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GENETIC ANALYSIS OF SOME ROSES CULTIVARS APPROPRIATE FOR S-E ROMANIA CLIMATE USING PCR - ISSR TECHNOLOGY

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Abstract

Current cultivated roses varieties were obtained through intensive cross-breeding between a limited number of species of genus Rosa (about 8 to 20 from 200 wild species) and for many of them there are no accurate information about their genealogy. The present study aims to determine the genetic diversity of 10 rose varieties, from Botanical Garden of Bucharest collection, which are not included in the official list of rose varieties from Romania, according ISTIS. Choosing these cultivars started from the need to identify rose varieties resistant to the climate in SE Romania with extended summer drought and winter frost. A number of 86 individuals were genotyped using PCR-ISSR technique. From a total of 23 tested ISSR primers, 11 were selected due to their capacity to produce informative band patterns. These primers generated 292 ISSR markers, with a mean of 26.54 bands/primer and a polymorphism percentage of 96.025%. The average values of polymorphic information content (PIC), resolving power (Rp) and marker index (MI) proved the discriminatory power of ISSR markers (i.e PIC varied between 0.22 and 0.43 and Rp between 7.81 and 17.2). Genetic diversity as revealed by Shannon index (I=0.45±0.0734), genetic differentiation index (G_{ST}=0.027) and estimate of gene flow (Nm=16.982) is relatively high. The AMOVA analysis showed that 67.36% of the total genetic variation resides within cultivars, which is similar with other shrub species. The UPGMA dendrogram built on ISSR markers grouped the 10 cultivars in two major clusters according with their origin and some of their phenotype characteristics. ISSR fingerprinting proved to be appropriated for the quantification of the level of genetic variation in the selected cultivars.

Key words*: rose cultivars, PCR-ISSR, genetic diversity, dendrogram.*

INTRODUCTION

Roses (*Rosa x hybrida)* are some of the most cultivated ornamental plant species, presenting an extremely large diversity of colors, growth shapes, and fragrances. It is estimated that there are between 170 and 300 Rose species (Panwar et al., 2015), but only 8-10 of them have been used for creating the commercial varieties grown today, which are more than 20,000 (Gudin, 2000). Genus Rosa is extremely difficult to classify due to a number of factors, such as the human intervention, extremely wide spreading area, polyploidy and complex inter-specific hybridization. Moreover, the constant emergence of new commercial varieties makes the use of precise identification techniques to be absolutely necessary, particularly to certify the uniqueness of the new varieties.

A number of comparative investigations have been carried out to explore which molecular technique is most suitable and most reliable for biotype/cultivar identification (Robichaud, 2006). DNA-based molecular marker systems are efficient and informative for the genetic analysis of roses because DNA polymorphism indicated by these markers is not affected by environmental conditions. Any choice of DNA marker method depends, among other factors, on the scale and purpose of cultivar identification. Thus, in recent years, different DNA markers have been used to estimate the levels of genetic diversity, genetic structure, dispersal rates and paternity in numerous plant

species (Aldrich, 1998; Streiff, 1999; Craft, 2002; Glaubitz, 2003; Tabbener and Cottrell, 2003; Christopolus, 2010; Potter et al., 2002). Among them, ISSR markers involve the use of microsatellite sequences as primers in PCR, thereby amplifying the DNA sequence located between two SSR repetitions. Due to the nature of the primer sequence (short di, tri or tetranucleotide tandem repeats) it is possible to obtain a large number of polymorphic fragments, which make these markers very useful in identification and discrimination of closely related varieties and in assessing phylogenetic relationships between species.

The present study aims to determine, based on ISSR markers, the genetic variation of 10 rose varieties from Botanical Garden of Bucharest collection, resistant to extended summer drought and winter frost, specific of climate in SE Romania. These cultivars are not yet included in the official list of rose varieties from Romania, according to National Institute for Variety Testing and Registration and the quantification of genetic diversity and the establishment of reletedness relations are very important for their molecular identification.

MATERIALS AND METHODS

Plant material

Plant material used in this study comprises a set of 10 rose cultivars, belonging to "English Garden" roses, "Floribunda", "Thea Hybrida" roses (Table 1). All 10 cultivars are gown and maintained in the "Dimitre Brândză" Botanical Garden rosarium.

