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Molecular Characterization of Romanian Rose Genotypes Based on RAPD Markers

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Abstract: RAPD markers were used to characterize 26 *Rosa* genotypes, of which 17 were obtained in Romanian breeding programs. All of the twenty decamer primers yielded scorable amplification patterns and generated polymorphic bands among the genotypes studied. RAPD is therefore a reliable procedure for distinguishing among *Rosa* cultivars and also for assessing the genetic similarity among different genotypes useful in breeding selection programs. This work is the first of its kind reported in Romania.

Keywords: genetic diversity, *Rosa* varieties, breeding programs, parentage analysis

INTRODUCTION

The roses (sp. *Rosa*) are one of the most important ornamental flowers in terms of economy and cultural history of humankind, due to their beauty, attractiveness and different usage areas (Caliskan and Agaoglu, 2009). More than 200 species are found in the Northern Hemisphere where thousands of cultivars (Gudin, 2000) are grown in temperate zones (Kruessmann 1981, Cairns 1993). Most modern roses are generally triploid or tetraploid hybrids derived from 7-10 wild diploid rose species and a few tetraploid species (Gudin, 2000, Zhang, 2003). Traditional characterization and identification of cultivars is made according to the morphological traits, which are influenced by the environmental conditions. Furthermore, the genetically close cultivars are harder to identify using only morphological characteristics as the genetic distance between the varieties decreases (Jan and Byrne, 1999, Esselink et al., 2003). In this situation, proper identification of *Rosa* cultivars is needed to ensure that the product is sufficiently genetically pure to meet the expectations of growers and distributors.

Accurate cultivar identification is also important in protecting the legal rights of breeders. For this reason, molecular marker techniques have been developed to ensure unequivocal identification. Even cultivars which are phenotypically extremely similar can be easily distinguished based on differences in their genomes. Furthermore, molecular identification techniques can be used at any stage of plant development and they are not affected by environmental factors.

RAPD technique has been successfully used to analyze DNA polymorphism in several species (Nybom and Bartish, 2000, Raina et al., 2001, Nybom, 2004, Sivolap et al., 1998). Besides, good results have been described using RAPDs in *Rosa* (Caliskan and Agaoglu, 2009, Torres et al. 1993, Debener et al. 1996, Walker and Werner 1997, Jan et al. 1999, Martin et al. 2001, Atienza et al. 2005, Kaur et al. 2007), but the Romanian rose cultivars were not previously characterized using molecular markers, thus representing a new approach in Romania.

MATERIALS AND METHODS

Plant material. The twenty six *Rosa* genotypes used in this study were obtained with the courtesy of Dr. Stefan Wagner from the Fruit Research & Development Station Cluj.

Tab. 1.

The twenty six *Rosa* genotypes used in this study, their colour, class, parentage, country of origin, creator and year of introduction (Wagner, 2002).

