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The efficiency of the genetic markers in the characterization of sea buckthorn varieties

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Abstract

Hippophae rhamnoides ssp. carpatica (sea buckthorn) is a perennial multipurpose plant, recently introduced in culture in Romania. The aim of the present study was to evaluate the genetic variation and the relatedness relations in 6 cultivated varieties of sea buckthorn from Romania using ISSR (Inter Simple Sequence Repeat) and RAPD (Random Amplified Polimorphic DNA) methods. The generated ISSR and RAPD markers showed a high power of discrimination as results from the analysis of PIC, AvIb and Rp values. The analysis of molecular variance (AMOVA) revealed that most of the genetic diversity occurs within varieties (87% for ISSR and, 90% for RAPD). The results on GST (0.0189 for ISSR primers and 0.0276 for RAPD) were in agreement with the intense gene flow between varieties (Nm=25.9450 for ISSR and Nm=17.5895 for RAPD) and demonstrated a reduced genetic differentiation. Both DNA markers grouped the 6 analyzed varieties into two clusters, the grouping pattern revealed by the UPGMA dendrograms being confirmed also by the PCO analysis.

Keywords: DNA markers, ISSR, RAPD, sea buckthorn, cultivars, molecular variation

1. Introduction

Sea buckthorn (*Hippophae rhamnoides* L.) is an important perennial plant from the temperate regions of Europe and Asia (RUAN & al.[1]), which is appreciated for many reasons: strong root system; in symbiosis with Frankia fixes the atmospheric nitrogen with 2-fold higher rate than soya (CHEN & al. [2]); young leaves have a high content in proteins and flavonoids (AHMAD & al. [3]); seeds are rich in β-caroten and vitamin E and, the most important part, the berries, have a high nutritional and therapeutic value (ZEB[4], SHAH & al. [5]).

The increased interest for sea buckthorn cultivation has generated in Romania the need for new varieties, adapted to the local environmental conditions. According to the current requirements regarding the export of seedlings, sea buckthorn varieties must be described in detail both phenotypically and genetically. Morphological traits have their limitations, since they are affected by environmental factors; in contrast, molecular methods provide valuable tools for the precise identification of the plant genotypes and the apportionment of their genetic variation (CHEN & al. [2]).

Several PCR based methods are frequently used for DNA fingerprinting, quantification of the genetic variation and establishment of the relatedness relationships between sea buckthorn

populations (RUAN & al. [6], SRIHARI & al. [7]), but RAPD and ISSR are the most common. Unlike RAPD, ISSR method uses longer primers, which require more precise annealing temperatures, further resulting in more accurate amplification patterns.

The main goal of this study was to estimate the genetic variation in 6 cultivated varieties of sea buckthorn from Romania based on the molecular data generated by ISSR and RAPD methods. Discrimination power of ISSR and RAPD markers was determined, as well as the possibility to reconstruct the genetic relationships among and between populations.

2. Matherials and methods

Plant material. Leaf tissue was sampled from 24 individuals belonging to 6 cultivated varieties of *H. rhamnoides* ssp. *carpatica* from Romania (varieties Auras, Diana, Ovidiu, Victoria originated from Danube Delta and varieties Serpenta and Tiberiu from Bacau region).

Genomic DNA was extracted using a modified CTAB method (DEHESTANI [8] & al). DNA was quantified using NanoVue V2.0.3 Plus Spectrophotometer (GE Healthcare) and then stored at -20°C for further analysis.

ISSR and RAPD methodology. Twenty two ISSR primers and seventeen RAPD primers (Integrated DNA Tehnologies, Inc. US) were screened for their ability to produce informative patterns in terms of the repeatability, scorability and ability to distinguish between cultivars (Table 1 and 2). Each reaction contained 2X Go Taq® Green Master Mix (Promega, U.S.A.), 10µM primer and 30 ng ADN, in a final reaction volume of 12 µl. The PCR program was: a) ISSR – 1 cycle of 4 min at 94°C; 40 cycles of: 45 s at 94°C, 1 min at 40-57°C (see Table 1), 2 min at 72°C; 1 cycle of 10 min at 72°C; b) RAPD – was performed as described by SIMON-GRUITA & al [9]. All PCR reactions were realized in a Corbett RESEARCH CG1-96 gradient thermal cycler. Upon the completion of the reaction, PCR products were visualized in 2% agarose gel electrophoresis in 1 X TBE buffer. The ISSR and RAPD assays were repeated three times for each primer, using the same reagents and procedures.