DNA extraction

Fresh, healthy leaf tissue from 86 individuals (on average 5 individuals/cultivar) was used for DNA extraction, applying modified CTAB method (Dehestani et Kazemi, 2007, Pavlusenco et al., 2015). DNA samples were further purified twice with an equal volume of chloroform:isoamylic alcohol (24:1) and CTAB solution (0.7M NaCl, 1% CTAB), precipitated with cold isopropanol and washed with 3M ammonium acetate solution and cold ethanol. Finally DNA was re-suspended in 0.1X TE (10 mM Tris pH=8, 1 mM EDTA pH=8). DNA was quantified spectrophotometric, using NanoVue V2.0.3 Plus Spectrophotometer (GE Healthcare) and then stored at -20°C for further analysis.

ISSR analysis

Twenty three ISSR primers (Integrated DNA Technologies, Inc. US) were screened for their ability to produce informative patterns in terms of the repeatability and ability to distinguish between cultivars (Table 2). Each reaction was performed in a final volume of 12 µl which contained 2X Go Taq® Green Master Mix (Promega, U.S.A.), 10 µM primer and 30 ng DNA. The PCR program consisted in an initial denaturation step (4 min at 94°C), 40 cycles of: 45 s at 94°C, 1 min at 40-57°C (Table 2), 2 min at 72°C and a final amplification step (10 min at 72°C). All PCR reactions were accomplished in a PTC 100 thermal cycler. PCR products were visualized in 2% agarose gel electrophoresis in 1 X TBE buffer and photographed with Canon PowerShot SX150IS.

Data Analysis

The electrophoretic ISSR profiles were analyzed with PyElph 1.3 software package (Pavel et Vasile, 2012). Each DNA band was treated as an independent character, being scored as present (1) or absent (0), resulting a binary data matrix which was used to compute primer banding characteristics such as: total number of bands (TNB), number of polymorphic bands (NPB), percentage of polymorphic bands (PPB), polymorphic information content (PIC), resolving power (Rp) and marker index (MI) (Anderson et al., 1993; Prevost et Wilkinson, 1999). The molecular data were further analyzed with POPGENE Version 1.31 (Yeh et al., 1999) and Nei's gene diversity (h), Shannon's information index (I) (Nei et al., 1979), coefficient of genetic differentiation among populations (GST), and gene flow estimates (Nm) were calculated. Also, Arlequin 3.5 (Excoffier et al., 2010) was used for the analysis of molecular variance (AMOVA). The matrix of genetic distances generated by PyElph program was implemented in MVSP 3.22 software package (Kovach, 2013) and used to perform cluster analysis and to construct an UPGMA dendrogram. Also, for a better representation of the genetic relationships among varieties, a principal coordinate analysis (PCO) was conducted.

Table 1. Details of rose genotypes representing "English", "Floribunda" and "Thea Hybrida" groups taken for genetic analysis using ISSR markers

No.	Genotype	Group	Patent/origin
1	Abraham Darby	English	Yellow Cushion‘ x Aloha‘
2	Heritage	English	seedling x (Iceberg PL x Wife of Bath)
3	Forever Young	Floribunda	Cindy de Meilland x Samantha
4	Rhapsody in Blue	Floribunda	seeds Summer Wine x pollen International Herald Tribune × [Blue Moon × Montezuma] × [Violacea × Montezuma]
5	Angela	Floribunda	Yesterday x Peter Frankenfeld
6	Acapella	Thea hybrid	Seedling × Seedling
7	Caprice de Meilland	Thea hybrid	seeds Tino Rossi × Rendez-Vous and pollen Sonia
8	Christoph Columbus	Thea hybrid	seeds Coppélia x pollen from Ambassador
9	Ingrid Bergman	Thea hybrid	Precious Platinum × Seedling
10	Red Berlin	Thea hybrid	Huibert W Olij (Olanda, 1997)

