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STUDIUL DIVERSITĂȚII GENETICE ȘI ANALIZA GRADULUI DE ASEMĂNARE LA UNELE SOIURI DE MĂR ȘI PRUN FOLOSIND MARKERI RAPD

STUDY OF GENETIC DIVERSITY AND ANALYSIS OF THE DEGREE OF SIMILARITY ON SOME APPLE AND PLUM VARIETIES USING RAPD MARKERS

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Abstract

Molecular evaluation of germplasm is an important step in breeding programs, and the application of molecular biological techniques has led to important results in terms of both within- and between-species variability of traits. The RAPD technique has been successfully used to reveal allelic polymorphism as well as to measure genetic similarity. In this study, the genetic diversity of 25 genotypes and cultivars for apple species and 26 genotypes and cultivars for plum species was assessed with six RAPD markers. All these cultivars belong to the *ex situ* collection of apple and the *ex situ* collection of plum at the Research Institute for Fruit Growing Pitesti. The average number of amplified bands was 19.2 for apple and 17.66 for plum. Statistical analysis of intraspecific allelic polymorphism was expressed using the PIC (Polymorphic Information Content) index, which takes into account the allelic frequency. Two statistical indices were used to quantify genetic diversity: the Shannon index and the Simpson index. The degree of similarity between varieties was analyzed using the NTSYSpc version 2.1. Following RAPD analyses, the allele sizes of the analyzed varieties were within the range quoted in the literature, the genetic profiles of the studied varieties suggesting a medium to high genetic diversity, except for markers OPBC-04 and OPBB-05 for plum species, which expressed a high genetic diversity. Genetic distances calculated based on polymorphism of migrated bands in agarose gel confirmed the known genealogies of the apple and plum varieties studied. Thus, the smallest genetic distance for apple species was found between 'Jonagold' and 'Golden Delicious', 'Pionier' and 'Rustic', 'Jonathan' and 'Idared', 'Wagener Premiat' and 'Granny Smith', 'Remar' and 'Aura', 'Romus 3' and 'Rome Beauty', and the largest between *Malus floribunda* and the other genotypes studied. In plum, the smallest genetic distance was found between 'Dani' and 'Tita', 'Roman' and 'Tuleu gras', 'Dara' and 'Haganta', 'Romanța' and 'Stanley', 'Anna Spath' and 'Renclod Violet', and the largest between 'Lama', 'Black Diamond' and the other genotypes studied.

Cuvinte cheie: măr, prun, diversitate genetică, grad de similaritate, tehnica RAPD, dendrogramă.
Key words: apple, plum, genetic diversity, degree of similarity, RAPD technique, dendrogram.

1. Introduction

The apple and plum germplasm reserve constitutes an invaluable stock of genetic resources needed for these species for current and future breeding works. The creation of new cultivars is an important requirement in the work of these programs, and for the conservation of biodiversity, it is necessary to know those genotypes with a high genetic diversity and presenting characteristics of interest both for the consumer (high fruit quality, food safety and security) as well as for the grower (resistance to certain diseases and pests, increased productivity, climatic adaptation, etc.). Obtaining cultivars and hybrids with polygenic resistance to diseases and pests bring benefits to both the grower and the consumer by reducing or even eliminating treatments with fungicides. The selection by applying molecular analysis techniques in hybrid populations allows the rapid identification of promising plants, shortening apple's classical breeding process (from 15 years to 9 years). Screening with the molecular markers is thus the optimal solution for hybrids selection at the sapling phase, and the method is recognized and applied in many apple breeding centers around the world.

Molecular screening of apple and plum cultivars in the *ex situ* collection of the Research Institute for Fruit Growing Pitesti will allow the selective choice of useful parents for the breeding programs and accelerate the breeding process by early identification of hybrids with characteristics of interest.

RAPD markers are efficient in terms of cost, the dominant character, rapid time to get results, and the possibility of investigating the whole genome. These markers are very useful in variety identification, paternity analysis, distinguishing phylogenetic relationships between related species.

