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SHORT COMUNICATION

Assessment of the genetic variability among some *Juglans* cultivars from the Romanian National Collection at S.C.D. P. Vâlcea using RAPD markers

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Abstract

The genetic diversity of 51 accessions belonging to Juglans genus maintained in the Romanian National Collection at the Station for Research and Development for Fruit Growth Valcea (S.C.D.P. Valcea) was assessed, using RAPD (Random Amplified Polymorphic DNA) markers. The 25 primers used in this study yielded scorable amplification patterns. The produced fingerprint distinguished the identical accessions, confirming their genetic identity and discriminated all the other accessions. Accessions representing Romanian homologated cultivars tend to group together according to their origin. The determined genetic variability was specific to a germplasm collection and to the high number of different accessions studied. The RAPD markers can be useful in developing DNA fingerprinting techniques to distinguish the valuable genotypes used in selection.

Keywords: RAPD markers, *Juglans* genus, genetic variability, identical accessions

Introduction

The walnut is a very important species in Romania, our country being considered one of the biggest walnuts production countries worldwide (Cociu *et al*. [1]).

Juglans genus includes approximately 40 species, grouped into three sections, *Dioscaryon Dode, Cardocaryon Dode and Thyoscaryon Dode. Juglans regia* L. belongs to *Dioscaryon Dode Section, while Juglans nigra* and *Juglans cinerea* belong to the *Thyoscaryon Dode Section* (Cociu *et al*. [1]).

Juglans regia L., also known as Persian walnut, is a very old species, which spreads from the northern region of Iran to Japan, from Greenland to Siberia and Burma (Bordeianu *et al*. [2]).

Juglans nigra L., or the black walnut, is prevalent in the United States of America, between the Atlantic Ocean and Texas (Forde [3]). It is grown for its superior quality wood and nuts, but also as an ornamental tree.

Juglans cinerea L. has its origin in Georgia and Arkansas (U.S.A.) and is the most resistant to cold from all the american walnut species. Due to the fine nutshell and to the quality of its nuts, some cultivars have been selected in the past decades (Forde [3]).

Walnuts are a very important source of nutrients, being rich in vitamins and also in Mg and Ca. The wood from *Juglans* species can be used to make furniture. For all these reasons, walnuts were cultivated on large territories in Romania in the past. In the last decades in Romania was seen a decrease of cultivated walnuts (Cociu *et al*. [1]). In order to increase the walnut production, new cultivars need to be introduced and also old cultivars to be conserved in genebanks.

Accurate and rapid cultivar identification and characterization is important in vegetatively propagated plant species both for breeding purposes and for proprietary rights

protection (Weising *et al*. [4]). UPOV (International Union for the Protection of New Varieties of Plants) has the mission to provide and promote an effective system of plant variety protection, with the aim of encouraging the development of new varieties of plants, for the benefit of society (http://www.upov.int/en/about/ [5]). In order to achieve this, it specifies descriptors for the analysis of plant varieties, among which there are also molecular markers.

In our study, we completed the phenotypic description of the *Juglans* accessions from the National Collection held at S.C.D.P. Valcea with a molecular characterization using RAPD markers.

RAPD markers, Random Amplified Polymorphic DNA, have the advantages of being easy to use, the experiments have a low cost and they cover the entire genome (Williams *et al* [6]). Also, they can be used as a first method of choice for screening the accessions in order to find duplicates in collections (Karp *et al*. [7]). RAPD markers have been used to assess the level of polymorphism in *Juglans* genus (Woeste *et al*. [8], Nicese *et al*., [9] 1998) and in other important fruit tree species, with interesting results (Hormaza *et al*. [10], Huang *et al*. [11], Goulao *et al*. [12], Solar *et al*. [13]).

The main purpose of this study was the molecular characterization of 51 accessions belonging to *Juglans* genus held at S.C.D.P. Valcea and the assessing of the genetic variability among them, hence the identification of duplicates.