RESULTS AND DISCUSSIONS

ISSR amplification. A total of 23 ISSR primers were tested, from which only 11 were selected, due to their capacity to produce informative band patterns. These primers generated 292 bands, from which 283 were polymorphic (Table 2). The total number of bands/primer varied between 17 (ISSR 4a) and 33 (ISSR-1a), with a mean of 26.54 bands/primer. The primer with the lowest degree of polymorphism was ISSR-2 (80.95%), while ISSR-1a, ISSR-7, UBC-807, UBC-808, UBC-823, UBC-827 and UBC-835a have had a 100% polymorphism (Figures 1 and 2). The ability of generated ISSR fragments to distinguish between individuals and cultivars was evaluated based on three parameters: a) PIC of a band, calculated according to Roldan-Ruiz et al., 2000, b) Rp that is based on the distribution of alleles within the sampled genotypes and was calculated according to Prevost and Wilkinson, 1999 and c) MI as proposed by Powell et al., 2015, which is the product between PIC and EMR (EMR=effective multiplex ratio, namely the product between the fraction of the polymorphic loci and the number of the polymorphic loci) (Table 2).

As shown in Table 2, the lowest PIC and Rp values were obtained for primer ISSR – 4b (PIC= 0.22, Rp = 7.81) and the highest for ISSR-4a (PIC = 0.43, Rp = 16.29) and ISSR-1a (PIC = 0.35, Rp = 17.20). Also, ISSR markers generated by ISSR-1a primer had the highest MI value (11.55), followed by UBC 808.

For assessing the genetic diversity of rose varieties, the *Nei* (h) index, which measures the heterozygosity level and *Shannon* (I) index were computed. Usually, for a correct estimation of genetic diversity both of these parameters should be over 0. In our study *h* has a value of 0.2843 (with 0.0606 standard deviation) and I = 0.4561 (0.0734 standard deviation) (Table 3), data that are in line with the scientific literature.

The G_{ST} value, 0.0287, indicating the degree of differentiation between varieties, is consistent with the high value of the *Nm* parameter (16.929), recorded usually for populations affected by an intens gene flow. The partitioning of the molecular variance (AMOVA), performed with *Arlequin 3.5.1.2* software, revealed that only a small percentage of the genetic variation resides among varieties, showing that the most of the genetic diversity occurs within varieties (67.36%) (Table 4).

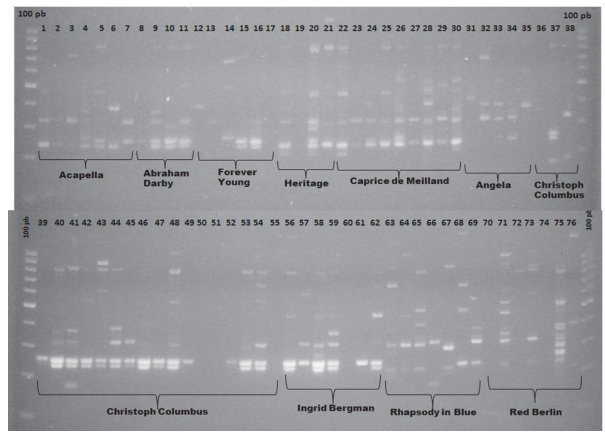

Figure 1. ISSR-4b primer PCR amplification profiles of all 10 rose cultivars. 100 bp ladder; Lanes 1-76 individuals from all the rose cultivars

Table 2. List of primers, their sequences, annealing temperatures, number of the amplified fragments, polymorphic information content and resolving power

Primer	Sequence (5`-3`)	°C	TNB	NPB	PPB	PIC	Rp	MI
ISSR 1a	$(AG)_8CT$	53	33	33	100	0.35	17.20	11.55
ISSR 2	$(GA)_8C$	52	21	17	80.95	0.34	12.16	7.14
ISSR 4a	$(AC)_8CT$	57	18	15	83.33	0.43	16.29	7.15
ISSR 4b	$(AC)_8TT$	53	27	27	100	0.22	7.81	5.94
ISSR 7	$(GA)_8ACC$	57	26	26	100	0.34	12.55	8.84
UBC 807	$(AG)_8T$	52	26	26	100	0.29	13.41	7.54
UBC 808	$(AG)_8C$		32	32	100	0.30	13.97	9.60
UBC 823	$(TC)_8C$		24	24	100	0.31	11.32	7.44
UBC 827	$(TC)_8G$		30	30	100	0.23	8.92	6.90
UBC 835a	$(AG)_8CC$	55	26	26	100	0.24	8.32	6.24
UBC 840a	$(GA)_8TC$	52	25	23	92	0.31	12.12	7.75
		Sum	**292**	**283**				
		Mean	**26.54**	**25.72**	**96.02**	**0.30**	**12.19**	**7.88**

