

All used primers are polymorphic.

Among the 62 identified loci, only 11 are monomorphic.

Cultivars 'Remar and 'Iris' are grouped apart from the rest of the cultivars, confirming that they are genetically similar, being created from the same genitor by seed irradiation.

In the second group in the dendrogram the closest cultivars are 'Ciprian' and 'Generos'.

The ISSR markers could be used for cultivars' molecular identification, allowing for duplicates elimination without reducing genetic variability in orchard collections.

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Tables and Figures

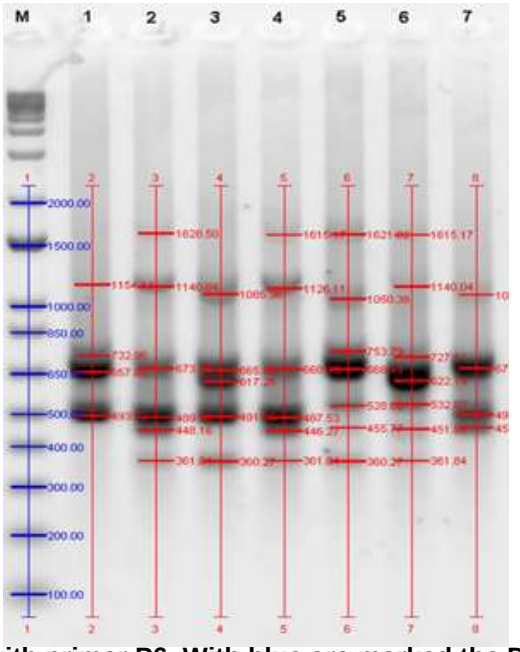

Fig. 1. PCR reaction with primer P6. With blue are marked the DNA fragments lengths of the marker 1 Kb Plus DNA ladder (M). With red are marker the lengths of PCR amplicons, measured with the software Quantity One. M. 1 Kb Plus DNA Ladder, 1. 'Florina', 2. 'Remar', 3. 'Ciprian', 4. 'Iris', 5. 'Rebra', 6. 'Generos', and 7. 'Redix'

Table 1. ISSR primers used in the study

ISSR Primers	DNA Sequence
P3	5'- $(AGC)_5GA$-3'
P4	5'- $(GT)_8TC$- 3'
P5	5'- $(TC)_8C$ -3'
P6	5'- $(CA)_8GC$ -3'

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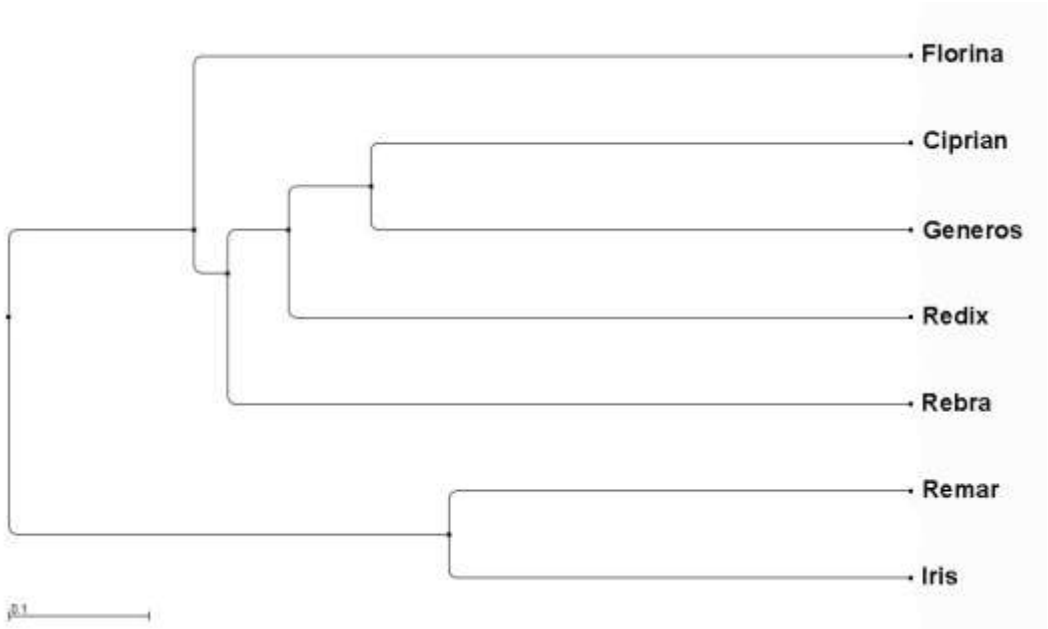

Fig. 2. Dendrogram based on the ISSR data generated with Bio-R software.

Table 2. Genitors of the genotypes in the study. *

Genotip	Genitori
Ciprian	Prima x Starkrimson
Florina	*M.floribunda* 821, Rome Beauty, Golden Delicious, Starking Simpson'S Giant Limb, Jonathan
Generos	(Parmain d'or x *M. kaido*) x (Jonathan x Frumos de Voinești)
Iris	Prima (iradierea semințelor)
Rebra	Florina x Idared
Redix	Goldspur x Prima (polenizare liberă)
Remar	Prima (polenizare liberă, iradierea semințelor)

*Preda et al., 2020

Table 3. Pair wise genetic dissimilarity estimates (Roger's Distance) among seven genotypes based on ISSR markers. The shortest genetic distance is highlighted with yellow, and the longest genetic distance is highlighted with blue.

Name	gFlorina	gRemar	gCiprian	gIris	gRebra	gGeneros	gRedix
gFlorina	0	0.70014	0.65679	0.71401	0.76696	0.64169	0.64169
gRemar	0.70014	0	0.64169	0.46442	0.70014	0.68599	0.71401
gCiprian	0.65679	0.64169	0	0.68599	0.62622	0.54233	0.57735
gIris	0.71401	0.46442	0.68599	0	0.68599	0.67155	0.70014
gRebra	0.76696	0.70014	0.62622	0.68599	0	0.61037	0.70014
gGeneros	0.64169	0.68599	0.54233	0.67155	0.61037	0	0.62622
gRedix	0.64169	0.71401	0.57735	0.70014	0.70014	0.62622	0