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GENETIC RELATIONSHIPS BETWEEN SEVERAL ROMANIAN PLUM VARIETIES USING RAPD MOLECULAR MARKERS

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Abstract

Plum (Prunus domestica L.) is the dominant fruit tree species in Romania and has an ancient tradition in growing all over the country. The assortment of the plum varieties has known a continuous increase in time, enhancing its genetic variability. In the current work, RAPD analysis was carried out in order to assess the genetic relationships between seven Romanian plum cultivars ('Brumării de Voinești', Record', 'Gemenea', 'Elena', 'Centenar', 'Silvia', and 'Pescăruș') existent in the germplasm collection of the Faculty of Horticulture, USAMV Bucharest, Romania. Five random decamer primers identified 28 polymorphic and 11 monomorphic RAPD loci. The constructed UPGMA dendrogram associated the cultivars studied into 2 clusters, one cluster with the varieties 'Record' and 'Gemenea', and another cluster grouping the rest of the varieties. The genetic fingerprints obtained following amplification with the RAPD markers, are specific for each variety, and may be used for molecular identification of the varieties in the germplasm collection.

Key words*: European plum, genetic variability, plum breeding, plum germplasm, genetic fingerprint.*

INTRODUCTION

European plum, (*Prunus domestica* L.), a hexaploid species (2n = 48), is the dominant fruit tree species in Romania and has an ancient tradition in growing all over the country. By the end of 2021, Romania ranked 6th in the European Search Catalogue for Plant Genetic Resources (EURISCO) Catalogue, based on the existing number of European plum accessions, after Switzerland, Russian Federation, Spain, Germany and United Kingdom (Sottile et al., 2022). In an effort to identify, conserve and evaluate plum genetic resources in Romania, two research centers host plum germplasm collections with over 1000 accessions: wild species, local populations, named cultivars, breeder's selections and rootstocks (Butac et al., 2019).

Current climate changes, resulting especially in drought and salinization of farmlands, are calling for development of new cultivars that are drought and salt resistant through faster breeding using molecular methods to assist classical breeding (Vicente, 2022).

Recent developments in sequencing techniques have made whole genome sequencing more affordable, but still there are challenges to be met for polyploid species. Since the European plum is a hexaploidy species, in the National Center for Biotechnology Information (NCBI) database no genome or genome assembly has been deposited as yet, and there are only 130 genes registered for *Prunus domestica* L. A draft genome assembly is available on Genome Database for Rosaceae (GDR) database (Callahan et al., 2021), so in the future more specific molecular markers such as Simple Sequence Repeats (SSRs) can be discovered. However, the assessment of genetic diversity can still be done with Random Amplification of Polymorphic DNA (RAPD) molecular markers (Iordachescu et al., 2021; Udriște & Bădulescu, 2019).

RAPD is a PCR technique developed in 1990 (Williams et al., 1990), that uses a single random decamer primer that will anneal to both DNA strands, and will amplify the sequences between the annealing sites (Babu et al., 2021). RAPD technique has multiple applications

besides the study of genetic diversity among cultivars, such as checking the genetic stability of plants grown in vitro, study of genetic relationships among cultivars, and cultivar identification within collections (Athanasiadis et al., 2013; Ben Tamarzizt et al., 2015; Iancu & Chivu, 2021; Li et al., 2022; Thakur et al., 2018; 2021; Yu et al., 2013).
The goal of the present study is to reveal the genetic variability and genetic relationships among Romanian plum accessions present in the USAMV of Bucharest orchard collection.

MATERIALS AND METHODS

Materials

Seven Romanian plum accessions enclosed in the collection of University of Agronomic Sciences and Veterinary Medicine of Bucharest, Romania, 'Brumării de Voinești', Record', 'Gemenea', 'Elena', 'Centenar', 'Silvia', and 'Pescăruș', were studied in the present research.

Genomic DNA extraction

Extraction of genomic DNA from young leaves was performed using the Innu PREP Plant DNA II KIT IPC 16 Kit (Analytik Jena) according to the manufacturer instructions. Frozen tissue was grounded to 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 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 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 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. DNA quality and quantity were checked with Nanodrop 1000 (Biorad).

RAPD

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. PCR reaction setup

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

Annealing temperature optimization (between 30 and 35°C) was done for all primers. PCR program consisted of an initial denaturation step of 2 min at 94°C, followed by 40 cycles of denaturation 15 sec at 94°C, annealing 15 sec at 30°C, and extension 30 sec 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. Decamers' nucleotide sequences

Decamer	Nucleotide sequence
P59	5'-GTTGGTGGCT-3'
P60	5'-GGGAACGTGT-3'
P61	5'-CCGTGACTCA-3'
P62	5'-CTTCCGCAGT-3'
P63	5'TGCCGAGCTG-3'

DNA amplicons from the RAPD reactions were separated on 1.5% agarose gel, visualized with the Pharox FX system (BioRad), and were measured using the Quantity One software (Version 4.6.9., BioRad).

Data analysis

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

RESULTS AND DISCUSSIONS

Initially, the annealing step was optimised in a gradient temperature PCR reaction, using 30.0,

30.9, 32.2, 34.1 and 35.0°C (Figure 1). All bands amplified were below 2000 bp. As the best clear bands were observed for all primers used at 30°C, further RAPD analysis of selected cultivars was done with this temperature at the annealing step. Yu et al. (2013) used for the annealing step a temperature of 44°C, however in their study the primers were 11 nucleotides long, so it is expected that the annealing temperature is higher.

