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ORIGINAL PAPER

Genomic DNA isolation from *Hippophaë rhamnoides* subsp. *carpatica* Rousi for RAPD fingerprinting

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Abstract

The assessment of the genetic diversity of Hippophaë rhamnoides L. species became an important prerequisite for its conservation and marker assisted breeding. The first step in this type of studies is represented by the optimization of genomic DNA extraction in order to get high quality DNA, suitable for generation of molecular markers, such as RAPD. Thus, a variety of extraction methods have been evaluated for their efficiency and it was noticed that the quantity and quality of isolated DNA varied considerably among the various extraction protocols. SDS protocol and the commercial kit Wizard Genomic DNA purification represented the best DNA extraction methods for sea buckthorn leaf tissue, when an enzymatic digestion prior to isolation using CelluACE^{TM}XG System kit was performed. For further RAPD and other PCR-based methods the most suitable DNA extraction is SDS + CelluACE^{TM}XG System, due to the quality of obtained band pattern.

Keywords: sea buckthorn, DNA extraction, RAPD

Introduction

The development of molecular markers analysis has greatly enhanced the speed and efficiency of plant breeding and offered specific advantages in genome mapping, DNA fingerprinting, genetic diversity and other plant genetic studies [1]. However, these studies are limited by the ability to isolate pure, intact and high quality DNA from complex and usually unknown chemical mixture. Difficulties encountered include DNA degradation due to the presence of either native DNases or secondary plant compounds, and the co-isolation of polysaccharide contaminants that inhibit further enzymatic reactions [2]. The secondary metabolites that are particularly abundant in fruit trees, shrubs and medicinal plants are not completely removed during classical extraction protocols, remaining as contaminants in the final DNA isolates [3]. Thus, polysaccharides make DNA non-amplifiable in the PCR reaction by inhibiting *Taq* enzyme activity [4] and polyphenols covalently bind to DNA, giving it a brown color and making useless for most research applications [5].

The feasibility of genetic variability studies based on DNA markers is often limited because intact DNA must be isolated from many individuals belonging to different sub-populations and populations [2]. Moreover, plants contain high amounts of many different substances and it is unlikely the existence of just one nucleic acid isolation method [6]. This study was designated to develop a rapid, inexpensive method for DNA isolation from sea buckthorn leaves and to evaluate these methods by RAPD fingerprinting [7]. RAPD markers are generated by PCR amplification of random genomic DNA fragments using decameric primers, and have the advantages of simplicity and the ability to detect relatively small

amounts of genetic variation [8]. Hence, it has been developed and widely used to study plant genomes since its invention, especially in assessing the phylogenetic relationships and genetic diversity, constructing genetic maps and identifying cultivars [9].

Therefore, to study the genetic diversity in and between the wild and the cultivated populations of sea buckthorn from Romania, the first step was the optimization (improvement) of DNA extraction in order to get high quality DNA, suitable for generation of molecular markers, such as RAPD.

Hippophaë rhamnoides L. is a unique medicinal and aromatic plant, belonging to the family of Elaeagnaceae, which has been used for centuries in different medicine fields. However, selection of improved varieties has only been carried out for the past 60 or so years, cultivated varieties being released at first from the former U.S.S.R., Mongolia, the former East Germany, and Finland. Today breeding work is conducted in Russia, China, Scandinavia, Canada, and other countries, including Romania; this species has attracted international attention as a new crop opportunity, because it is said to have "momentous economic potential" and is predicted as the "next major health food" [10].

Sea buckthorn yields various important products such as leaves, oil, juices etc. with tremendous medicinal and pharmacological applications [11],[12], because its vitamin content is much higher than in any other cultivated fruit or vegetable. Also, sea buckthorn is rich in carotenoides, flavonoids, steroids, tannins, minerals, enzymes, amino acids, essential oils, and essential fatty acids [13]. The fruits have high contents of pulp oil and oil soluble bioactive compounds, such as tocopherols, tocotrienols, carotenoids, and plant sterols. The leaves contain considerable amounts of proteins (averaging 20-21%) [14] and triacylglycerols, free fatty acids, carotenoids, aldehydes, and triterpenes, which exhibit pharmacological properties. Tannins, flavonoids and other secondary metabolites have also been isolated from sea buckthorn leaves [15], [16]. During the isolation of DNA from plant tissues like leaves of *H. rhamnoides* many of these compounds act as inhibitors, deteriorating the quality and yield of DNA.