Many studies have been carried for the apple and plum cultivars, and important results have been obtained using RAPD markers, including with those used by us in this study (Casas et al., 1999; Kolle et al., 1993; Liu et al., 2007; Mehdi et al., 2012; Melih et al., 2015; Shahin et al., 2017; Shimada et al., 2001; Yan et al., 2015).

The aim of this work was to study the genetic variation among different genotypes of apple and plum and to determine the genetic similarities among accessions using RAPD markers.

2. Material and methods

Apart from the apple cultivars 'Florina', 'Granny Smith', 'Prima', 'Idared', 'Jonagold', 'Jonathan', 'Rome Beauty', 'Wagener Premiat', 'Golden Delicious' and 'Belle de Boskoop' and the plum cultivars 'Anna Spath', 'Haganta', 'Black Diamond', 'Čačanska Lepotiča', 'Early Rivers', 'Lama', 'Renclod Violet', 'Renclod Althan', all the others are Romanian cultivars, created in the main apple breeding centers in Romania (Pitești, Voinești, Bistrița), respectively local varieties (Table 1, Table 2). To obtain an optimal DNA extraction, both in terms of concentration and ratio of absorbance's (A260/A280), the leaf's samples were treated with liquid nitrogen and milled with the help of a press, semi-automatic HOMEX 6, which is ideal for tissue extraction. For optimal processing, purification, performance and high yield, the Isolate II Plant DNA Extraction Kit (Bioline) was used providing two different Lysis protocols. The standard protocol recommends Lysis Buffer PA1 based on the well-established CTAB procedure, and the second protocol recommends Lysis Buffer PA2 (SDS-based), which requires subsequent precipitation of protein with potassium acetate (Precipitation Buffer PL3). Amplifications were performed in a Termocycler PCR model FG-TC01, amplification programs were optimized for each primer. The reaction mix was made using 2 x MyTaq™ Red Mix in 0.2 ml tubes containing 15 µl final reaction volume, of which 10 µl reaction mix (MyTaq RedMix), 1 µl of each primer, 1 µl DNA (30 ng) and 3 µl ultrapure water. The amplification reaction was performed using six RAPD markers according to the following protocols: OPAC-11 marker (initial denaturation step at 94°C for 3 min. followed by 44 cycles of 20 sec. at 94°C, 44 sec. at 35°C, 1 min. at 72°C and final extension 7 min at 72°C); OPBB-05 marker (initial denaturation step at 94°C for 5 min, followed by 45 cycles of 1 min. at 94°C, 1 min. at 37°C, 2 min. at 72°C and final extension 5 min at 72°C); OPBC-04 marker (initial denaturation step at 94°C for 3 min, followed by 5 cycles of 1 min. at 92°C, 1 min. at 39.5°C, 2 min. at 72°C, 45 cycles of 30 sec. at 94°C, 45 sec. at 37.5°C, 2 min. at 72°C and final extension 7 min. at 72°C); OPBD-01 and OPBD-04 markers (initial denaturation step at 93°C for 5 min, followed by 45 cycles of 1 min. at 93°C, 1 min. at 36°C, 1 min. at 72°C and final extension 7 min at 72°C); OPBA-20 marker (initial denaturation at 94°C for 3 min, followed by 5 cycles of 1 min. at 92°C, 1 min. at 39.5°C, 2 min. at 72°C, 45 cycles of 30 sec. at 92°C, 45 sec. at 37.5°C, 2 min. at 72°C and final extension with 7 min at 72°C) (Table 3).

