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IDENTIFICATION OF GENETIC DIVERSITY AMONG *Arnica montana* L. GENOTYPES USING RAPD MARKERS

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Abstract. *Arnica montana* L. is one of the most important herbal plants used in medicine, pharmaceutical and cosmetic industry. The number of studies performed with molecular markers on arnica genotypes is very limited. Because of this fact the aims of presented examination were optimization of protocols DNA isolation from fresh leaves of *A. montana* and identification of genetic diversity among this plant genotypes. In presented study to obtain pure DNA Plant & Fungi DNA Purification Kit (EURx) were used. To clean obtained DNA long and slow electrophoresis and isolation DNA from gels were used. *A. montana* genotypes were analyzed using 40 RAPD primers (Operon Technologies), out of which 12 produced high number of polymorphic and repeatable fragments. In total, selected primers produced 120 fragments, among them 111 (92.5%) were polymorphic. The genetic similarity matrices were produced based on RAPD using the Dice's coefficient. RAPD based genetic similarity was estimated between 0.535 and 0.945. The highest genetic similarity was estimated among GA17 and GA18 genotypes, which are closely located on the obtained dendrogramme.

Key words: DNA polymorphism, medicinal plant, molecular analysis

INTRODUCTION

Identification of plant species and estimating genetic diversity among species, cultivars and populations of medicinal plant is very important for standardizing herbal medicine. Genetic tools that use polymerase chain reaction provide objective and reliable method to achieve this goal [Li and Dao 2011, Okoń et al. 2013]. The DNA fingerprinting technique of random amplified polymorphic DNA (RAPD) has become one of the most effective methods to estimating genetic diversity [Wiliams et al. 1990, Hosokawa et al. 2000]. Because of many advantages like high speed, low cost and requirement of small amount of template DNA, RAPD were used many times for estimating genetic

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diversity of different herbal plants [Khanuja et al. 2000, Kapteyn et al. 2002, Hadian et al. 2007 Okoń and Surmacz-Magdziak 2011].

Arnica montana L. is one of the most important herbal plants used in medicine, pharmaceutical and cosmetic industry [Bilia et al. 2006]. The various pharmaceutical properties results of many compounds such as alcohols, tannin, flavonoids, and sesquirterpenic lactones, especially helenalin [Macedo et al. 2004]. Specimens containing extracts from *A. montana* reduce pain, swelling and discoloration resulting from bruises, possess potent antioxidant and antiradical abilities, they also reduce the symptoms of hand osteoarthritis [Widrig et al. 2007, Gawlik-Dziki et al. 2011].

The number of studies performed with molecular markers on *Arnica* genotypes is very limited. There is only one publication concerning the use of molecular markers to estimate genetic diversity among arnica genotypes on the molecular level [Pop et al. 2008]. Because of this fact the aims of presented research were optimization of DNA izolation protocol from fresh leaves of *Arnica montana* and identification of genetic diversity among *A. montana* genotypes.

MATERIALS AND METHODS

Plant material and DNA extraction. 24 genotypes of *Arnica montana* collected from experimental plot in Felin (University of Life Sciences in Lublin) were analyzed.

For effective isolation of DNA from fresh tissue two different methods were used. In the first experiment DNA was isolated using CTAB method [Doyle and Doyle 1987], in the second experiment genomic DNA was extracted with GeneMATRIX Plant & Fungi DNA Purification Kit (EURx). Isolated DNA was additionally purified using long (3 h) and slow (80V) electrophoresis in 2% agarose gel. After electrophoresis DNA was isolated from agarose gel using MiniElute Gel Extraction Kit (Qiagen).