Taking into consideration all of this, we considered that the assessment of the genetic diversity of this dioecious species became an important prerequisite for its conservation and improvement. Thus, a variety of extraction methods have been evaluated for their efficiency and it was noticed that the quantity and quality of isolated DNA varied considerably among the various extraction protocols. In order to obtain appropriate DNA for further amplifications, some improvements of these methods were made and, finally, the PCR-RAPD was optimized to produce clear, scorable amplicons, useful for the assessment of genetic distance and differentiation in and between sea buckthorn populations.

Material and methods

Plant material

Leaf tissue was collected during summer of 2009-2010 years from eighteen populations of *Hippophaë rhamnoides* subsp. *carpatica* Rousi (eleven in native state and seven cultivated varieties). After collection, the leaves were transferred to the laboratory on ice and then stored at -20 ºC. Between two and five individuals per population were analyzed, including both males and females.

DNA extraction

For DNA isolation different protocols were tested: two variants of the classical methods SDS [17] and CTAB [18] and two commercial kits, Wizard Genomic DNA Purification Kit (Promega) and ZR Plant/ Seed DNA kit (Zymo Research). Taking into

account the initial results, the two methods which provided the best results (DNA extraction using SDS method and Wizard Genomic DNA Purification Kit) were supplemented through an additional enzymatic treatment with CelluACE^{TM}XG System kit (Promega). Only the modified protocols which offered the best results are presented.

SDS modified technique

Reagents: lyses buffer (100mM Tris, pH=8, 50mM EDTA pH=8, 500mM NaCl, 02% β-merchapthoethanol); 20% SDS; 5M potassium acetate; chloroform:isoamylic alcohol (24:1 v/v), 0.1X TE (10mM Tris, 1mM EDTA).

Method: approximately 1g of leaf material was cut and sliced in to small pieces and placed in a 30ml nalgene tube containing 2000 µl CXG buffer, 100 µl CXG enzymatic mix (CelluACE^{TM}XG System kit Promega) and porcelain balls for mechanical grinding (15min); after the balls were removed, the mix was incubated for 4 h at 37^{0}C and then 3000 µl lyses buffer (preheated at 65^{0}C) and 200 µl 20% SDS were added and incubate again for 10 min. at 65^{0}C; then 100 µl 5M potassium acetate were added; the probes were incubated on ice for 20 min. and centrifuged (1200rpm/5min/4^{0}C); the DNA from the supernatant was precipitated with ice cold ethanol, by incubating the tubes on ice for 30min; the precipitated DNA was rehidratated in 100 µl 0.1X TE and then RN-ase solution was added; deproteinisation step was realized by adding 110 µl chloroform:isoamylic alcohol; after a centrifugation step (1100 rpm/5min/4^{0}C) the supernatant was transferred in to a clean tube and 50 µl 5M NaCl and 200 µl ice cold isopropanole were added; precipitated DNA was washed twice with 70% ethanol and then rehydrated with 0.1X TE.

Wizard Genomic DNA Purification modified technique

Method: approximately 300mg of leaf material was cut and sliced in to small pieces and placed in a 30ml nalgene tube containing 2850 µl CXG buffer, 150 µl CXG enzymatic mix (CelluACE^{TM}XG System kit Promega) and porcelain balls for mechanical grinding (4h at 50^{0}C); then the balls were removed, tubes centrifuged and the supernatant transferred in to a clean tube; to this, 600 µl nucleic lyses solution were added and the probes were incubated for 15min at 65^{0}C; then the tubes were centrifuged again and over the transferred supernatant 6 µl RN-ase solution were added; after the incubation (15 min./37^{0}C), 200 µl of protein precipitation solution were added, the tubes were kept on ice for 5 min and then centrifuged; the supernatant was transferred in to a clean tube and the deproteinisation step was performed twice; the DNA was precipitated with cold isopropanol, washed with 70% ethanol, air dried and rehydrated with 0.1X TE.

Verification of quality and quantity of DNA isolates

Spectrophotometrical analyses of the concentration and purity of extracted DNA were made with Specord 40-Analitikjena spectrophotometer, reading the absorbance between 200-300nm against a control sample.

The agarose gel electrophoresis analyses for determining the integrity of DNA were made on a 0.8% agarose in TBE Buffer pH 8.0 (0.089M Tris, 0.089M Boric Acid, 0.02M EDTA).