PCR product evaluation was performed by 2% high-resolution agarose gel electrophoresis (Cleaver) and 1X TBE buffer (stock solution prepared from 10X TBE buffer), staining with RedSafe Nucleic Acid Staining. For fragment size estimation, a 50 bp DNA ladder (HyperLadder™ 50bp) was loaded into the gel, allowing visualization of fragments between 50 and 2000 bp. Migration was performed at 100 V for one hour. Visualization of the results was done with high-quality imaging system and Uvitec Cambridge Essential analysis software. The degree of similarity between the varieties, both for the apple species and for the plum species, was determined by analyzing the dendrogram made with the NTSYSpc version 2.1 software (Marti et al., 2012; Rohlf, 1994; Rohlf, 2005).

Statistical analysis of allelic intraspecific polymorphism was expressed using the PIC index (Content of polymorphic information), which takes into account the allelic frequency, and is calculated using the mathematical expression: 2f (1-f) (Roldán-Ruiz et al., 2000; Soengas et al., 2006), where "f" is the frequency of the amplified allele (band present), and (1-f) is the frequency of the null allele (band absent) (Erturk and Akcay, 2010). Two statistical indices were used to quantify genetic diversity: the Shannon index (Shannon and Weaver, 1949) and the Simpson index. The calculation of the Shannon index was performed with the mathematical expression:

- $\sum_{i=1}^{n} pi * \ln pi \Leftrightarrow - \sum_{i=1}^{n} {}^{n}/_{N} * ln\, n/N$,

and of the Simpson index with the mathematical expression:

- $\frac{\sum_{i=1}^{n} n*(n-1)}{N*(n-1)}$, where:

- n - the number of alleles in a locus, for the total number of varieties;
- N - the total number of alleles from all loci, for the total number of varieties (Shannon et al., 1948; Simpson, 1960).

3. Results and discussions

Molecular variability is useful both for highlighting genetic diversity and for revealing the degree of similarity between varieties belonging to the same species.

Statistical analysis with RAPD markers OPAC-11 (Fig. 1), OPBD-04, OPBC-04, OPBB-05 and OPBD-01 showed a moderate informative polymorphism and a medium to high genetic diversity in the apple species (Table 4). For plum species, markers OPAC-11, OPBD-04, OPBD-01 and OPBA-20 also

indicated moderate polymorphism and medium to high genetic diversity, while markers OPBC-04 (Fig. 2) and OPBB-05 expressed a high genetic diversity and moderate polymorphism (Table 5).

The genetic distances calculated based on the polymorphism of the bands migrated in the agarose gel confirmed the known genealogies of the studied apple and plum varieties. Thus, the smallest genetic distance for the apple species was found between the cvs. 'Jonagold' and 'Golden Delicious', 'Pionier' and 'Rustic', 'Jonathan' and 'Idared', 'Wagener Premiat' and 'Granny Smith', 'Remar' and 'Aura', 'Romus 3' and 'Rome Beauty', and the largest between *Malus floribunda* and the other cultivars studied, genetic similarity values varying from 0.61 to 0.86 (Fig. 3). In the plum species, the smallest genetic distance was found between the cvs. 'Dani' and 'Tita', 'Roman' and 'Tuleu gras', 'Dara' and 'Haganta', 'Romanța' and 'Stanley', 'Anna Spath' and 'Renclod Violet', and the largest between the varieties 'Lama', 'Black Diamond' and the other cultivars taken in this study, genetic similarity values varying from 0.55 to 0.9 (Fig. 4).

