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GENETIC DIVERSITY EVALUATION OF THE SPECIES *PULMONARIA RUBRA* (*BORAGINACEAE*)

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SUMMARY

Genus Pulmonaria is represented in our country by the species *Pulmonaria rubra*, *Pulmonaria angustifolia*, *Pulmonaria officinalis* and *Pulmonaria montana*. *Pulmonaria rubra* is endemic for the Carpathians and Balkans Mountains and it presents two varieties: *Pulmonaria rubra ssp. eurubra* and *Pulmonaria rubra ssp. filarszkyana*, the latter having a small areal in the northen part of the country. Some authors consider *P. rubra ssp. filarszkyana* to be a full state species (*Pulmonaria filarszkyana*) while others question the existence of two different species. It is difficult to distinguish the two species solely on the base of morphologic characters which may be the effect of an individual polymorphism. By analysing the molecular data derived from chloroplastic DNA (cpDNA) sequences we manage to delimit those two species on a genetic and habitat level.

Keywords: *Boraginaceae* family, *Pulmonaria* sp., chloroplastic DNA, PCR - RFLP, chloroplastic microsatellites

INTRODUCTION

The current state of knowledge of our country's flora is appreciated at the international level by the existence of some improperly defined species, with overlapping characters and serious dificulties in their analysis. Unfortunately, the floristic evaluation and analysis in our country is limited to the traditional methods based on morphology, histology,

anatomy, ecology. Therefore, despite the huge amount of data and valuable scientific works gathered over time, there is a strong need for a review of these problematical species in order to facilitate theoretical or practical studies. Present international tendencies combine traditional methods of evaluation and modern methods based on genetic studies, so new branches in biology have appeared, such as molecular taxonomy, molecular ecology, phylogenetics. For our country, in order to become competitive at the international level and to use its patrimony of endemic and relict species, it is imperative to introduce in the botanical studies the new methods of molecular biology.

The *Boraginaceae* family includes about 120 genera with approximately 2000 cosmopolitan species met from the tropical to the temperate region, with a strong concentration in the Mediterranean area and less common in the Antarctic area. The family belongs to the Euasteridae clade I [1] and is considered to be paraphiletic. The relations within and between the major groups of the family are not completely understood, mostly due to the insufficient phylogenetic analysis. Molecular phylogenetic studies performed on the family focused on the division of the family [2, 3, 4], delimitation and intergeneric classification of the *Boragineae* group [2, 5] and development of microsatellites for some species such as *Echium vulgare*, *Cynoglossum officinale* [6, 7].

One of the species belonging to the *Boraginaceae* family, which poses determination problems is *Pulmonaria rubra* whose morphological characters have been interpretated differently by authors over the time. Classic plant taxonomy is based on the qualitative and quantitative morphologic characters. Most of the qualitative characters are influenced by the ecological factors (such as temperature, radiations, amount of precipitation) and therefore they present variation over the year. This is the reason why the authors had differently interpreted the morphological particularities of this species.

The Carpathic species *Pulmonaria rubra* Schott has been considered in some reviews [8, 9] as a unique species with two infrataxa: subspecies *rubra* and subspecies *filarszkyana*. Moreover, Borza in “Conspectus florae Romaniae” considers *P. filarszkyana* as a variety of the species *Pulmonaria rubra* [10]. In “Flora Europaea”, *Pulmonaria rubra* ssp. *filarszkyana* is trated as a different species (*Pulmonaria filarszkyana*) and not as a subspecies of *Pulmonaria rubra* [11]. Prodan [12] and Ciocârlan [13] in “Flora ilustrată a României”, appreciate *Pulmonaria filarszkyana* as a full state species and also an endemic species for the North-East Carpathians.

The morphological characters which distinguish the two species are minute and difficult to recognize in the field and may occur from an individual polymorphism and not from interspecific differences.

Pulmonaria rubra – ovate basal leaves, hibernating, abruptly incrusted in the leaf-stalk. The leaves and inflorescence branches have long bristly hair, mixed with thin and short hair and long glandular hair. The stem leaves are slightly decurrent.

Pulmonaria filarszkyana – the basal leaves are narrow lanceolate, non-hibernating, covered with short, soft hair. The stem leaves are lanceolate, with small leaf-stalk and they are decurrent on the stem.

Regarding the ecology, coenology and chorology of these species we need to mention that *Pulmonaria rubra* and *Pulmonaria filarszkyana* vegetate in middle and high mountain floor (1000 - 1400 m), on brown, acid soils and brown crypto-podzolic soils, moist to wet, with acid reaction, in spruce fir woods (*Picea abies*) or spruce fir mixed with beech (*Fagus sylvatica*). *Pulmonaria rubra* is frequent in the central and southern part of the Oriental Carpathians, Meridional Carpathians and Apuseni Mountains [14, 15, 16, 17]. *Pulmonaria filarszkyana* is found only in the northern part of the Oriental Carpathians: Rodna Mountains, Maramures Mountains [14, 18].

The purpose of this work is to clear up the taxonomic position of the species *Pulmonaria rubra* using molecular markers such as RFLP and chloroplastic microsatellites.