PCR – RAPD analysis

PCR – RAPD analysis was carried out in a final volume of 30 µl, containing: 3µl buffer (5x Green Go Taq Flexi Buffer), 1,2 µl dNTP (10mM), 1,2 µl $MgCl_2$ (25mM), 2 µl Primer, 0,4 µl Taq polymerase, 3 µl diluted 1/50 DNA and sterile water; PCR program was: 4 min./94^{0}C initial denaturation, followed by 40 cycles of 1 min. at 94^{0}C, 1 min. at 36^{0}C and 2 min. at 72^{0}C, a final extension step of 10 min at 72^{0}C was included.

Results

In order to obtain DNA suitable for genetic diversity analysis of *H. rhamnoides* L., initially four extraction methods (CTAB, SDS, Wizard Genomic DNA Purification Kit and ZR Plant/Seed DNA kit), commonly used for plant tissues, were tested [19], [20], [21], [22]. The obtained results, verified electrophoretically and spectrophotometrically for purity and quantity, were unsatisfactory, especially for CTAB method and ZR Plant/Seed kit. Different degrees of smeared bands on agarose gel (Fig. 1) indicated high-levels of protein and polysaccharide impurities on the isolated samples. Also, the DNA purity calculated by the ratio A260/280 for protein contamination and A260/A230 for carbohyhrates impurification confirms the reduced efficiency of the first three methods for *H. rhamnoides* leaf tissue (Tabel 1).

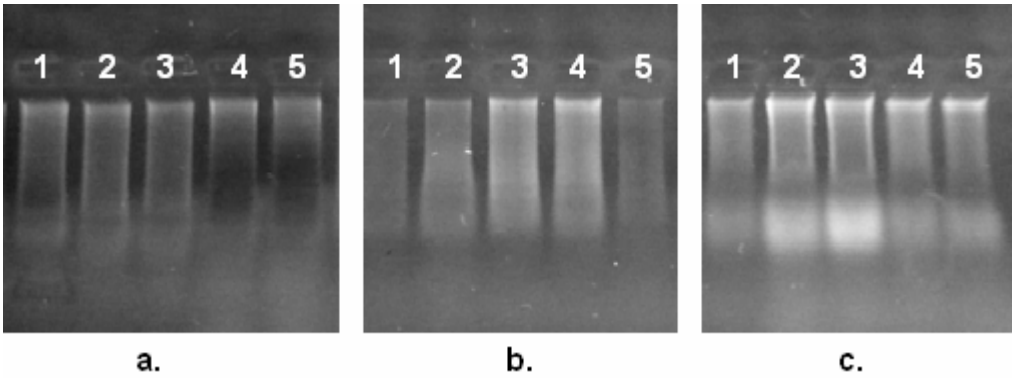

Figure 1. Agarose gel electrophoresis of genomic DNA extracted with three different methods: a. CTAB method [18], b. Wizard Genomic DNA Purification Kit (Promega), c. SDS method [17]

Table 1. Quality and quantity of genomic DNA extracted with different methods (mean values)

Extraction method	A260/A230	A260/A280	DNA concentration (μg/mL)
CTAB	1.542	1.507	0.056
SDS	1.143	1.100	2.280
SDS*	2.032	1.795	2.280
Wizard Genomic DNA kit	0.608	1.165	0.582
Wizard Genomic DNA kit*	1.853	1.890	1.979
ZR Plant/ Seed DNA kit	-	-	No DNA

*samples digested with CelluACE™ XG System prior to isolation

SDS method and Wizard Genomic DNA Purification kit provided better results compared to the other two methods (Fig. 1) so, several improvements and changes of the initial protocols were necessary. The grinding of the leaves in liquid nitrogen was replaced by an enzymatic treatment with CelluACE^{TM}XG System kit, especially designated for this type of plant tissue. This commercial kit contains cellulase, other carbohydrases and enzymes, which digest the leaf tissue in acid buffer at high temperature. Because plants significantly differ regarding DNA content and other compounds, the incubation time is species-specific, and usually is determined experimentally. The incubation time for most leaf tissue is 2-3 hours, but for fibrous leaves is greater than 3-4 hours. For *H. rhamnoides* the optimum incubation time at 50^0 C is 4 hours and it was determined gradually, starting with 2 hours, time period which was insufficient for this species.

Another change targeted (involved) the RNA contamination, observed on the gel electrophoresis. In order to solve this problem the amount of RN-ase was increased and also the reaction time.

Figures 2-3 show the electrophoretical vizualization of genomic DNA from *H. rhamnoides* extracted with SDS method and Wizard Genomic DNA Purification kit after the digestion with CelluACE^{TM}XG System kit. It can be observed that the extracted DNA is unfragmented and in an appropriate amount. The calculated purity and concentration of DNA (Table 1) confirm the agarose gel electrophoresis results.