In the apple species, the 'Jonathan' cv. is the parent for many of the cultivars studied, the inheritance of the characters expressed with the RAPD markers mentioned above, being able to be observed by establishing the degree of similarity for the cultivars with this common parent. The small genetic distance between 'Jonathan' cv., as a parent and 'Idared' cv., indicates segregation of most of the characters from this parent. For the 'Jonagold' cv., which has the 'Jonathan' and 'Golden Delicious' cvs. as parents, the degree of similarity indicates segregation of the majority of characters from the 'Golden Delicious' parent. According to the dendrogram, the 'Rustic' cv. is very close to its 'Pionier' parent. 'Rebra' is also close to the 'Rustic' and 'Pionier' cvs., the small genetic distance can be explained by inheriting identical characters from the parent 'Florina', common to the two cvs., 'Rebra' and 'Rustic'. 'Aura' and 'Remar' cvs. have a common parent, the 'Prima' cv., the proximity between the two cultivars indicating segregation of most of the characters from this parent. 'Romus 5' is a smaller genetic distance from the 'Wagener Premiat', 'Granny Smith', 'Parmen Auriu', 'Remar' and 'Aura' cvs., than from one of its parents, 'Romus 3'. 'Prima' cv., common parent for 'Romus 5', 'Remar' and 'Aura' cvs., would explain the proximity between the three cultivars. 'Florina' cv., one of the parents of 'Rebra', 'Rustic', 'Valery' cvs., has a higher degree of similarity to 'Domnesc' and 'Belle de Boskoop' cvs., compared to the three cultivars for which it is the parent. The greatest genetic distance was found between *Malus floribunda* and the cultivars taken in this study.

In the plum species, the 'Tuleu gras' cv. is a common parent for following cultivars: 'Centenar', 'Dani', 'Roman' and 'Tita'. The 'Roman' cv. is closer to this parent than the 'Dani' and 'Tita' cvs., which, from the dendrogram analysis, the two cultivars have the same common characters. The 'Centenar' cv. inherited predominantly characters from the 'Early Rivers' parent and less from the other parent, 'Tuleu gras'. The 'Romanța' cv. has a higher degree of similarity to the 'Stanley' parent than has the Jojo cv., but both cultivars, 'Romanța' and 'Jojo', are located at a small genetic distance from each other. The closeness between the cultivars 'Topend' and 'Haganta' show the segregation of some characters from their common parent 'Čačanska Najbolja'. The 'Record cv. is close to the 'Renclod Violet' cv., which is its parent. The 'Scolduș' cv., a local variety, is located at a great genetic distance from all other cultivars, and the 'Lama' and 'Black Diamond' cvs. have the biggest differences.

4. Conclusions

The RAPD markers used in this study show high reliability for the two species, obtaining amplified fragments between 200 bp and 3000 bp and highlighting dominant characters in a large number of loci distributed throughout the genome.

Based on genetic distances studied from the analysis of the dendrograms it was possible to observe the degrees of kinship between the varieties with common parents, but also the predominance of the character segregation coming from one of the parents.

The medium to high genetic diversity, as it looks at statistical indices, confirms the importance of using these varieties in breeding programmes.

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Tables and Figures

Table 1. Apple biological material

No.	Cultivar	Genitors, Origin
1	Aura	Prima x BN 33/39, Romania
2	Belle de Boskoop	Unknown parents, Netherlands
3	Cretesc	Local variety, Romania
4	Cretesc auriu	Local variety, Romania
5	Domnesc	Local variety, Romania
6	Delicios de Voinesti	Golden delicious x Creţesc de Vâlcea, Romania
7	Florina	(Jonathan x (Starking Delicious x (Golden Delicious x F2 26829-2-2)), France
8	Generos	(Parmain d'or x *M. kaido*) x (Jonathan x V 53-39-2) x (Frumos de Voinesti x V 60-6-51), Romania
9	Golden Delicious	Unknown parents, USA
10	Granny Smith	Unknown parents, Australia
11	Idared	Jonathan x Wagener, USA
12	Jonathan	Unknown parents, USA
13	Jonagold	Jonathan x Golden Delicious, USA
14	*Malus floribunda*	
15	Pionier	(Jonathan X Verzişoare) x Prima, Romania
16	Parmen Auriu	Old cultivar, England
17	Romus 3	Hybrid interspecific, F4, Romania
18	Romus 5	Romus 3 x Prima, Romania
19	Rebra	Prima x Florina, Romania
20	Rustic	Florina x Pionier, Romania
21	Rome Beauty	Unknown parents, USA
22	Wagener Premiat	Unknown parents, USA
23	Remar	Gama radiation of Prima seeds (o.p.), Romania
24	Valery	Florina x Golden Spur, Romania
25	Verzișoare	Local variety, Romania