MATERIALS AND METHODS

Plant material

Plant material used for our study has been gathered from different areas; leaves have been collected and conserved in silica gel tubes, in order to ensure the drying of the vegetable material without the destruction of DNA.

DNA isolation

Total genomic DNA was isolated using an adapted variant of the Doyle & Doyle's protocol [19]. The quality of the DNA has been tested using agarose electrophoresis gel 1%.

PCR - RFLP technique (Polymerase Chain Reaction - Restriction Fragment Length Polymorphism)

Since 1985, restriction fragment length polymorphism has emerged as a very powerful tool for genetic analysis in plants. RFLP, like many other phenomena, was first identified in microbes, then utilized in humans and later flourished in plants. The ability to detect variation directly at the DNA level makes RFLP a very important tool in plant genetics. Secondly, RFLPs have an extremely nigh resolution power for detection of genetic variation compared to other existing technologies; hence they can be directly integrated into the existing breeding technologies with considerable ease.

The steps of the PCR - RFLP technique are the following: the amplification of polymorphic DNA fragments using PCR; DNA digestion with endonucleases; electrophoretic separation of the obtained fragments according to their size and finally polymorphism detection. The technique used in this study was adjusted from the one used in oaks by Demesure *et al.* 1995 [20], Fineschi *et al.* 2002 [21] and Petit *et al*, 2002 b [22].

The amplification of the DNA fragments susceptible to be polymorphic was carried out in an Eppendorf thermocycler (Mastercycler gradient) and the quality of DNA was tested by agarose gel electrophoresis.

In order to identify the chloroplastic haplotypes we used a set composed of three pairs of primers [trnD/trnT (DT), trnC/trnD (CD) and psaA/trnS (AS)]. These primers are complementary with different informative regions highly conserved from the chloroplastic DNA and they permit the amplification of variable non-informative regions [20].

The characteristics of the used primers and the sequence of the nucleotides are shown in the following table:

Table I. The primers used in the study of three chloroplastic loci [20]

Primer	Name of the primer	Nucleotide sequence	Melting temp.
Pair 1 DT	trnD [tRNA-Asp (GUC)]	5′ - ACC AAT TGA ACT ACA ATC CC - 3'	54.5° C
	trnT [tRNA-Thr (GGU)]	5' - CTA CCA CTG AGT TAA AAG GG - 3'	
Pair 2 CD	trnC [tRNA-Cys (GCA)]	5' - CCA GTT CAA ATC TGG GTG TC - 3'	58°C
	trnD [tRNA-Asp (GUC)]	5' - GGG ATT GTA GTT CAA TTG GT - 3'	
Pair 3 AS	psaA[PSI(P700 apoproteinAl)]	5' - ACT TCT GGT TCC GGC GAA CGA A - 3′	57.5°C
	trnS[tRNA-Ser(GGA)]	5' - AAC CAC TCG GCC ATC TCT CCT A - 3'	

The reaction mix per sample contains buffer solution 1X, dNTPs: dATP, dGTP, dTTP and dCTP with 0.1 mM concentration, $MgCl_2$ 2.5 mM concentration, each of the primers with a concentration of 0.2 mM, *Taq*Polymerase 0.75 units per reaction (all the reagents supplied by Promega), DNA (5 µl of 5:100 DNA dilution) and sterile UV/UP water until the final volume of 25 µl per PCR tube.

The PCR programs used were the following:

Table II. PCR program used for chloroplastic loci amplification

Step no.	Steps	Temperature	Time
1.	Polymerase activation	94°C	5 minutes
2.	Denaturation	94°C	50 seconds
3.	Annealing:	55°C (DT), 58°C (CD and AS)	50 seconds
4.	Extension	72°C	2 minutes
5.	30 repeats of steps 2-4		
6.	Final extension	72°C	10 minutes
7.	Conservation	4°C	∞

The amplification products are viewed by gel electrophoresis (1% concentration) using a marker (Smart Ladder) to confirm the size of the PCR products.

The digestion of the amplification products is achieved by the restriction endonucleases as follows: the PCR products obtained with the trnD-trnT (DT) and trnC-trnD (CD) primers are digested with *Taq*I for 3 hours at a temperature of 65°C while the PCR products obtain with the psaA/trnS (AS) primer are digested with restriction endonuclease *Hin*fI for 5 hours at a temperature of 37°C. The reaction mix per sample (30 µl) is formed by: 1 unit of restriction endonuclease, 5 units of digestion product, buffer 1X and UV/UP water.

The DNA fragments obtained after digestion were separated on a polyacrylamide gel (8%), in an electric field, at 300 V in 1X TBE buffer. A common vertical electrophoresis apparatus was used: APELEX-H.D. "VERTIGEL" (20 x 20 cm), and the polyacrylamide gel was maintained at a relatively steady temperature, 21°C.

Polymorphism detection was made manually, using silver staining [23]. The polymorphism evaluation was carried out in the UV or visible spectrum.