Genotype	**Colour**	**Class**	**Parentage**	**Country of Origin/Creator/Year**
Ambassador	Orange	Hybrid Tea	Unknown x Whisky	France, Meilland, 1977
Aroma	Brick red	Floribunda	Rusticana x Lavender Dream	Romania, G. Roman and S.Wagner, 2009
Bonica 82	Pink	Floribunda	(rosa Sempervirens x Mlle. M. Caron) x Picasso	France, Meilland, 1981
Bordura de nea	White	Floribunda	Bonica x Incandescent	Romania, S. Wagner, 1995
Candy Rose	Pink	Floribunda	complex hybrid (7 parents)	France, Meilland 1980
Dame de Coeur	Carmin red	Hybrid Tea	Peace x Independence	Belgium, Lens, 1958
Foc de Tabara	Fire red	Floribunda	Paprika x Coup de Foudre	Romania, S. Wagner, 1970
Cluj 2010	Yellow to red	Floribunda	Sport of Foc de Tabara	Romania, S. Wagner and Angela Bokor, 2010
Golden Elegance	Golden yellow	Hybrid Tea	Sport of Ambassador	Romania, S. Wagner, 1995
Incandescent	Brick red	Hybrid Tea	Bond Street x Dame de Coeur	Romania, S. Wagner, 1991
Judit	Yellow, pink	Floribunda	Rosabunda x Circus	Romania, S. Wagner, 1996
La Sevillana	Brick red	Floribunda	complex hybrid	France, Meilland, 1978
Lavender Dream	Lilac pink	Floribunda	Yesterday x Nastarana	Netherlands, G. P. Ilsink, 1984
Luchian	Brick red	Floribunda	Paprika x Coup de Foudre	Romania., S. Wagner and Palocsay, 1972
Paprika	Carmin red	Floribunda	Marchenland x Red Favorite	Germany, M. Tantau, 1958
Pascali	White	Hybrid Tea	Queen Elisabeth x White Butterfly	Belgium, Lens, 1963
Perla Transilvaniei	Light mauve	Floribunda	Rusticana x Lavender Dream	Romania, G. Roman and S. Wagner, 2003
Petrina	Pure pink	Floribunda	Bonica x Lavender Dream	Romania, G. Roman and S. Wagner, 2004
Rosabunda	Light pink	Floribunda	Frankfurt am Main x Maria Callas	Romania, S. Wagner, 1979
Rosadoll	Carmin red with white	Floribunda	Rusticana x Lavender Dream	Romania, G. Roman and S. Wagner, 2005
Rosalinda	Pure pink	Floribunda	Rosabunda x Foc de Tabara	Romania, S. Wagner, 1994
Rusticana	Pale pink	Floribunda	Candy Rose x Yesterday	Romania, S. Wagner, 1995
Simfonia	White	Hybrid Tea	Mount Shasta x Pascali	Romania, S. Wagner, 1978
Violet	Mauve with white eye	Floribunda	Rusticana x Lavender Dream	Romania, G. Roman and S.Wagner, 2009
Yesterday	Lilac pink	Floribunda	(Phillys Bide x Sheperd's Delight) x Ballerina	United Kingdom, Harkness, 1974
Zburlici	Dark pink	Floribunda	Bonica 82 x La Sevillana	Romania, S. Wagner, 1995

Only nine genotypes were not results of Romanian *Rosa* breeding program, the other 17 being created by Stefan Wagner (Tab. 1).

DNA extraction Young leaves were collected in early summer and immediately stored at -80ºC prior to DNA extraction. Total DNA was extracted using the protocol developed by Lodhi *et al.* (1994) and modified by Pop *et al.* (2003). Two pieces of one cm^2 of leaf tissue were ground to fine powder in liquid nitrogen in an Eppendorf tube. 700 µL of 65ºC preheated extraction buffer (100 mM Tris-HCl, 20 mM sodium EDTA, pH=8, 1,4 M NaCl, 2 % (w/v) CTAB, 2% PVP, 5mM ascorbic acid and 4mM DIECA, the last three components being added to the extraction buffer just before the heating at 65^0C on the water bath) were added to the tube. The tube was then incubated at 65ºC for 25 minutes. The lysate was extracted with 700 µL of chloroform/isoamyl alcohol (24:1) and centrifuged for 15 min at 11000 rpm in a desktop centrifuge. In order to precipitate the nucleic acids, the aqueous fraction was mixed with an equal volume of 5M NaCl and then with 600 µL of ice cold 96% ethanol. The nucleic acid precipitate was washed two times in 76% ethanol and air dried before being resuspended in 50 µL TE buffer (10 mM Tris-HCl pH 8.0, 1mM disodium EDTA). The concentration and purity of extracted DNA were determined using a Nanodrop ND-1000 Spectrophotometer. DNA was diluted to 50 ng/µL and used for PCR amplification.

DNA amplification and electrophoresis conditions PCR amplification reactions were carried out as described by Williams *et al.* (1990). 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 Corp., Madison, WI, USA), 2,5 µL $MgCl_2$ (Promega Corp., Madison, WI, USA), 0,5 µL dNTP mix (Promega Corp., Madison, WI, USA), 0,5 µL RAPD primer (Microsynth, Balgach, Switzerland), 0,2 µL GoTaq polymerase (Promega Corp., Madison, WI, USA). DNA amplification was carried out in a 96 Well Gradient Palm-Cycler CG1-96 (Corbett Research, Sydney, Australia) 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 Corp., Madison, WI, USA). Gels were visualized on a UV light Biospectrum AC Imaging System (UVP BioImaging Systems, Upland, CA) after staining with 0,5 µg/µl Ethidium Bromide for 25 min.