RAPD analysis. PCR reactions were performed according to the RAPD method described by Williams et al. [1990] with modifications. Reaction mixtures contained 1 × PCR Buffer (10 mM Tris pH 8.8, 50 mM KCl, 0.08% Nonidet P40) (Fermentas), 160 µM of each dNTP, 530 pM oligonucleotide primer, 1.5 mM $MgCl_2$, 70 ng of template DNA, 0.5 U Taq DNA Polymerase (Fermentas) in a final reaction mixture of 15 µl. Amplification were carried out in Biometra T1 thermal cycler programmed for 3 min in 94°C of initial denaturation, 44 cycles: 94°C – 45 s, 37°C – 45 s, 72°C – 45 s, with final extension at 72°C for 7 min. A negative control was added in each run. In order to check reproducibility the selected primers were tested two times on the same sample.

Amplification products were separated by electrophoresis on 1.5% agarose gels containing 0.1% EtBr (1.5 h, 120V). Fragments were visualized under UV transiluminator and photographed using PolyDoc System. GeneRuler^{TM} 100bp DNA Ladder Plus was used to establish molecular weight of the products.

Data analysis. RAPD products were scored as present (1) or absent (0) from the photographs. Only bright and reproducible products were scored. The level of polymorphism of the primer (polymorphic products/total products) and relative frequency of polymorphic products (genotypes where polymorphic products were present/ total num-

ber of genotypes) [Belaj et al. 2001] were calculated. Resolving power of the primer was calculated using the formula: Resolving power (Rp) = Σ Ib (band informativeness). Band informativeness was calculated for each band scored by the primer individually. Ib = 1 – [2(0.5 – p)], p is the proportion of occurrence of bands in the genotypes out of the total number of genotypes [Prevost and Wilkinson 1999].Banding patterns (the different combination of bands obtained for each primer) were scored. Relative frequency of banding patterns was also calculated.

Genetic pairwise similarities (SI-similarity index) between studied genotypes were evaluated according to Dice's formula after Nei and Li [1979]. A cluster analysis was conducted using the distance method UPGMA (Unweighted Pair-Group Method with Arithmetic Mean) in the NTSYS program [Rohlf 2001]. Clustering was verified by bootstrapping. PCA analysis was performed using PAST software. Statistical analysis was performed in the program PAST [Hammer et al. 2001].

RESULTS AND DISSCUSSION

Nowadays DNA analysis has become routine technique to estimate genetic diversity in many plant species [Wilkinson 1999, Anthony et al. 2004, Prakash and van Staden 2007, Prevost and Okoń et al. 2012] including medicinal plants [Khanuja et al. 2000, Hadian et al. 2007]. A large number of medicinal plant species produce different secondary metabolites which can hamper the DNA isolation procedures. Because of this fact optimization of DNA isolation protocols is often necessary as a primary step in molecular analysis of medicinal plant [Tan and Yiap 2009]. Because *A. montana* produce many different secondary metabolites the first step of presented study concerned on identification of effective method of DNA isolation from fresh tissue. DNA extracted with CTAB method characterized bad purity and quality. DNA pellets were sticky and have brown or green color. After electrophoresis in UV light many pollutants around of DNA were observed. After PCR with selected RAPD primers no amplification products were observed.

In the second experiment isolated DNA characterized better purity and acceptable quality than obtained using CTAB method, but after PCR no amplification product were observed too. Because of fact that pollutants migrated in agarose gels to purify obtained DNA long and slow electrophoresis and isolation DNA from gels were used. After that procedure DNA characterized small amount but purity and quality were sufficient to conduct PCR.

Second part of the experiment was estimating genetic diversity among *A. montana* genotypes using RAPD method. Randomly amplified polymorphic DNA is a technique, which identified relatively high number of polymorphic products [Kizhakkayil and Sasikumar 2010, Okoń and Surmacz-Magdziak 2011].