Data Analysis. The electrophoretic ISSR and RAPD profiles were analyzed with PyElph 1.3 software package (PAVEL & al. [10]). The DNA fragments were scored as present (1) or absent (0), each band being treated as an independent character and the resulting binary data matrix was used to compute primer banding characteristics such as: total number of bands (TNB), number of polymorphic bands (NPB), percentage of polymorphic bands (PPB), polymorphism informative content (PIC), resolving power (Rp) and average of band informativeness (AvIb) (ANDERSON & al. [11], PREVOST & al. [12]). The molecular data were further analyzed with POPGENE Version 1.31 (YEH & al. [13]) and Nei's gene diversity (h), Shannon's information index (I) (NEI & al. [14]), coefficient of genetic differentiation among populations (GST), and gene flow estimates (Nm) were calculated. Also, Arlequin 3.5 (EXCOFFIER & al. [15]) was used for the analysis of molecular variance (AMOVA).

The genetic distance matrix generated by PyElph program was implemented in MVSP 3.22 software package (KOVACH [16]) and used to perform cluster analysis and to construct two separate UPGMA dendrograms based on the ISSR and RAPD data. For a better representation of the genetic relationships among varieties, a principal coordinate analysis (PCO) was conducted.

3. Results

The 22 ISSR primers generated a total of 477 markers, 463 of which being polymorphic (Table 1). The number of scorable markers produced per primer ranged from 10 (primer HB 15) to 29 (primer UBC 881), with an average of 21.68. An example of an ISSR pattern

obtained with the primer UBC 818 is shown in Figure 2. The percentage of polymorphism varied between 80.00 to 100, with a mean value of 95.78 across all the genotypes (Table 1).

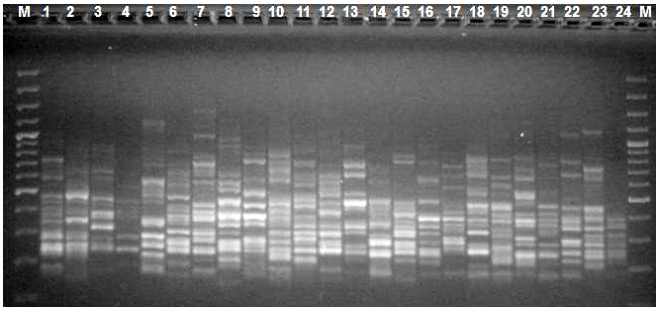

Figure 1. ISSR profiles using primer UBC 818: M=100 bp plus DNA Ladder (Thermo Scientific), 1-4*Tiberiu*, 5-8 *Diana*,9-12 *Ovidiu*, 13-16 *Victoria*, 17-20 *Serpenta*, 20-24 *Auras*