Table 2. Plum biological material

No.	Cultivar	Genitors, Origin
1	Anna Spath	Unknown parents, Germany
2	Black Diamond	Chino-Japanese cv. (*Prunus salicina*)
3	Čačanska lepotica	Wangenheim x Pozegača, Serbia
4	Centenar	Tuleu gras x Early Rivers, Romania
5	Dani	Tuleu gras x Grase românești, Romania
6	Dara	Unknown parents, Romania
7	Early Rivers	Unknown parents, England
8	Gras ameliorat	Grase românești self pollination, Romania
9	Grase românești cl. 205	Grase românești clone, Romania
10	Haganta	Čačanska Najbolja x Valor, Germany
11	Jojo	Ortenauer x Stanley, Germany
12	Lama	Hybrid 9-250 (*P. cerasifera* var. *pissardii*) open pollination, Belarus
13	Renclod Violet	Stones from Reine Claude Verte, Hungary or Czech Republic
14	Renclod Althan	Stones from Reine Claude Verte, Hungary or Czech Republic
15	Record	Renclod violet open pollination, Romania
16	Roman	Tuleu gras x Early Rivers, Romania
17	Romanța	Stanley x Vâlcean, Romania
18	Scolduș	Local variety, Romania
19	Stanley	d'Agen x Grande Duke, USA
20	Timpurii de Țurlesti	Local variety, Romania
21	Tita	Tuleu gras, irradiated stones, Romania
22	Topend	Čačanska Najbolja x Valor, Germany
23	Tuleu gras	Unknown parents, Romania
24	Vinete românești	Local variety, Romania
25	Vinete românești cl. 300	Clone in Vinete românești, Romania
26	Zamfira	Anna Spath x Renclod Althan, Romania

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Table 3. List of primers used for amplification

Marker	Primer sequence (5'→3')	Annealing temperature (°C)
OPAC-11	CCTGGGTCAG	35
OPBD-04	TCGGGTGTTG	36
OPBC-04	CCACGTGCCA	37,5 (for 45 cycles) 39,5 (for 5 cycles)
OPBB-05	GGGCCGAACA	37
OPBD-01	TCACTCGCTC	36
OPBA-20	GAGCGCTACC	37,5 (for 45 cycles) 39,5 (for 5 cycles)

Table 4. Statistical results for allelic polymorphism and genetic diversity in apple species

Marker	Total no. of amplified fragments	PIC*	Shannon index**	Simpson index (D)***	Simpson diversity index****
OPAC-11	21	0.3068952	2.7166495	0.07112874	0.92887126
OPBD-04	19	0.3008	2.724592	0.067538	0.932462
OPBC-04	20	0.32512	2.732745	0.062739	0.937261
OPBB-05	19	0.317642	2.320724	0.061609	0.938391
OPBD-01	19	0.2944	2.505277	0.086232	0.91377

*value>0.25 - moderately informative;
**H > 3 - high genetic diversity;
***D = 0 - infinite diversity; D = 1 - lack of diversity;
**** 1-D - value Є [0,1]

Table 5. Statistical results for allelic polymorphism and genetic diversity in plum species

Marker	Total no. of amplified fragments	PIC*	Shannon index**	Simpson index (D)***	Simpson diversity index****
OPAC-11	13	0.33358	2.40373	0.091343	0.908657
OPBD-04	12	0.25789	2.361015	0.096479	0.903521
OPBC-04	26	0.324306	3.093566	0.024188	0.975812
OPBB-05	25	0.348317	3.075102	0.047699	0.952301
OPBD-01	16	0.28791	2.53554	0.086269	0.913731
OPBA-20	14	0.2574	2.359469	0.10044	0.89956

*value>0.25 - moderately informative;
**H > 3 - high genetic diversity;
***D = 0 - infinite diversity; D = 1 - lack of diversity;
**** 1-D - value Є [0,1]