Data analysis Gel images were analyzed using TL120 software (Nonlinear Dynamics, Newcastle upon Tyne, UK). PCR reactions were repeated two times and only reproducible bands were scored as present (1) or absent (0) and data entered into a binary matrix. Genetic distance between accessions was calculated using Nei and Li/Dice coefficient of similarity (Nei and Li, 1979). Cluster analysis was conducted with FreeTree software (Pavlicek *et al.*, 1999) using an UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm (Hampl *et al.*, 2001) and the dendrogram was visualized using TreeView software (Page, 1996). A synthetic outgroup was used for dendrogram rooting and bootstrap analysis was performed in 1000 repetitions.

RESULTS AND DISCUSSIONS

DNA extraction. The DNA quantity obtained varied between 150 ng/µl and 1200 ng/µl and and the A_{260}/A_{280} readings were between 1,70-1,91.

DNA amplification with RAPD primers. A total of 20 decamer primers from

Operon Technologies (synthesized by Microsynth) were used to amplify DNA extracted from the *Rosa* genotypes used in this study (Tab. 2). All the primers yielded scorable amplification patterns.

The total number of bands obtained with all the primers was 279, from which 237 were polymorphic. The average number of bands obtained per primer was 14, from which 12 were polymorphic, representing a percent of 84.9 %. The molecular weight of the bands ranged from 250 bp to 2800 bp. Primer OPA 04 generated the most polymorphic bands, 31, while primer OPB 09 generated the least polymorphic bands, 5, respectively. These results are similar to those presented by Caliskan and Agaoglu (2009).

Tab. 2.

The sequence of the RAPD primers used for amplification, the number of visible bands, the percent of polymorphic bands obtained and the range of the molecular weight of the bands

No.	Primer name	Primer sequence (5'- 3')	Visible bands (no.)	Percent of polymorphic bands	Range of molecular weight of bands (bp)
1	OPA 01	CAGGCCCTTC	29	100	650-2100
2	OPA 03	AGTCAGCCAC	7	85.7	740-1700
3	OPA 04	AATCGCGCTG	31	100	390-2500
4	OPA 09	GGGTAACGCC	10	80.0	250-2200
5	OPA 11	CAATCGCCGT	8	87.5	340-2800
6	OPAB 11	GTGCGCAATG	24	91.7	650-2800
7	OPAL 20	GAACCTGCGG	22	95.5	420-1700
8	OPB 09	TGGGGGACTC	9	55.6	280-1700
9	OPB 11	GTAGACCCGT	11	81.8	320-2300
10	OPB 17	AGGGAACGAG	15	93.3	290-2020
11	OPB 18	CCACAGCAGT	13	69.2	270-2600
12	OPC 02	GTGAGGCGTC	15	80.0	320-2700
13	OPC 14	TGCCTGCTTG	10	70.0	280-2500
14	OPD 20	ACCCGGTCAC	8	100.0	290-2200
15	OPE 14	TGCGGCTGAG	9	77.8	300-2600
16	OPF 02	GAGGATCCCT	9	66.7	350-2400
17	OPF 13	GGCTGCAGAA	17	64.7	290-2700
18	OPF 20	GGTCTAGAGG	12	83.3	270-2600
19	OPG 07	GAACCTGCGG	11	72.7	300-2500
20	OPH 02	TCGGACGTGA	9	77.8	320-2700

The results obtained using only six primers (OPA 04, OPA 01, OPAB 11, OPA 03, OPAL 20 and OPB 17) that yield 128 bands with 96 % polymophism produced a unique fingerprint for each of the 26 analyzed *Rosa* cultivars, allowing a clear identification of each genotype. The fingerprint for each genotype is defined by multiple markers (presence and/or absence of RAPD bands) presumably at multiple loci, this being of important value for cultivar characterization. Therefore, a set of six primers seems large enough to represent phylogenetic relationships among the studied *Rosa* cultivars. Our results agree with earlier studies using RAPDs (Baranek *et al.*, 2006, Casas A.M. *et al.*1999, Moreno and Trujillo, 2005). For example, 31 plum cultivars could be distinguished from each other by using only three primers (Ortiz *et al.*, 1997) and 18 peach cultivars could be distinguished from each other by using only six primers (Zhen-Xiang *et al.*, 1996).