Table 1. Details on the ISSR primers and amplification results

Primer	Sequence(5`-3`)	Tm (°C)	TNB	NPB	PPB	PIC	Rp	AvIb
ISSR 1a	(AG)8CT	53	21	20	95.23	0.24	12.08	0.24
ISSR 1b	(AG)8TT	50	26	24	92.3	0.29	9.24	0.16
ISSR 4a	(AC)8CT	57	26	26	100	0.02	10.74	0.2
ISSR 4b	(AC)8TT	53	21	21	100	0.26	10.16	0.23
ISSR 7	(GA)8ACC	57	23	23	100	0.25	7.66	0.17
UBC 807	(AG)8GT	52	21	20	95.23	0.26	13.83	0.32
UBC 808	(AG)8C		14	12	85.71	0.13	6.91	0.24
UBC 809	(AG)8G		23	23	100	0.32	14.24	0.3
UBC 811	(GA)8C	53	25	24	96	0.38	17.49	0.35
UBC 812	(GA)8A	50	17	14	82.35	0.13	8.58	0.25
UBC 816	(CA)8T	55	21	21	100	0.27	12.24	0.27
UBC 817	(CA)8A	54	27	26	96.29	0.34	19.49	0.36
UBC 818	(CA)8G	49	26	26	100	0.39	22.08	0.42
UBC 823	(TC)8C	52	26	26	100	0.39	14.33	0.27
UBC 827	(TC)8G		21	21	100	0.24	6.58	0.15
UBC 835a	(AG)8CC	55	17	17	100	0.24	8.16	0.24
UBC 835b	(AG)8TC	52	23	23	100	0.25	7.83	0.17
UBC 840a	(GA)8CT		23	21	91.30	0.29	10.83	0.36
UBC 840b	$(GA)_8$	40	14	13	92.85	0.19	10.28	0.32
UBC 881	CCCT(CCCCT)2C	54	29	29	100	0.38	18.41	0.32
HB 11	(GT)6CC	45	10	8	80	0.06	2.16	0.10
HB 15	(GTG)3GC		23	23	100	0.31	10.24	0.30

Tm- annealing temperature, TNB- total number of bands, NPB- number of polymorphic bands, PPB- percentage of polymorphic bands, PIC- polymorphism informative content, Rp- resolving power, AvIb- average of band informativeness

Out of the 260 reproducible bands generated by the 12 RAPD selected primers from the 17 initially tested, 243 were polymorphic (Table 2). The number of bands per primer ranged from 17 (primer OPB-11, OPC-6) to 27 (primer OPB-18). Figure 2 presents the RAPD profile for primer OPD 3. The percentage of polymorphism varied between 78.95 and 100, with an average of 93.05 (Table 2).The PIC values varied from 0.02 to 0.39 with an average of 0.25 for ISSR primers and between 0.19 and 0.39, with a mean of 0.26 for RAPD markers. The AvIb values were similar for both ISSR and RAPD primers (Table 1and Table 2), ranging

from 0.10 to 0.42 for ISSR and from 0.15 to 0.38 for RAPD. Higher differences were recorded for Rp values, this parameter showing a minimum value of 2.16 (HB 15) and a maximum of 22.08 (UBC 818), with an average of 11.52 for ISSR, and a minimum of 5.74 (OPC-02) and a maximum of 19.33 (OPD-3), with a mean of 10.33 for RAPD (Table 1and 2).

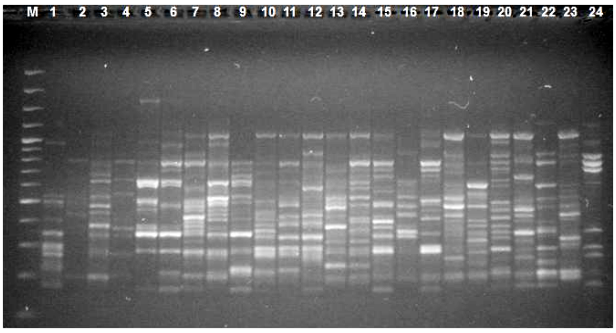

Figure 2. RAPD profiles using primer OPD-3: M=100 bp plus DNA Ladder (Thermo Scientific), 1-4*Tiberiu*, 5-8 *Diana*, 9-12 *Ovidiu*, 13-16 *Victoria*, 17-20 *Serpenta*, 20-24 *Auras*