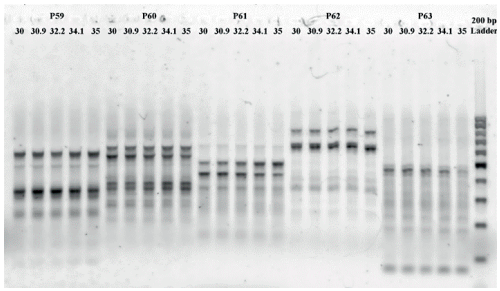

Figure 1. Effect of annealing temperature on RAPD amplification patterns for each primer used. On the right is present the 200 bp Ladder (Invitrogen). Numbers represent the annealing temperature in °C

The five random decamer primers (Table 3) identified a total of 27 polymorphic and 12 monomorphic RAPD loci.

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

Decamer	Polymorphic loci	Monomorphic loci	Amplified fragments sizes (bp)*
P59	4	2	1200, 1150,1100, 900, **620, 430**
P60	5	2	2000, **1200**, 1050, 950, 700, **630**, 520
P61	1	2	**950, 800**, 450
P62	8	2	1500, 1450, **1230**, 1220, 1050, 900, **820**, 700, 620, 550
P63	9	4	1200, **1000, 900**, 800, 750,650, 550,540,**450**, 350, 250, 200, **150**

*Fragments written with bold letters represent monomorphic loci.

P61 decamer is the least desirable decamer to be used, as it amplified only three DNA fragments, out of which only one was polymorphic. The P62 and P63 decamers amplified 10, respectively 13 DNA fragments with 8, respectively 9 polymorphic loci, making them the best candidates from this set of markers for further fingerprinting studies. The number of loci amplified by each primer was lower than those obtain by Athanasiadis et al. (2013), however in that study was observed the polymorphism of three different plum species, *P. domestica, P. insititia* and *P. cerasifera*.

The markers used have provided specific "fingerprints" for each genotype, allowing for molecular identification.

NAME	Brumării de Voinești	Record	Gemenea	Elena	Centenar	Silvia	Pescăruș
Brumării de Voinești	0.00	0.69	0.77	0.58	0.72	0.69	0.64
Record	0.69	0.00	0.58	0.67	0.75	0.61	0.67
Gemenea	0.77	0.58	0.00	0.79	0.72	0.79	0.69
Elena	0.58	0.67	0.79	0.00	0.64	0.47	0.61
Centenar	0.72	0.75	0.72	0.64	0.00	0.69	0.75
Silvia	0.69	0.61	0.79	0.47	0.69	0.00	0.54
Pescăruș	0.64	0.67	0.69	0.61	0.75	0.54	0.00

Figure 2. Roger distances calculated with the Bio-R software. The shortest Roger's genetic distance between cultivars is marked with green, and the longest is marked with blue

The calculated Roger's genetic distances are represented in Figure 2. The most closely related are the cultivars 'Silvia' and 'Elena', whereas the most distantly related are the accessions 'Silvia' and 'Gemenea' and 'Elena' and 'Gemenea', as it can be also observed from the dendrogram presented in Figure 3.

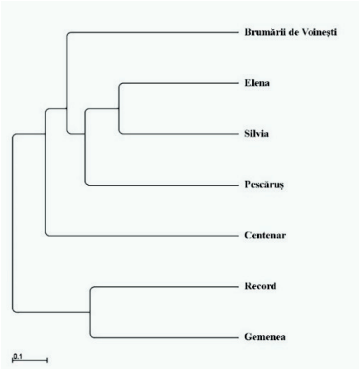

Figure 3. Dendrogram based on the RAPD data generated with the Bio-R software

The constructed UPGMA dendrogram (Figure 3) associated the accessions studied into two clusters, one cluster with the genotypes 'Record' and 'Gemenea', and another cluster grouping the rest of the cultivars. 'Record' is a cultivar resulted from the free pollination of 'Renclod violet' at the SCDP Voinești Research Station (Table 4). Since 'Gemenea' is a local population present in the same area, so

it is possible that 'Gemenea' or another closely related population to be the pollen donor.
Interestingly, the most closely related are the genotypes 'Silvia' and 'Elena', even though they have different genitors. Further studies with additional markers and eventually whole genome sequencing may unravel the similarity between these two accessions.

Table 4. Accession's origin

Accession	Origin
Elena	Tuleu gras x Stanlay, SCDP Bistrița*
Silvia	Renclod Althan x Early Rivers, SEA Mărculești*
Pescăruș	Renclod Althan x Wilhemina Spath, SEA Mărculești*
Centenar	Tuleu gras x Early Rivers, SEA Mărculești*
Record	Natural pollination Renclod violet - SCDP Voinești*
Brumării de Voinești	Local population - Voinești region
Gemenea	Local population - Voinești region

*(Ștefan et al., 2018)

CONCLUSIONS

RAPD analysis of the seven accessions from the USAMV of Bucharest revealed that:

- All random decamer primers identified both polymorphic and monomorphic RAPD loci.
- P62 and P63 are the most suitable markers to be used in discriminating the accessions.
- The genetic fingerprints obtained following amplification with the RAPD markers, are specific for each variety, and may be used for molecular identification of the varieties in the germplasm collection.
- The RAPD analysis using five decamers provided enough data to construct a UPGMA dendrogram, demonstrating the genetic variability among the cultivars studied.

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