Materials and Methods

Plant material

The 51 genotypes used in this study (Annex 1) were obtained from the collection maintained at S.C.D.P. Valcea, Romania, some being part of a walnut breeding program developed at this institution, some representing old cultivars, some representing homologated cultivars, some representing selections from natural populations and some representing cultivars in process of homologation.

Annex 1. The 51 walnut accessions used, their name, origin and species

No.	Name	Origin	Species
1	Argeşan C1	Romania, homologated cultivar, selection from Pitesti region	J. regia
2	Argeşan C1	Romania, homologated cultivar, selection from Pitesti region	J. regia
3	Ferjean	France	J. regia
4	Fernette P1	France, Franquette x Lara hybrid	J. regia
5	Fernor P1	France, Franquette x Lara hybrid	J. regia
6	Franquette	France	J. regia
7	Franquette C1	France	J. regia
8	Germisara C5	Romania, homologated cultivar, selection from Hunedoara region	J. regia
9	Germisara C5	Romania, homologated cultivar, selection from Hunedoara region	J. regia
10	Hartley C1	USA	J. regia
11	J. cinerea P1	unknown	J. cinerea
12	J. nigra variety Laciniata P2	unknown	J. nigra
13	J. nigra variety Laciniata P1	unknown	J. nigra
14	J. regia varietaty purpurea P2	unknown	J. regia
15	J. regia variety pendula	unknown	J. regia

16	J. regia variety purpurea P1	unknown	J. regia
17	Jupîneşti P1	Romania, homologated cultivar obtained at ICDP Pitesti, selection from Arges region	J. regia
18	Jupîneşti P2	Romania, homologated cultivar obtained at ICDP Pitesti, selection from Arges region	J. regia
19	Lara C1	France	J. regia
20	Leopold P1	USA	J. nigra
21	Leopold P2	USA	J. regia
22	Mihaela P1	Romania, homologated cultivar obtained at ICDP Pitesti, selection from Arges region	J. regia
23	Mihaela P2	Romania, homologated cultivar obtained at ICDP Pitesti, selection from Arges region	J. regia
24	Muscelean C1	Romania, homologated cultivar obtained at ICDP Pitesti, selection from Pitesti region	J. regia
25	Muscelean C3	Romania, homologated cultivar obtained at ICDP Pitesti, selection from Pitesti region	J. regia
26	O2	Selection from Caucaz region	J. regia
27	Roxana P1	Romania, homologated cultivar obtained at ICDP Pitesti, selection from Arges region	J. regia
28	Roxana P2	Romania, homologated cultivar obtained at ICDP Pitesti, selection from Arges region	J. regia
29	Secular C1	Romania, homologated cultivar	J. regia
30	Secular C2	Romania, homologated cultivar	J. regia
31	Serr	USA	J. regia
32	Supergiant	USA	J. regia
33	Valcor C1	Romania, homologated cultivar obtained at S.C.D.P. Valcea	J. regia
34	Valcor C2	Romania, homologated cultivar obtained at S.C.D.P. Valcea	J. regia
35	Valmit C2	Romania, homologated cultivar obtained at S.C.D.P. Valcea	J. regia
36	Valmit C3	Romania, homologated cultivar obtained at S.C.D.P. Valcea	J. regia
37	Valrex C3	Romania, homologated cultivar obtained at S.C.D.P. Valcea	J. regia
38	Valrex C4	Romania, homologated cultivar obtained at S.C.D.P. Valcea	J. regia
39	Velnița C2	Romania, homologated cultivar obtained at SCDP Iasi, selection from Iasi region	J. regia
40	Velnița C8	Romania, homologated cultivar obtained at SCDP Iasi, selection from Iasi region	J. regia
41	Vina P1	USA	J. regia
42	VL 102 H P1	Selection from Horezu region	J. regia
43	VL 202 PO C1	Selection from Pausesti – Otasau region, cultivar in process of homologation	J. regia
44	VL 202 PO C1	Selection from Păuşeşti – Otăsău region, cultivar in process of homologation	J. regia
45	VL 202 PO P1	Selection from Pausesti – Otasau region, cultivar in process of homologation	J. regia
46	VL 44 B P2	Selection from Valcea region, cultivar in process of homologation	J. regia
47	VL 52 B	Selection from Valcea region, cultivar in process of homologation	J. regia
48	VL 54 B P1	Selection from Valcea region, cultivar in process of homologation	J. regia
49	VL-1P3	Hybrid, selection from population of seeds	J. regia
50	VL-1P4	Hybrid, selection from population of seeds	J. regia
51	VL-1P5	Hybrid, selection from population of seeds	J. regia