Table 2. Details on the RAPD primers and amplification results

Primer	Sequence (5`-3`)	Tm (°C)	TNB	NPB	PPB	PIC	Rp	AvIb
OPB 11	GTAGACCCGT	36	17	16	94.11	0.19	6.91	0.20
OPB 18	CCACAGCAGT		27	25	92.59	0.28	16.24	0.33
OPBB 14	GTGGGACCTG		19	15	78.95	0.19	11.08	0.29
OPC 1	TTCGAGCCAG		24	23	95.83	0.35	12.74	0.31
OPC 2	GTGAGGCGTC		19	17	89.47	0.18	5.74	0.15
OPC 4	CCGCATCTAC		25	25	100	0.32	10.66	0.21
OPC 6	GAACGGACTC		17	14	82.35	0.17	6.24	0.24
OPC 8	TGGACCGGTG		21	20	95.23	0.29	10.08	0.31
OPD 1	ACCGCGAAGG		21	21	100	0.21	6.74	0.18
OPD 3	GTCGCCGTCA		25	25	96.15	0.39	19.33	0.38
OPD 8	GTGTGCCCCA		25	23	92	0.27	10.66	0.21
OPD 11	AGCGCCATTG		19	19	100	0.23	7.58	0.30

Tm- annealing temperature, TNB- total number of bands, NPB- number of polymorphic bands, PPB- percentage of polymorphic bands, PIC- polymorphism informative content, Rp- resolving power, AvIb- average of band informativeness

The values of h and I (Table 3) were slightly different, depending on the molecular method used (ISSR or RAPD), but in both cases they indicated high levels of genetic variation within varieties and a low degree of genetic differentiation among them. The highest h and I (0.3426 and, respectively, 0.5220) were recorded for RAPD data, which were consistent with GST value (0.0276) and with Nm value (17.58) from the same assay (Table 3).

AMOVA confirmed the results that only a small percentage of the genetic variation reside among varieties (13 in the case of ISSR data and 10 for RAPD's) and that the most of the genetic diversity occurs within varieties (87% and, respectively, 90%) (Table 4).

Table 3. Genetic variability across all the 6 varieties of *H. rhamnoides*

Primers	Nei's genetic diversity (h)	Shannon's information Index (I)	G_{ST}	Estimate of gene flow (Nm)
ISSR	0.3116 (0.0893)	0.4862 (0.1039)	0.0189	25.9450
RAPD	0.3426 (0.0912)	0.522 (0.1048)	0.0276	17.5895

G_{ST} – coefficient of genetic differentiation among populations

Table 4. Summary of analysis of molecular variance (AMOVA)

Source of variation	d.f.		Sum of squares		Variance components		Percentage	
	ISSR	RAPD	ISSR	RAPD	ISSR	RAPD	ISSR	RAPD
Among pop.	5	5	525.333	272.208	9.898 Va	4.245 Va	13.13	10.18
Within pop.	18	18	1178.5	674.25	65.472 Vb	37.458 Vb	86.87	89.82
Total	**23**	**23**	**1703.83**	**946.458**	**75.37083**	**41.70417**		
Fixation Indices FST:	ISSR - 0.13133, RAPD - 0.10181		Significance tests (1023 permutations)					

d.f. – degrees of freedom

For establishing the relatedness relationships between varieties, two separated UPGMA dendrograms, based on ISSR and RAPD data were plotted. (Figures 3 and 4). Both DNA markers grouped the 6 analyzed varieties into two clusters, but the structure differs from one method to another.

In both dendrograms, Tiberiu grouped outside the two clusters, even if it has the same geographic origin as Serpenta. Moreover, Serpenta grouped together with Auras and Victoria, which originated from Danube Delta. So, it can be appreciated that the geographic distance had little influence on the genetic variability and relationships between these Romanian sea buckthorn cultivars.