°C = annealing temperature, TNB=total number of bands; NPB = number of polymorphic bands, PPB = percentage of polymorphic bands, PIC = polymorphic information content, Rp = Resolving power, MI = marker index

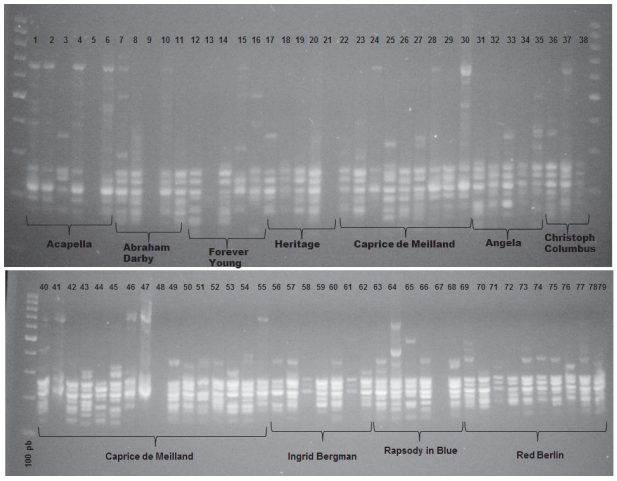

Figure 2. ISSR-840a primer PCR amplification profiles of all 10 rose cultivars: 100 bp ladder; Lanes 1-76 individuals from all the rose cultivars

Table 3. Genetic diversity parameters

	h	I	G_{ST}	*Nm*
Average	0.2843	0.4561	0.0287	16.9292
s.d.	0.0606	0.0734		

h = *Nei* genetic diversity, *I* = *Shannon* index, G_{ST} = interpopulation differentiation coeficient, N_m = gene flow, s.d. = standard deviation

The genetic distance matrix obtained after de analysis of ISSR data was used to build the UPGMA dendrogram and to reconstruct the relatedness relations between rose cultivars (Figure 3). The 10 roses varieties were grouped in two major clusters, in one being included Ingrid Bergman (I), Rhapsody in Blue (B), Red Berlin (R) şi Christoph Columbus (C) varieties and second comprising Angela (U), Caprice de Meilland (M), Heritage (H), Forever Young (F) şi Abraham Darby (A), while Acapella had clustered outside of this two major groups.

PCO analysis, conducted with the appropriate application of *M.V.S.P ver.3.2.2*, confirmed the clustering model outlined by the UPGMA dendrogram (Figure 4 a and b).

Table 4. AMOVA molecular variance values for the analysed rose varieties

Source of variation	f.d.	Sum. Sq.	Percentage of variation
Among pop.	9	1235.858	32.64
Within pop.	76	2099.224	67.36
Total	**85**	**3335.081**	**100**

f.d. = freedom degree

DNA fingerprinting with the 11 ISSR markers (selected from the 23 ones tested) was found to be efficient to distinguish different rose cultivars. Thus, molecular markers obtained by PCR-based techniques can play an important role in the analysis of genetic diversity in ornamental plants which generally have a very complex and controversial origin. The percentage of polymorphism reported in this study varied from 80.95 to 100, with a mean value of 96.02 which is in concordance with others researches on rose cultivars using ISSR or RAPD markers.

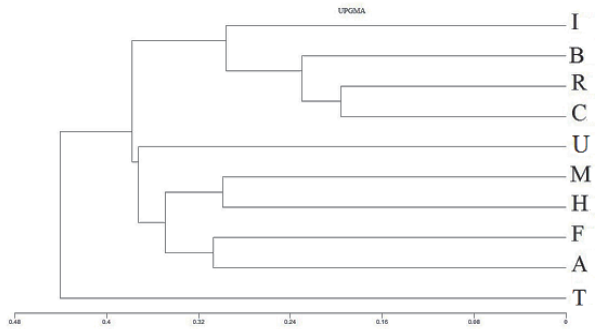

Figure 3. *UPGMA d*endrogram for 10 rose cultivars: T-Acapella, A-Abraham Darby, F-Forever Young, H-Heritage, M-Caprice de Meilland, U-Angela, C-Christoph Columbus, I-Ingrid Bergman, B-Rhapsody in Blue, R-Red Berlin

For example, a similar study based on ISSR markers conducted in India on cultivars from "Thea Hybrida" and "Floribunda" groups revealed a level of polymorphism of 94% (Panwar et al., 2015). A slightly lower percentage of polymorphism (90.3%) was determined for the roses from Taif region (Saudi Arabia) **(**El-Assal et al., 2014).