In presented studies *A. montana* genotypes were analyzed using 40 RAPD primers (Operon Technologies), out of which 12 produced high number of polymorphic and repeatable fragments. In total, selected primers produced 120 fragments. The number of fragments ranged from 6 to 14, with an average 10 per primer as well as 5 bands per genotype. Among the 12 primers T2b had the lowest primer diversity 66.7%, six prim-

Table 1. Characteristic of selected RAPD primers

No.	Primer	Sequence 5`-3`	Number of products			Number of banding patterns		Frequency of banding patterns	Primer diversity%	Frequency of poly morphic products	Resolving power of the primer
			total	poly morphic	mono morphic	total	specyfic				
1	A18	AGGTGACCGT	12	11	1	11	8	0.73	91.7	0.59	12.16
2	D07	TTGGCACGGG	10	10	0	9	4	0.44	100	0.55	9.08
3	D16	AGGGCGTAAG	11	11	0	14	5	0.36	100	0.60	13.25
4	U280	CTGGGAGTG G	10	8	2	10	5	0.50	80	0.53	6.71
5	T01	GGGCCACTCA	9	9	0	12	8	0.67	100	0.52	8.33
6	U225	CGACTCACAG	7	7	0	11	4	0.36	100	0.57	8.00
7	U250	CGACAGTCCC	8	8	0	9	3	0.33	100	0.58	9.25
8	U532	TTGAGACAGG	9	8	1	14	8	0.57	88.9	0.53	7.5
9	G3	CCAGTACTTC	12	11	1	14	7	0.50	91.7	0.59	12.16
10	G5	AACCCGGGAA	12	10	2	17	12	0.71	83.3	0.69	12.5
11	U18	GGGCCGTTTA	14	14	0	23	21	0.91	100	0.63	17.66
12	T2b	CTACACAGGC	6	4	2	9	5	0.56	66.7	0.65	3.75
total			120	111	9	153	90				
average/primer			10	9.25	0.75	12.75	7.5				
average/genotype			5	4.63	0.38	6.37	3.75				

ers scored 100%. Most of them did not produce monomorphic bands. Among the total 120 amplified products 111 (92.5%) were polymorphic. The number of polymorphic bands amplified by single primer ranged from 4 to14 with an average 9.25 per primer and 4.63 per genotype (tab. 1).

Relative frequency of polymorphic bands ranged from 0.04 (polymorphic band present only in one genotype of the 24 studied) to 0.96 (polymorphic band absent only in one genotype of the 24 studied). In general the average frequency generated by single primer was high (0.58) and varying from 0.46 to 0.69 (tab. 1). The average frequency of polymorphic products was previously calculated for tea genotypes [Chen et al. 2005] and was similar to average frequency obtained in present study and amount to 0.47. High variability in the frequency of polymorphic bands observed also Belaj et al. [2001]. These results suggested that analyzed material characterized high level of polymorphism.