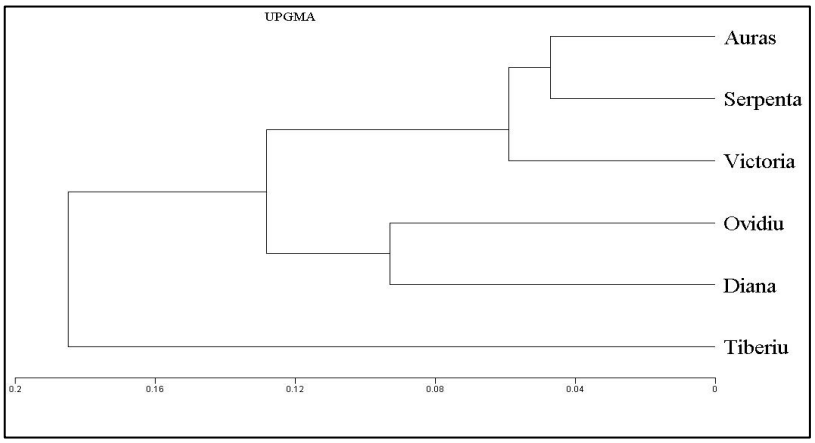

Figure3. UPGMA dendrogram based on the ISSR data

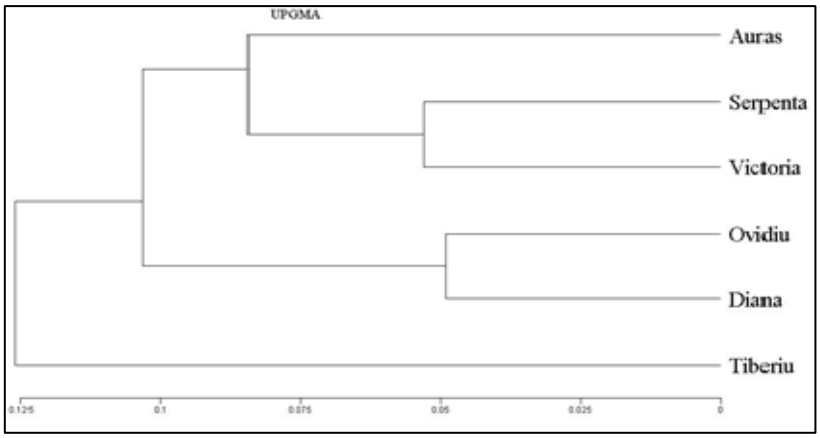

Figure 4. UPGMA dendrogram based on the RAPD data

PCO analysis (Figures 5 and 6) confirmed the grouping pattern revealed by the UPGMA dendrograms and the lack of genetic differentiation between cultivars.

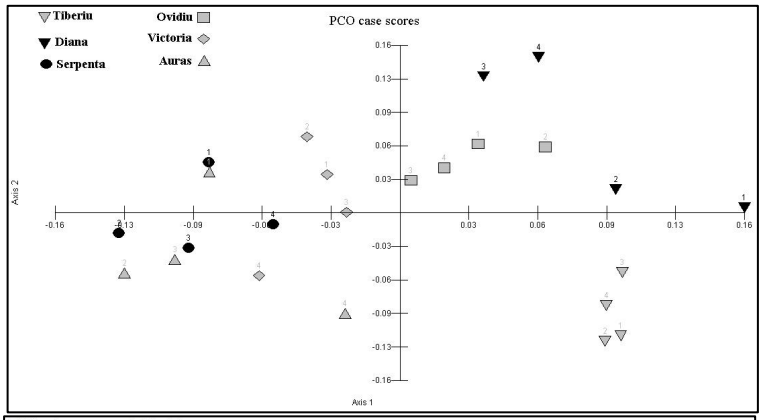

Figure 5. PCO graph based on the ISSR data

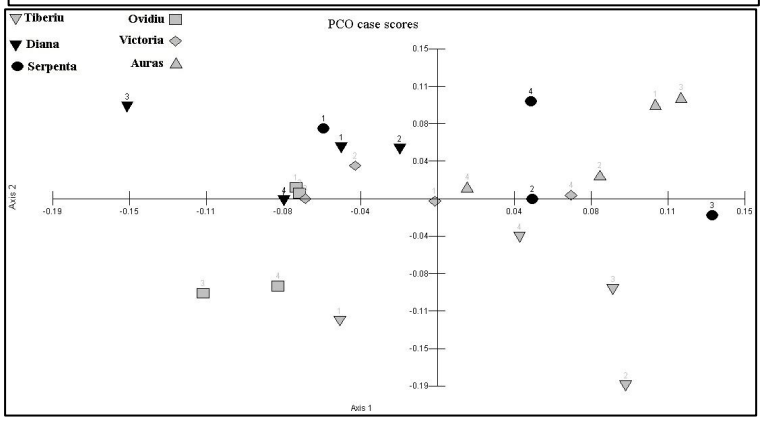

Figure 6. PCO graph based on the RAPD data

3. Discussion

The growing interest for commercial cultivation of sea buckthorn calls for precise phenotypic and molecular characterization of cultivars. Because of this, the efficiency of ISSR and RAPD methods in DNA fingerprinting and quantification of the genetic diversity was compared on 6 sea buckthorn varieties patented in Romania.