There is a correlation between the primer sequence and the polymorphism percentage, the markers with AG and TC motives generating the highest level of polymorphism comparing with GA motives. These findings on AG motives are sustained by many literature data (Panwar et al., 2015).

The computed PIC values (average 0.30) are in the normal range for dominant markers (0-0.5) (Muthusamy et al., 2008) as well as the other parameters used for describing the discriminating power of the ISSR markers.This fact allowed to use the ISSR data for UPGMA dendrogram construction. The 10 rose cultivars were grouped in two major clusters according with their origin and some of their phenotype characteristics. It can be inferred that the varieties with none to mild fragrance have clustered, even subclustered, together (for example like Red Berlin with Christoph Columbus).

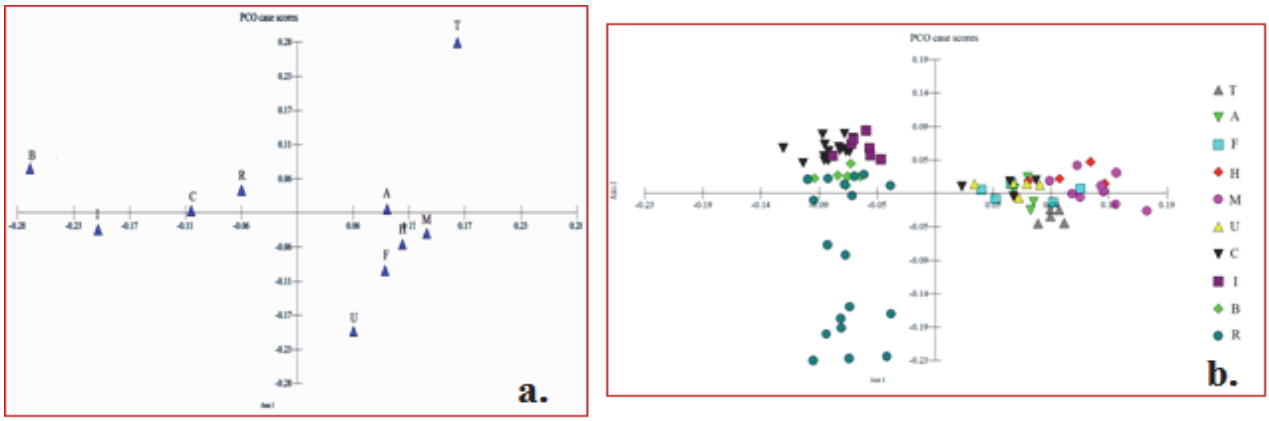

Figure 4. *PCo* analysis: a. for the 10 roses varieties under study; b. for each analysed individual: T-Acapella, A-Abraham Darby, F-Forever Young, H-Heritage, M-Caprice de Meilland, U-Angela, C-Christoph Columbus, I-Ingrid Bergman, B-Rhapsody in Blue, R-Red Berlin

The results of the AMOVA analysis revealed that the main genetic variation is at intra-populational level, which is similar with those recorded for the plant species with short life cycle like *Camellia sinensis* (Ji et al., 2011), *Rhizoma coryalyis* (Qiu et al., 2009), *Rheum officinale* (Wang et al., 2012*)* and *Torreya jackii* (Li et al., 2007).

CONCLUSIONS

The ISSR method proved to be appropriated for the accurate identification and drawing of the molecular passport of the roses varieties, for the confirmation of cultivar origins, and quantification of their genetic diversity. In the present study, ISSR markers allowed the precise DNA fingerprinting of ten rose cultivars able to survive in prolonged drought or frost conditions like those from SE of Romania.

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