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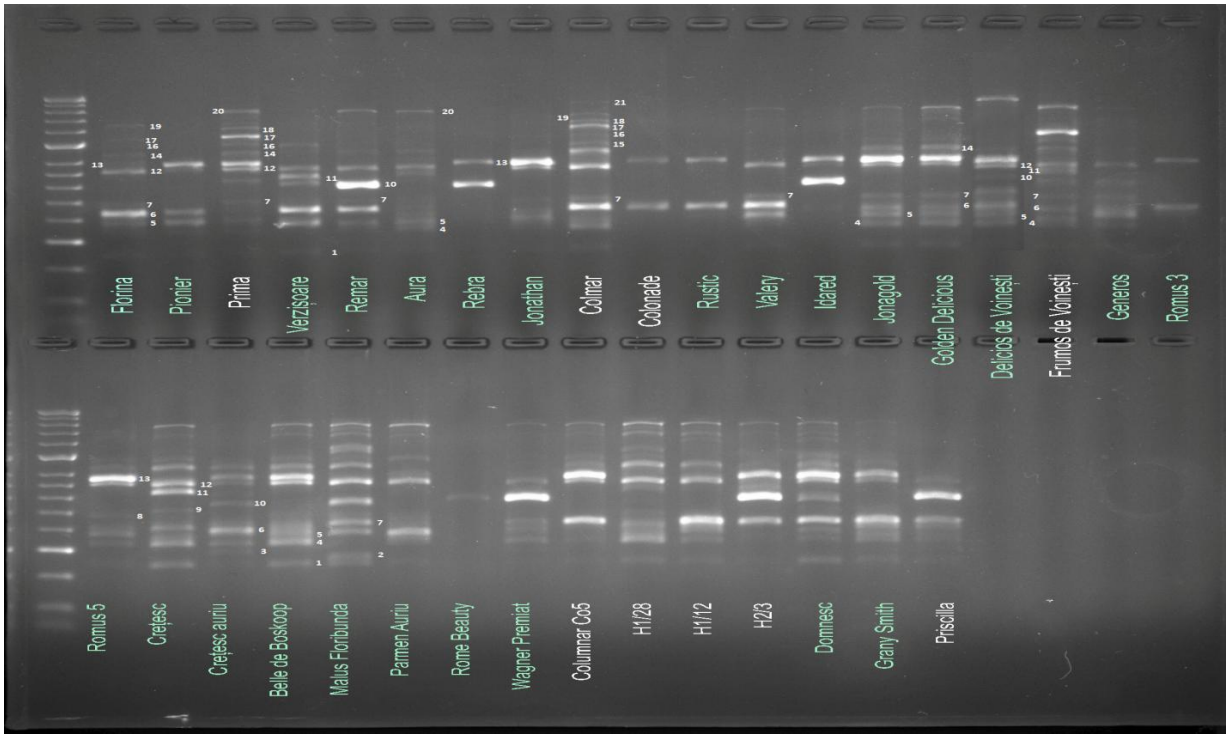

Fig. 1. The electrophoretic profile obtained with the OPAC-11 marker in apple species