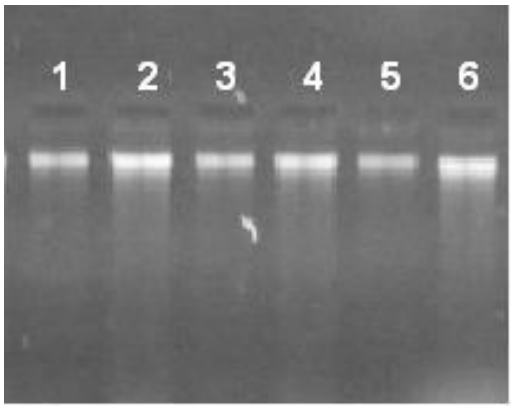

Figure 2. Agarose gel electrophoresis of genomic DNA extracted by SDS method after enzymatic treatment with CelluACE^{TM}XG System kit

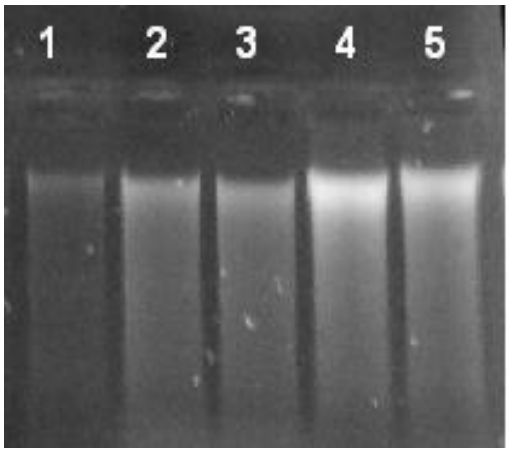

Figure 3. Agarose gel electrophoresis of genomic DNA extracted by Wizard Genomic DNA Purification kit after enzymatic treatment with CelluACE^{TM}XG System kit

To assess the quality of DNA, the RAPD fingerprinting [7] using three different decameric primers (OPC15, OPD18, OPA11 Bio Basic. Inc.) was performed. Initially, the amplification conditions were those mentioned by Ercisli [23], who studied the genotype diversity of different sea buckthorn populations from Turkey, but no band pattern was obtain. So, the annealing temperature was increased from 35^0 C to 36^0 C, according to Singh [24]. Because the results were still unsatisfactory, and the concentration of the DNA isolates was very high, compared to those mentioned in the literature [23], DNA at different dilutions was tested (Figure 4). The best results were obtained after the dilution of the DNA initial concentration at 1/50. (Figure 5).

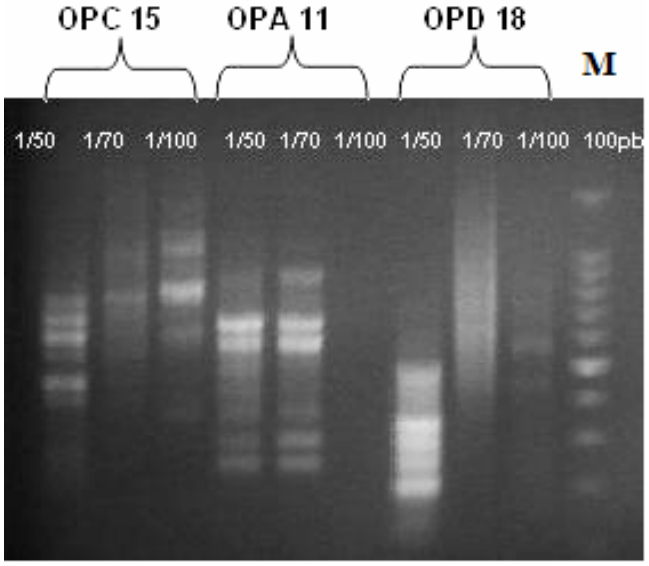

Figure 4. RAPD pattern banding for three decameric primers using different DNA template dilutions, M=100 bp molecular marker