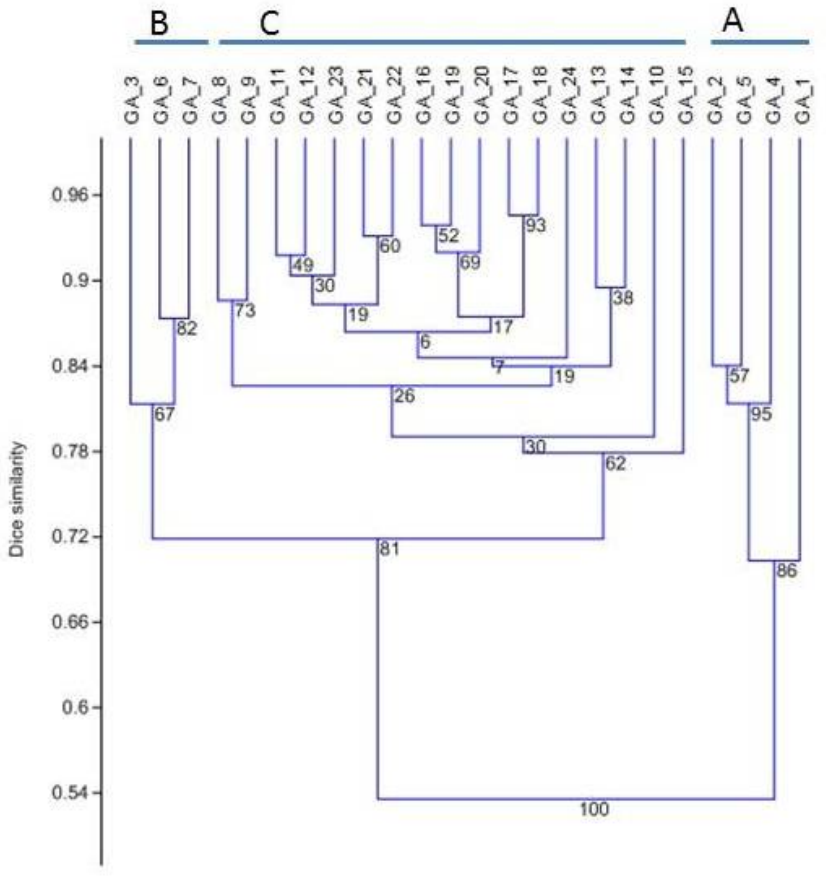

Fig. 1. Dendrogram of *A.montana* genotypes constructed using UPGMA method

The resolving power of the 12 RAPD primers ranged from 3.75 to 17.7 (tab. 1). Primers with high resolving power are used for the molecular diagnosis of any species from the mixed population [Prevost and Wilkinson 1999]. In presented study RAPD primers possessed high resolving power values were able to distinguish all analyzed genotypes, and could be potentially used for identifying them from any mixed population. In the past a similar approach has been successfully used for molecular diagnosis of potato cultivars [Prevost and Wilkinson 1999], *Rhus* species [Prakash et al. 2007] and *Jatropha* genotypes [Tatikonda et al. 2009].

The selected primers yielded 153 banding patterns. The number of banding patterns ranged from 9 to 23, with an average 12.75 per primer and 6.37 per genotype. Relative frequency of the banding patterns varied from 0.04 to 0.46. Average frequency of the banding patterns was very low (0.13) (tab. 1). Belaj et al. [2001] calculated relative frequency of banding patterns for olive (*Olive europea* L.), which varied from 0.02 to 0.88 and similar like in present study average frequency of banding patterns was very low (0.2). Okoń and Surmacz-Magdziak [2011] identify 208 banding patterns for 12 RAPD primers used in analysis of 20 chamomile genotypes. Relative frequency of banding patterns obtained by authors was very low and ranged from 0.05 to 0.3.

The genetic similarity matrices were produced based on RAPD using the Dice's coefficient. RAPD based genetic similarity was estimated between 0.535 and 0.945. The mean genetic similarity calculated at 0.886.