DNA isolation

Young leaves from the studied accessions were collected in spring and then stored at -80°C prior to DNA extraction. Total DNA was extracted using the protocol described by Lodhi *et al*. [14] and modified by Pop *et al*. [15]. The concentration of the extracted DNA was assessed using a Nano Drop ND 1000 spectrophotometer and was later diluted to 50 ng/µl with nuclease-free water (Promega) for PCR amplification.

DNA amplification and electrophoresis conditions

PCR amplification reactions were carried out as described by Williams *et al.* [6]. Reaction mixtures (25 µL total volume) consisted of 250 ng DNA, 9.3 µL distilled H_20 for PCR reactions, 2 µL PVP (poly vinyl pyrrolidone), 5 µL GoTaq Flexi green buffer (Promega), 2.5 µL $MgCl_2$ (Promega), 0.5 µL dNTP mix (Promega), 0.5 µL RAPD primer (Microsynth, Balgach, Switzerland), 0.2 µL GoTaq polymerase (Promega). DNA amplification was carried out in a 96 Well Gradient Palm-Cycler CG1-96 (Corbett Research) programmed for 1 cycle of 3 min at 95ºC, followed by 45 cycles of 1 min at 93ºC, 1 min at 34ºC and 1 min at 72ºC. After a final incubation for 10 min at 72ºC the samples were stored at 4ºC prior to analysis. The PCR amplified products were size fractionated by migration on a 1.4% agarose (Sigma-Aldrich) gel in 1X TAE Buffer (242 g Tris Base (MW=121.1), 57.1 mL Glacial Acetic Acid, 100 mL 0.5 M EDTA) at 0.29 V/cm^2 for 2 hours. The molecular marker used was 100bp DNA Step Ladder (Promega). Gels were visualized on a UV light Biospectrum AC Imaging System (UVP BioImaging Systems) after staining with 0.5 µg/µl Ethidium Bromide for 25 min.

Data analysis

Gel images were analyzed using TL120 software (Nonlinear Dynamics). Amplified bands were scored present (1) or absent (0) and data entered into a binary matrix. The genetic distance between accessions was calculated using Nei and Li/Dice coefficient of similarity (Nei and Li [16]). Cluster analysis was conducted with an UPGMA (Unweighted Pair Group Method with Arithmetic mean) algorithm using FreeTree software (Hampl *et al*. [17]) and a dendrogram was constructed, using the TreeView software (Page [18]). Its consistency was assessed using bootstrap method in 1000 repetitions. A synthetic outgroup was used for dendrogram rooting.

Results

DNA extraction

The DNA quantity obtained varied between 161.88 ng/µl (VL-1P5) and 3331.91 ng/µl (Mihaela P1) and its purity varied between 1.68 (Lara C1) and 1.99 (Vina P1).