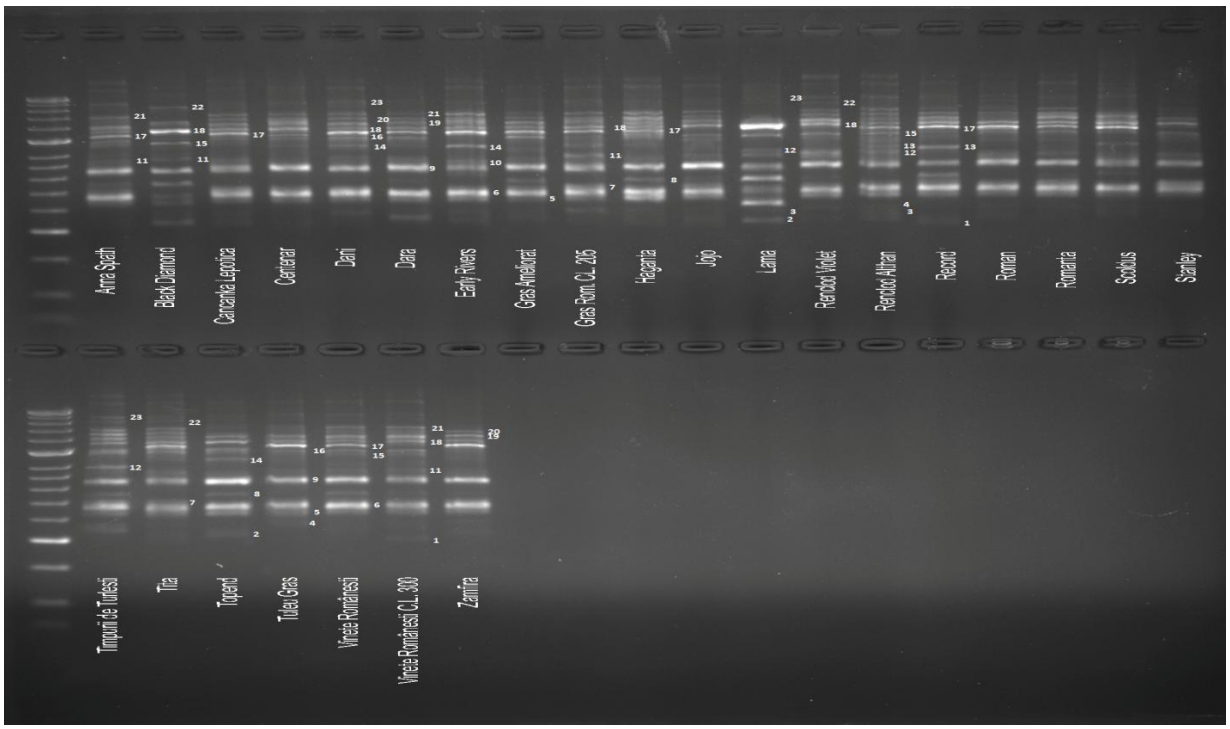

Fig. 2. The electrophoretic profile obtained with the OPBC-04 marker in plum species

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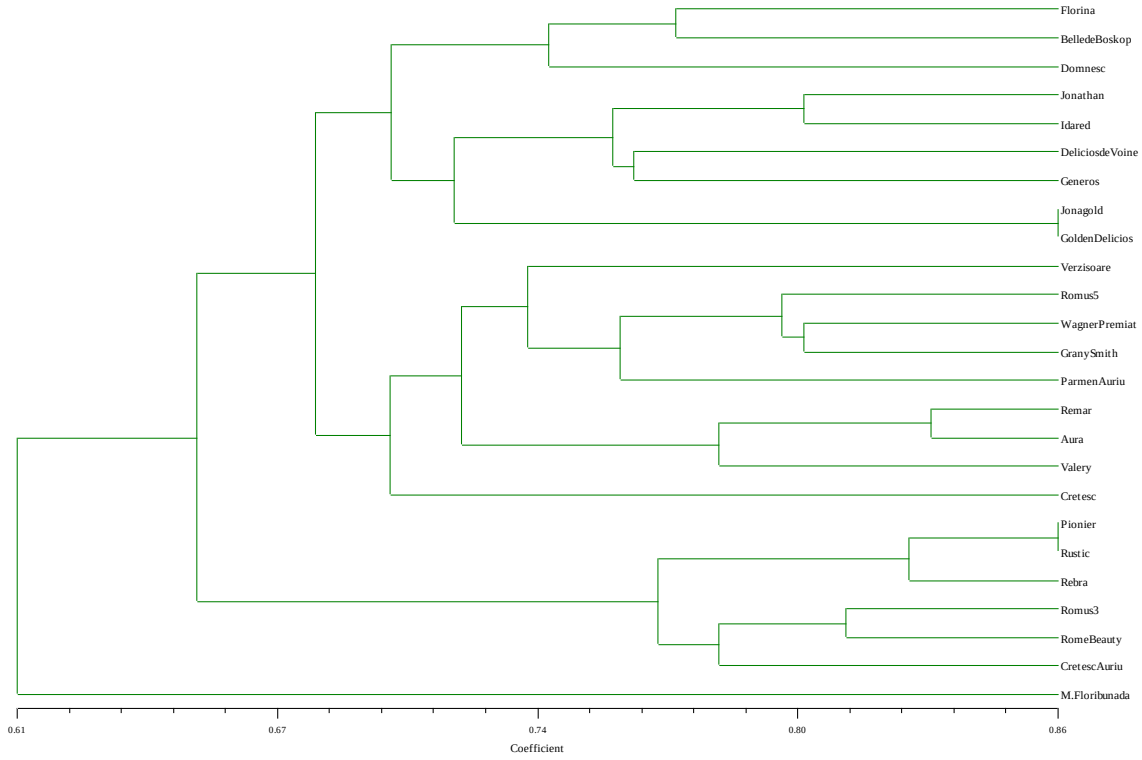