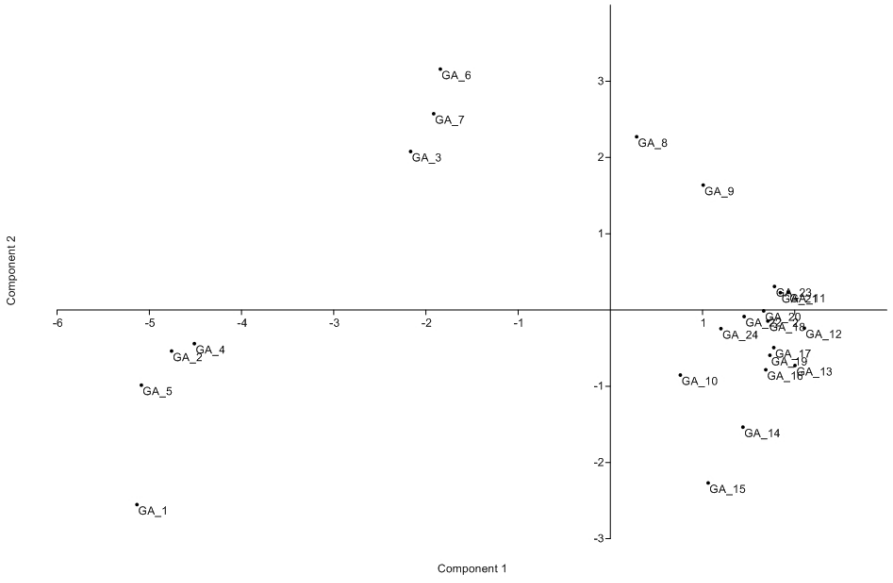

Fig. 2. Principal component analysis (PCA) of *A. montana* genotypes

Genetic similarity matrix was applied for cluster analysis through UPGMA method (fig. 1). The 24 genotypes could be grouped into three major groups based on major bootstrapping. Group A contained 4 genotypes GA1, GA2, GA4 and GA5. Group B

contained three genotypes GA3, GA6 and GA7. The rest of genotypes located in C group. On the edge of this cluster placed two genotypes: GA10 and GA15. The highest genetic similarity was estimated among GA17 and GA18 genotypes, which were closely located on the obtained dendrogram.

Relationships among 24 analyzed genotypes of *Arnica montana* were revealed by principal component analysis (PCA) (fig. 2). The PCA analysis led to comparable results to that obtained by UPGMA clustering. *A. montana* genotypes formed four distinct groups. Two distinct from each other and from the others groups correspond to A and B UPGMA clusters. The biggest and the tightest group resembled C cluster on the dendrogram. GA10 and GA15 individuals formed separate group. For the RAPD data, the first three principal components explained 68.7% of the total variation, with PC-1, PC-2 and PC-3 accounting for 50.2, 11.1 and 7.4% of the total variation, respectively.

CONCLUSIONS

1. Presented experiment showed that isolation of pure DNA from *A. montana* plants is very difficult. The good way to obtain good quality of DNA is using long and slow electrophoresis and isolation of DNA from agarose gels.

2. Present study demonstrated that RAPD markers provide a useful and effective method to estimate the genetic diversity among *A. montana* genotypes.

3. Analyzed *A. montana* genotypes characterized quite high genetic similarity. The highest genetic similarity was estimated among GA17 and GA18 genotypes, which were closely located on the obtained dendrogram.

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ANALIZA ZRÓŻNICOWANIA GENETYCZNEGO WŚRÓD GENOTYPÓW *Arnica montana* L. ZA POMOCĄ MARKERÓW RAPD

Streszczenie. *Arnica montana* L. jest jedną z najcenniejszych roślin zielarskich wykorzystywanych w medycynie, farmacji i przemyśle kosmetycznym. W dostępnej literaturze liczba doniesień związanych z analizą molekularną arniki jest znikoma, dlatego też celem prezentowanych badań była optymalizacja procesu izolacji DNA ze świeżych liści oraz identyfikacja zróżnicowania genetycznego oparta na markerach RAPD. W prezentowanej pracy w celu uzyskania czystego DNA do izolacji wykorzystano zestaw DNA Plant & Fungi DNA Purification Kit (Euro) oraz oczyszczanie za pomocą długiej elektroforezy w żelu agarozowym. Spośród testowanych 40 starterów RPAD do analiz wybrano 12 generujących stabilne i polimorficzne wzory prążków. Wyselekcjonowane startery amplifikowały 120 fragmentów, spośród których 111 (92,5%) było polimorficznych. Wykorzystujac markery RAPD utworzono matryce podobieństwa genetycznego. Średnia wartość podobieństwa analizowanych genotypów wynosiła 0.886. Najwyższy współczynnik podobieństwa genetycznego oszacowano pomiędzy genotypami GA17 i GA18, które ulokowały się blisko siebie na uzyskanym dendrogramie.

Słowa kluczowe: analizy molekularne, polimorfizm DNA, rośliny lecznicze

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