The high level of identified polymophism may reflect the diverse genetic pool of *Rosa,* as similar results have been reported using RAPDs in other species (Mulcahy *et al.*,

1993, Casas *et al*., 1999, Monte-Corvo *et al*., 2000). Althought the occurence of non-parental bands has been reported by some authors in previous studies with RAPDs (Hunt and Page, 1992; Riedy *et al*., 1992; Aruna *et al*., 1993; Ayliffe *et al*., 1994; Pooler and Scorza, 1995, Scott *et al*., 1992) and different explanations have been suggested, it was not the case in our study, as all the cultivars shared common bands with at least one of the parents.

The calculated genetic distances among the studied genotypes varied between 0.154 (between Lavender Dream and Rosadoll) and 0.629 (between Petrina and La Sevillana), with an average of 0.451. Considering the origin of the studied rose cultivars, this can be explained by the number of common shared parents.

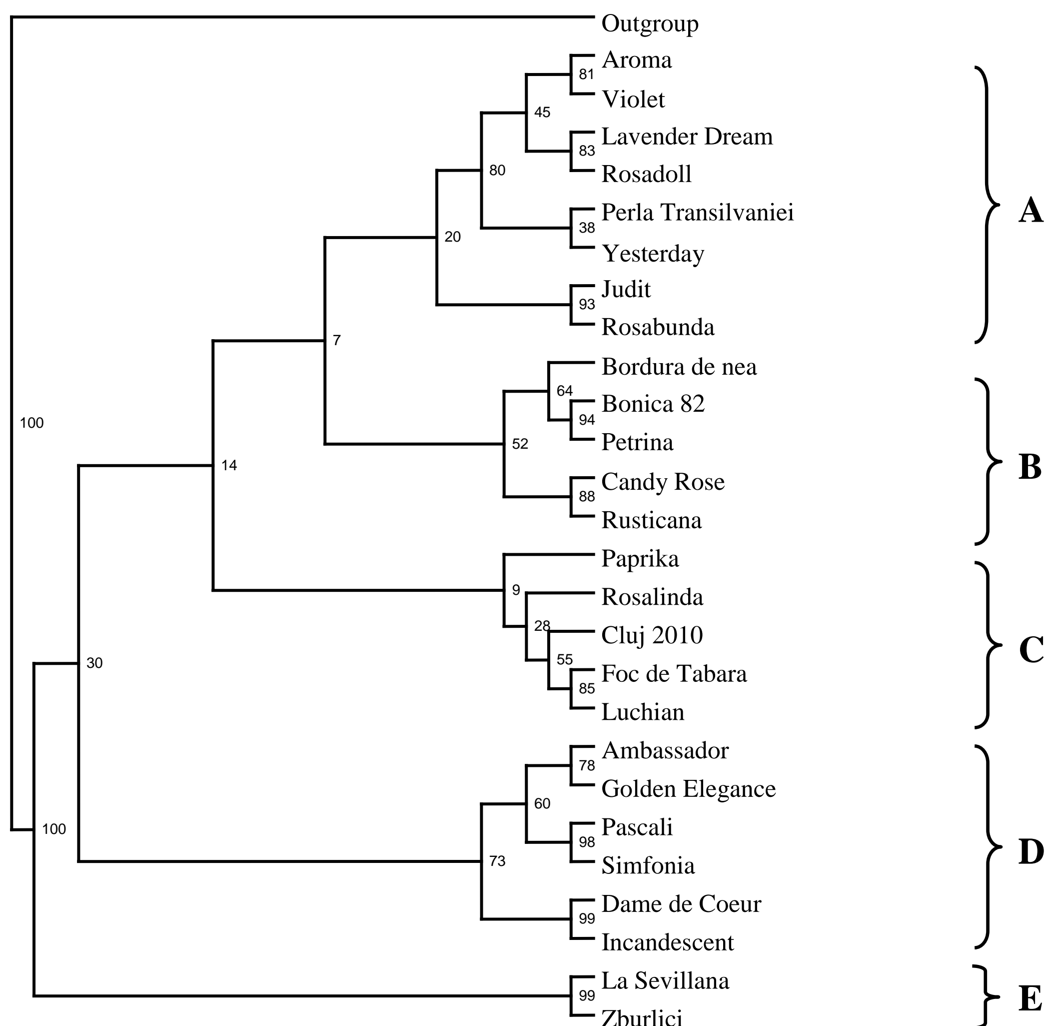

Fig. 1. Dendrogram built using UPGMA analysis of Nei Li/Dice coefficient of similarity between *Rosa* genotypes