DNA amplification with RAPD primers

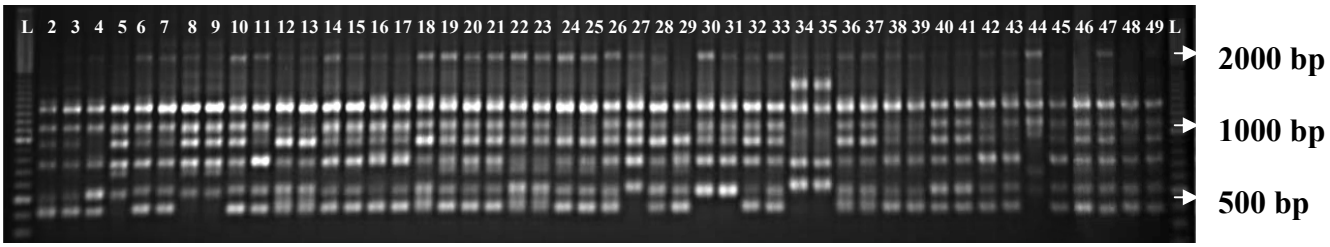
A total of 25 decamer primers from Operon Technologies (synthesized by Microsynth) were used to amplify DNA extracted from the 51 *Juglans* genotypes used in this study. All the primers yielded scorable amplification patterns (Table 1).

Table 1. Primers used for differentiation of the 51 analyzed *Juglans* accessions

No.	Primer	No. of polymorphic bands
1	OPA 01	8
2	OPA 03	14
3	OPA 04	15
4	OPA 06	15
5	OPA 09	13
6	OPA 11	12

7	OPA 20	12
8	OPAB 11	12
9	OPAL 20	13
10	OPB 08	10
11	OPB 10	16
12	OPB 11	9
13	OPB 17	12
14	OPC 02	8
15	OPC 08	13
16	OPC 14	10
17	OPC 15	16
18	OPD 16	11
19	OPE 14	16
20	OPF 02	16
21	OPF 20	15
22	OPFI 0	16
23	OPH 02	10
24	OPH 12	9

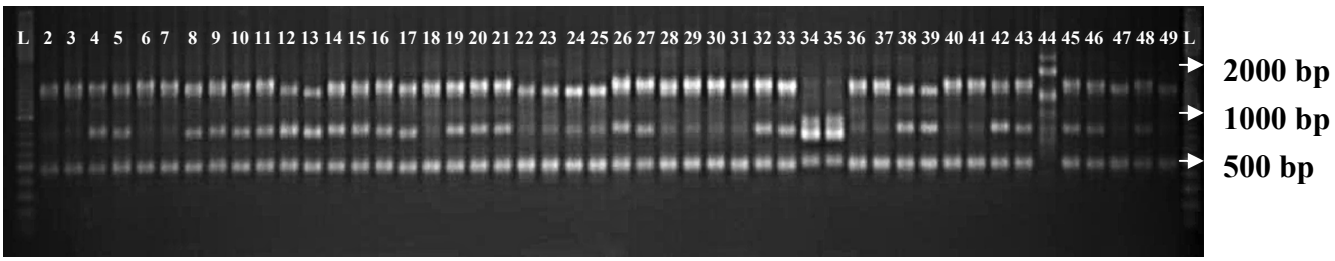
Figure 1 and Figure 2 show bands resulted from DNA amplification in 48 *Juglans* accessions using OPC 02 primer and OPA 01 primer, respectively .



L – ladder
bp – base pairs

Fig. 1. Amplification products obtained with OPC 02 primer in *Juglans* genus accessions

Primers OPC 15, OPF 02, OPE 14 and OPFI0 generated the most polymorphic bands, 16, while primer OPA 01 generated the least polymorphic bands, 8. A total number of 311 polymorphic bands was generated, with an average of 12.4 bands/primer (Table 1).



L – ladder
bp – base pairs

Fig. 2. Amplification products obtained with OPA 01 primer in *Juglans* genus accessions

The calculated genetic distances among the studied accessions varied between 0 (identical accessions) and 0.5 (between *Juglans cinerea* accession and Supergiant accession), with an average of 0.2.

In Figure 3 is represented the dendrogram generated using TreeView software, based on the genetic relationships between some of the *Juglans* accessions, calculated using Nei Li/Dice coefficient with FreeTree software.