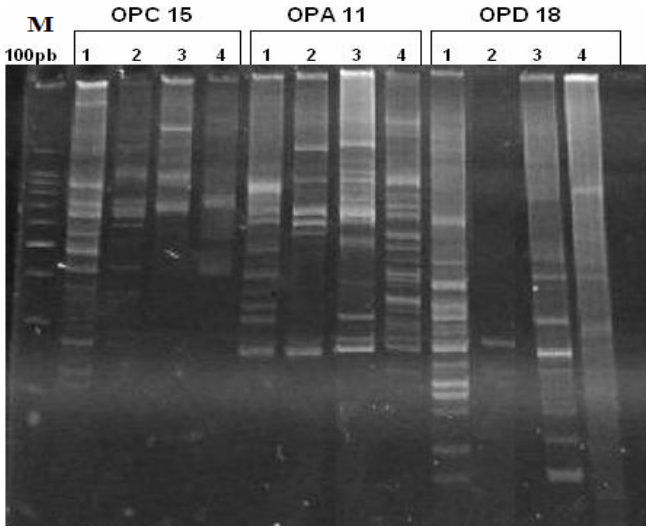

Figure 5. RAPD pattern banding for three decameric primers using different DNA extracted by SDS method after enzymatic treatment with CelluACE^{TM}XG System kit**.** M = 100 bp molecular marker, Lanes 1-4 represent four different populations of sea buckthorn (DNA template diluted 1/50)

Discussion

The leaves of sea buckthorn have a complex biochemical composition, and, as a consequence, the DNA isolation is very difficult. For example, flavonoids, a large group of phenolic compounds with several well documented biological activities beneficial to human health, are one of the most important constituents of sea buckthorn leaves [16]. The high concentration of flavonoids has a negative impact on the efficiency of the DNA isolation. Additionally, the high protein content and other secondary metabolites such as tannins, also alter the quality of the DNA extraction.

Our analysis revealed that the DNA quantity, degradation (integrity) and contamination (with both protein and carbohydrates) in the individually extracted samples from sea buckthorn leaf tissue varied among each extraction method. The CTAB method and

the commercial kit ZR Plant/Seed DNA were less efficacious, although the cationic detergent hexadecyltrimethylammonium bromide (CTAB) is commonly used for solubilizing plant tissue and isolation of DNA [2]. The explanation could be the high levels of secondary metabolites (polysaccharides, flavonoids) which determine many interference problems [25].

Because the results obtained with SDS and Wizard Genomic DNA purification protocols were satisfactory regarding the DNA amount and its integrity, the procedures were optimized by making several changes when compared to standard protocols. The digestion with CelluACE^{TM}XG System prior to isolation had favorable effect on the quality of the extraction, and a large part of contaminants were removed. Thus, the averaged A260/A280 ratio was 1.795 for SDS + CelluACE^{TM}XG System method, and 1.887 for Wizard Genomic DNA purification kit + CelluACE^{TM}XG System, indicating low levels of contaminating proteins [26]. Also, the values of A260/230 ratio, 2.032 for the first method and, respectively 1.851 for the second one, indicated a reduced polysaccharides and polyphenols contamination. While, the RNA residues observed on the initial extractions were removed by an additional treatment with RNase.

Both methods (SDS and commercial kit) provided comparable genomic DNA isolates, although, with the shortest time spent for a single extraction, the Wizard Genomic DNA purification kit seemed to be the most convenient. This aspect has a special signification for further studies on genetic variability in and between sea buckthorn populations, when a large number of samples must be process in a short period of time.

In order to evaluate the quality of extracted DNA for further variability studies involving *Hippophaë rhamnoides* L populations (cultivated and spontaneous) we applied the RAPD technique, using three different decameric primers on the DNA isolates.

The high sensitivity and low reproducibility of RAPD technique imposed several changes of the initial used protocol [23] in both PCR-components (template DNA), as well as in amplification cycles, especially the annealing temperature. The optimized reaction conditions, respectively 36^0C for primer annealing and 1/50 diluted template DNA, produced clear, scorable amplified products. It was found that the SDS + CelluACE^{TM}XG System method generate DNA that provide more reproducible RAPD results than does DNA generated by Wizard Genomic DNA purification kit and other extraction methods.

Conclusions

SDS protocol and the commercial kit Wizard Genomic DNA purification represented the best DNA extraction methods for sea buckthorn leaf tissue, when an enzymatic digestion prior to isolation using *CelluACE^{TM}XG System* kit was performed. In this way, it was eliminated the grinding of leaves using liquid nitrogen and also the strong contamination with flavonoids, other phenolic compounds, polysaccharides and proteins was reduced.

Although the quality and quantity of the isolated DNA verified by electrophoresis and spectrophotometry, were very similar in both protocols, for further RAPD and other PCR-based methods the most suitable DNA extraction is SDS + CelluACE^{TM}XG System, due to the quality of band pattern. In future, we will use RAPD markers to estimate the level of genetic diversity of *Hippophaë rhamnoides* L. from the Romanian spontaneous and cultivated populations, to assess inter- and intrapopulational relationships and to reveal possible relationships between genetic distances and geographic distances among populations.

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