Fig. 3. Dendrogram for apple species. Degree of similarity between cultivars

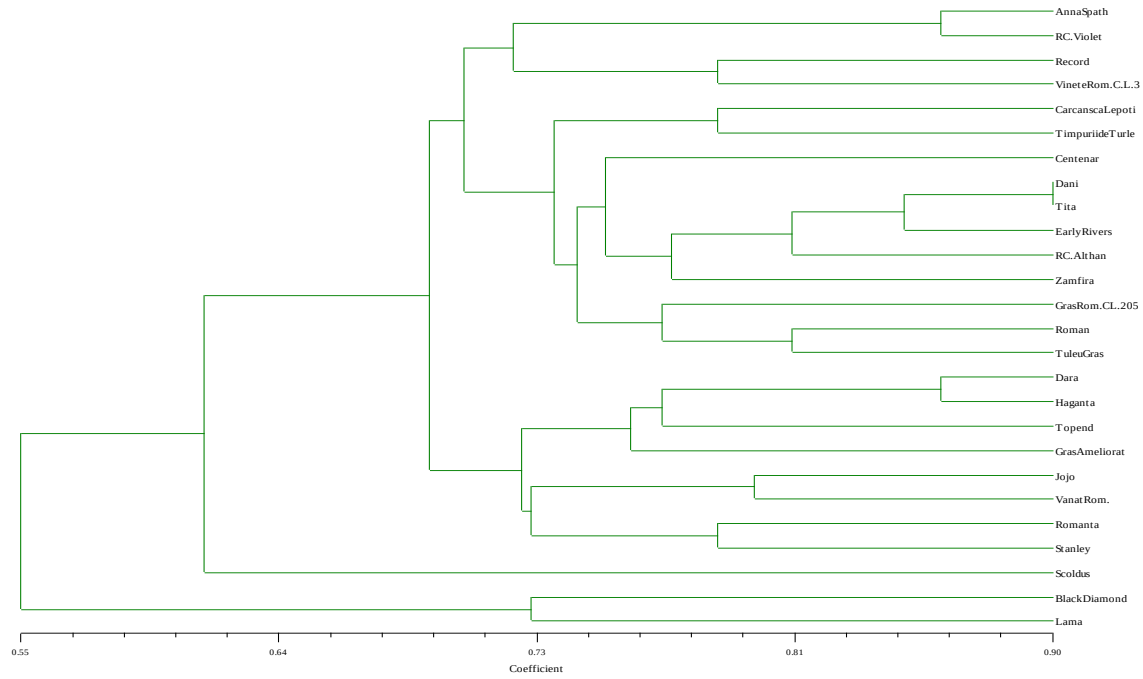

Fig 4. Dendrogram for plum species. Degree of similarity between cultivars