The ISSR and RAPD primers do not have species specificity and do not require a previous knowledge on genome sequence (ERCISLI & al. [17], THUL & al. [18], WANG & al. [19]). Thus, the 22 ISSR and 12 RAPD primers used in the present work were selected based on other studies carried out on different plant species, wild or cultivated (BARTISH & al. [20], RUAN & al. [6], MUTHUSAMY & al. [21], KAYIS & al. [22]).

The sequence of the ISSR used primers indicated that in *H. rhamnoides* microsatellites consist mainly in the dinucleotide repeats (AG)n, (CA)n, (TC)n, (AC)n, and in the pentane-cleotide repeat (CCCCT)n. Also, the primers based on the poly (CA) and poly (AC) motifs revealed, on average, more polymorphism (20-26 bands/primer), than the primers based on any other motifs used in the present investigation.

The amount of polymorphism detected by both methods was high and accounted for more than 93%. This value was similar with the results obtained for *H. rhamnoides* populations from China and other countries (CHEN & al. [2], LI & al. [23]), analyzed by ISSR method. Higher differences compared with literature data were recorded for RAPD method, the revealed polymorphism being usually lower, around 80% (SHENG & al. [24], RANA & al. [25]). This could be attributed to the randomic nature of RAPD primers.

The computed values of PIC, Rp and AvIb did not show any obvious differences among methods or compared to the literature data. For example, average PIC values (0.25 and, respectively 0.26) are in the normal range for dominant markers (0-0.5) (MUTHUSAMY & al [21]).

Also, the h and I parameters were very similar with those reported by CHEN (0.305 and respectively 0.468). Significant differences were observed in terms of the differentiation coefficient, which in our study was much smaller (0.0189 and respectively 0.0276) compared with the results of the above mentioned authors. However, another study on the sea buckthorn populations from the NW and NE of China (TIAN & al. [26]) found that GST = 0.0679, showing that the highest molecular variance resides within populations. Our results on GST were in agreement with the intense gene flow between varieties (Nm=25.9450 for ISSR and Nm=17.5895 for RAPD) and with AMOVA which highlighted the prevalence of intravarietal genetic variance over the intervarietal one. The reduced genetic differentiation could have several causes: (1) the 6 varieties belong to the same subspecies (ssp. carpatica) and have recent origin and almost similar geographic provenance (i.e. four of them from Danube Delta), (2) sea buckthorn is a woody dioecious species, wind pollinated, with outcrossing mating system. The analyzed varieties confirm that population differentiation is very restricted in the long-lived woody species, such plants possessing more genetic variation within populations and less among populations (CHEN & al. [2]).

The genetic relationships revealed by the UPGMA dendrograms shown a very strong relatedness between varieties regardless of the molecular method used. The composition of the two resulting clusters was similar, and only minor differences in cluster I were recorded.

Taking into consideration that primers with high Rp values, together with high PIC values are more suitable for genotype precise identification (DUARTE-DELGADO & al. [27]) it can be assumed that, for this study, the primers which provided more information regarding the genetic variation of the 6 sea buckthorns cultivars were the ISSR primers UBC 818, UBC 817, UBC 881, UBC 811. Regarding RAPD primers, only OPD-3 and OPBB-14 were appropriate for genetic diversity analysis. However, the use of the RAPD markers should be done with caution due to the great differences between independent studies caused by the increased sensitivity to the amplification conditions.

4. Conclusions

The results of the present study proved the higher utility of the ISSR markers compared to RAPD's in investigating the genetic diversity and the genetic relationships of the *H. rhamnoides* ssp. *carpatica* cultivated varieties. They are powerful and sensitive tools for the precise identification of cultivars, especially when at molecular level the differentiation between varieties is very low, as in the case of the present study.

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