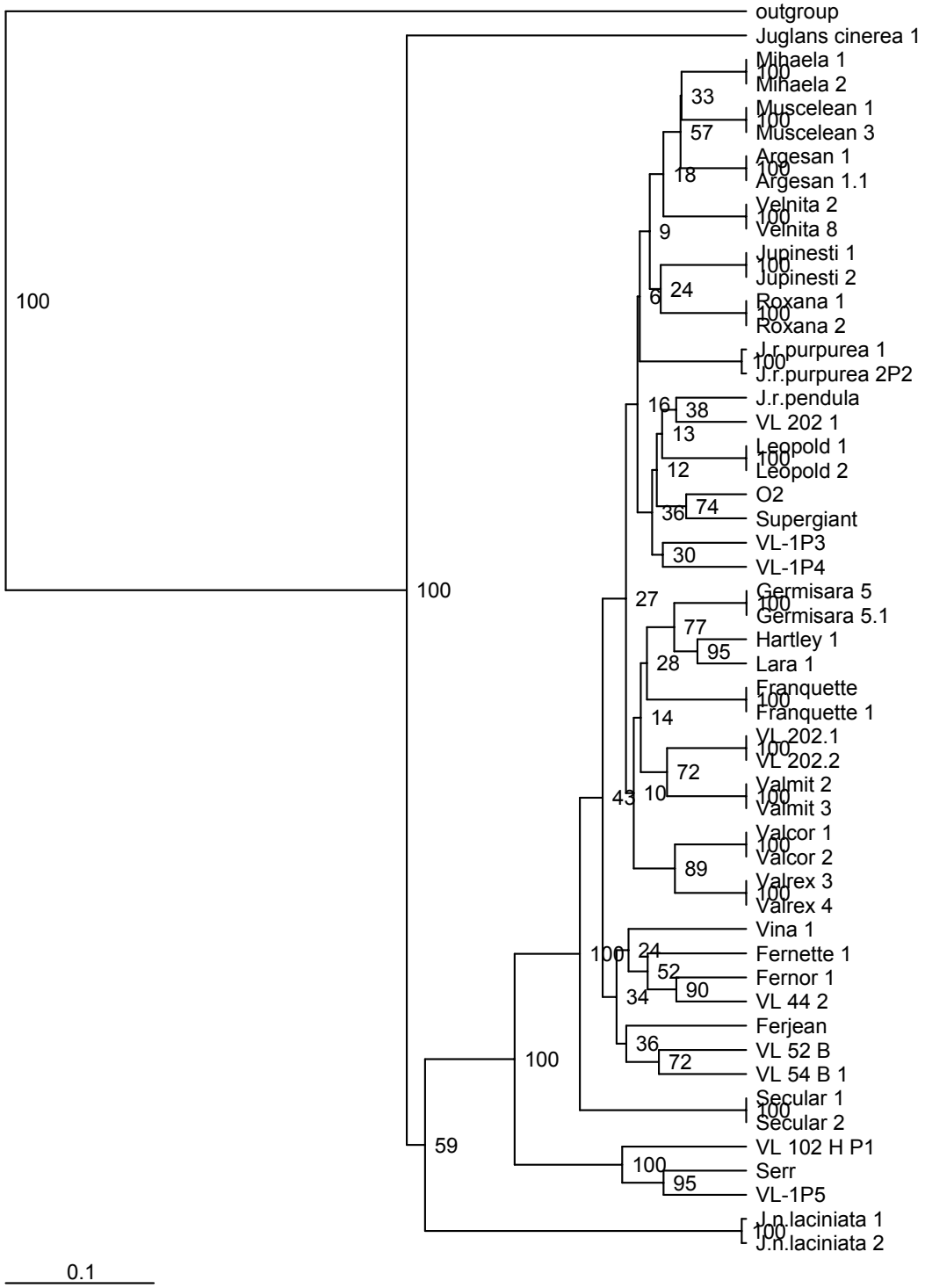


Fig. 3. UPGMA dendrogram generated using TreeView software, based on the genetic relationships between some of the *Juglans* accessions, calculated using Nei Li/Dice coefficient with FreeTree software

Discussion

The presence of the different patterns generated by RAPD primers shows variance between the accesions from the genetic point of view. This difference will be further analyzed using other types of molecular markers (Simple Sequence Repeats-SSR) in order to obtain a more precise molecular characterization of the studied genotypes.