The UPGMA dendrogram is presented in Fig. 1, the numerical values showed near its nodes being the bootstrap values. The RAPD primers used clustered the genotypes according to their origin, into five main groups (A-E). The bootstrap values of 100 were characteristic of solid nodes (the Outgroup clustered separately from all the *Rosa* genotypes and the group made of La Sevillana and Zburlici (E) clustered separately from the other *Rosa* groups). All the cultivars sharing common parents were grouped together and with at least one of the

parents (Nicese *et al*., 1998).

All the Hybrid Tea roses clustered together, as a separate group (D) between the Floribunda genotypes, showing their common origin (Floribunda roses were developed by crossing Hybrid Teas with Rosa multiflora. Ambassador was clustered with Golden Elegance, the last being a sport of the first, Pascali with Simfonia and Dame de Coeur with Incandescent, the last four having Queen Elisabeth as a common ancestor.

Group C includes Paprika and all the other cultivars to which the aforementioned is a common ancestor (Luchian and Foc de Tabara are both the result of a crossing between Paprika and Coup de Foudre, Cluj 2010 is a sport of Foc de Tabara and Rosalinda comes from the cross Rosabunda x Foc de Tabara). The genetic distances between Paprika and the other cultivars in its group range from 0.36 (between Paprika and Luchian) to 0.42 (between Paprika and Cluj 2010, this sport being the most distant from the common ancestor of this group) with an average of 0.447 (Nicese *et al*., 1998).

Group B is divided into two subgroups, one consisting of Bonica and its descendants, Bordura de Nea and Petrina, respectively, and the other of Candy Rose and Rusticana, the last being a descendant of Candy Rose.

Group A is divided into a subgroup including Yesterday and its descendants, Perla Transilvaniei, Rosadoll, Aroma and Violet all being selected from crossing Lavender Dream x Rusticana and another subgroup consisting of Judit and Rosabunda, the first being selected from a cross Rosabunda x Circus. The genetic distances between Yesterday and is descendants range from 0.28 to 0.36, the closest being Perla Transilvaniei and Lavender Dream and the furthest being Violet.

As been previously reported with other crop species, (Aruna *et al*., 1993; Dunemann *et al*., 1994; Dweikat *et al*., 1993; Hallden *et al*., 1994) in our study we observed a fairly close relationship between the known pedigree and the genetic similarity obtained with RAPDs. This is of great importance in breeding *Rosa* cultivars, as the origin of the progenies can be tested.

CONCLUSIONS

The used markers allowed us to unequivocally distinguish all the *Rosa* cultivars analyzed. Six primers (OPA 04, OPA 01, OPAB 11, OPA 03, OPAL 20 and OPB 17) completely and reliably separated all genotypes. The ability to identify a cultivar using just six primers demonstrates that RAPD markers are suitable for sure, easy, quick and inexpensive identification of rose cultivars.

RAPD technique can detect enough polymorphism to differentiate among rose genotypes, even among cultivars closely related because of their common parents (Aroma, Violet, Perla Transilvaniei) and also between the sports of different cultivars (Cluj 2010, Golden Elegance).

RAPD markers were able to group rose cultivars according to their origin and variety and determine genetic similarities between them. A clear separation between Floribunda and Hybrid Tea varieties was obtained in this study confirming the morphological classification of the roses.

We have presented a procedure to type rose genotypes by RAPD analysis. In particular, we have genetically identified rose cultivars of Romanian origin, providing a valuable tool for plant breeders.

RAPD is therefore a reliable and a relatively simple procedure to study genetic relationships among *Rosa* cultivars, which can be useful in current rose breeding programs,

allowing the identification of new cultivars as well as the assessment of the genetic similarity among different genotypes.

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