The number of bands generated following RAPD analysis agrees with earlier studies made in other species (Galderisi *et al.* [19], Goulao *et al*. [12], Solar *et al.* [13], Casas *et al*. [20]), but is superior to the number of bands obtained by Nicese *et al*. [9] due to a superior genetic variability among the studied genotypes.

The high level of detected polymorphism is specific to a germplasm collection and to the high number of different accessions studied which belonged to three different species and had different geographic origins.

Identical accessions were detected in the collection and they corresponded to the phenotypic description of identity. These accessions can be seen in Figure 3 grouped together as terminal nodes with zero branch lengths. The other accessions were unequivocally identified by the 25 RAPD markers used, thus conferring a specific fingerprint due to the multiple genetic loci analyzed with the set of markers.

The calculated mean genetic distance was equal to 0.27 and the biggest calculated distance was equal to 0.502, between *Juglans cinerea* accession and Supergiant accession, these belonging to two different species, *Juglans cinerea* and *Juglans regia,* respectively. The genetic distance between the identical accessions was equal to 0. The mean distance was calculated using only one accession from each group of identical accessions. The mean genetic distance was influenced by the common genetic background of many accessions, thus having a middling value.

The UPGMA clustering analysis separated the studied accessions into eight groups, as can be seen in Figure 3. The accessions belonging to *Juglans regia* species were grouped together, separated by the accessions from *Juglans cinerea* species and *Juglans nigra* species. The outgroup was used in order to root the dendrogram, but also *Juglans cinerea* separated itself as an outgroup.

All the accessions that represented homologated cultivars obtained at ICDP Pitesti were grouped together in one cluster, this including also Velnita acessions, which is a cultivar homologated at SCDP Iasi and also *Juglans regia* variety purpurea accessions. This could be explained by the fact that these cultivars share the same genetic background.

One accession representing a selection from local populations from Pausesti – Otasau region was grouped together with Leopold accessions in the same cluster as *Juglans regia* variety pendula accessions, O2 accession, Supergiant accession and VL 1P3 and VL 1P4 accessions, a separate cluster from the one containing the other two accessions representing selections from local populations from Pausesti – Otasau region.

The accessions representing homologated cultivars obtained at SCDP Valcea all are grouped together in the same cluster containing also some French and American cultivars and also selections from Pausesti – Otasau region.

The accessions representing Fernette and Fernor cultivars clustered together due to their origin, both being obtained by a cross between Franquette x Lara cultivars. The accessions representing Ferjean and Vina cultivars were in the same cluster, together with some accessions representing selections from Valcea region.

The studied accessions were grouped mainly according to their origin, with some exceptions. The fact that the accessions representing Fernette and Fernor cultivars did not cluster with the accession representing Lara cultivar can be explained by the occurrence of non-parental bands, reported also by Hunt and Page, [1] 1992, Riedy *et al*., [1] 1992, Aruna *et al*., [1] 1993, Ayliffe *et al*., [1] 1994 and Nicese *et al*., [1] 1998). Different explanations have been suggested, such as formation of heteroduplex molecules between alternate RAPD alleles, competition for primer binding sites, somatic rearrangements or mutations within the primer binding sites or inside the amplified fragments (Nicese *et al*. [9]).

The accessions that represented Romanian homologated cultivars clustered mainly according to their origin, while the accessions that represented cultivars in the process of homologation clustered with different other accessions, possibly due to their genetic instability, common in cultivars being in the process of homologation.

Our results agree with earlier studies using RAPD at *Juglans* species, RAPD markers revealing the genotypic diversity of *Juglans* (Nicese *et al*. [9]). RAPD is therefore a reliable procedure for distinguishing between different *Juglans* accessions cultivated at S.C.D.P. Valcea. The collected data will be useful in developing DNA fingerprinting techniques for routine use in the orchard, to distinguish the valuable genotypes used in selection.

The identical accessions will be further preserved in the collection as duplicates, the RAPD markers used being able to confirm their identity.

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