The SSR (Single Sequence Repeat) technique

The method consists in amplifying simple repetitive sequences using specific primers. For these short sequences the digestion of the amplified product is not longer necessary. The amplified product is denaturated before the segregation of fragments by electrophoresis, therefore the separated DNA fragments used to assess polymorphism are single-stranded.

Six pairs of universal primers were used in order to assess polymorphism at the cpDNA simple repetitive sequences level, primers described by Weising & Gardner in 1999 [24]:

Table III. Presentation of primers used for the amplification of chloroplastic microsatellites

Primer	Sequence	Base number	Annealing temperature	Size of PCR product
ccmp2 [F]	gatcccggacgtaatcctg	19	50°C	166-234
ccmp2 [R]	atcgtaccgagggttcgaat	20		
ccmp3 [F]	cagaccaaaagctgacatag	20	50°C	89-119
ccmp3 [R]	gtttcattcggctcctttat	20		
ccmp4 [F]	aatgctgaatcgaygaccta	20	51°C	115-220
ccmp4 [R]	ccaaaatattbggaggactct	21		
ccmp6 [F]	cgatgcatatgtagaaagcc	20	50°C	93-111
ccmp6 [R]	cattacgtgcgactatctcc	20		
ccmp7 [F]	caacatataccactgtcaag	20	49°C	129-151
ccmp7 [R]	acatcattattgtatactctttc	23		
ccmp10 [F]	ttttttttttagtgaacgtgtca	22	50°C	91-300
ccmp10 [R]	ttcgtcgdcgtagtaaatag	20		

The reaction mix per sample contains: 1X buffer, dNTPs of 0.1 mM concentration, $MgCl_2$ of 2.5 mM concentration, primers, each of 0.5 mM concentration, 0.25 units of *Taq*Polymerase (reagents provided by Promega), DNA (5 µl of 5:100 dilution) and UV/UP water to the final volume of 25 µl per PCR tube.

The DNA fragment amplification was carried out in an Eppendorf thermocycler (Mastercycler gradient).

The PCR program is the following:

Table IV. PCR program used to amplify chloroplastic microsatellites

Step number	Step	Temperature	Time
1.	Polymerase activation	95ºC	5 minutes
2.	Denaturation	94ºC	1 minute
3.	Annealing:	50 - 54ºC	1 minute
4.	Extension	72ºC	1 minute
5.	25 repeats of steps 2-4		
6.	Final extension	72ºC	8 minutes
7.	Conservation	4ºC	∞

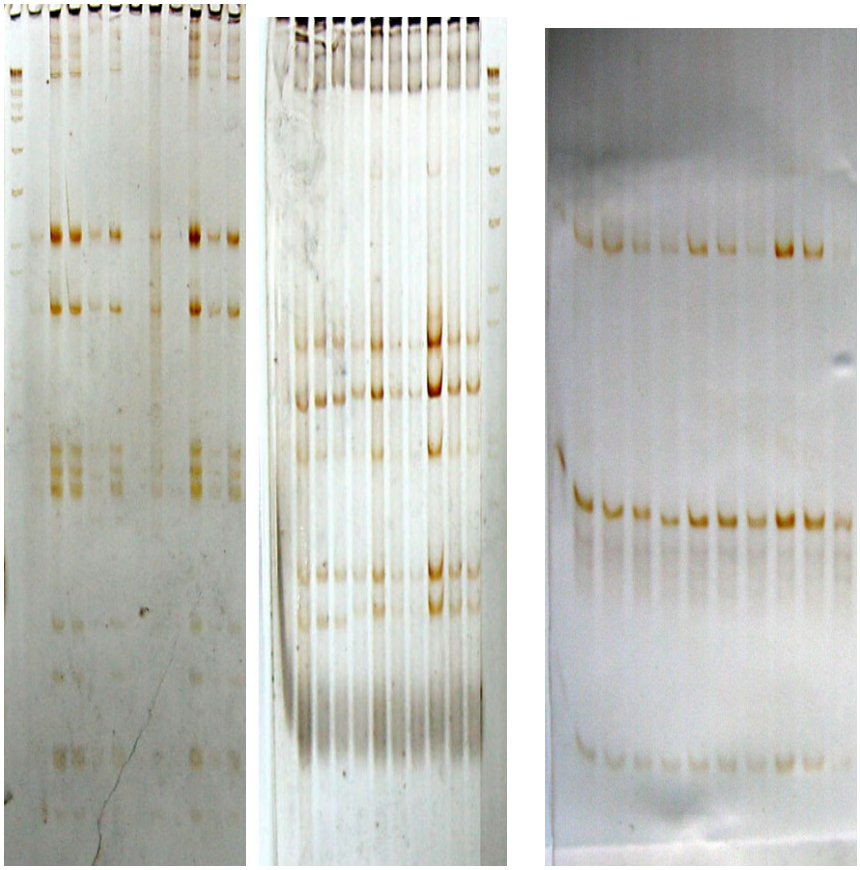
The DNA fragments obtained by means of PCR were separated on a polyacrylamide gel (7%), of 0.35 mm, in an electric field, at 1500 - 2000 V. The electrophoresis used for this method is specific to sequencing and has the following dimensions: 50 x 20cm. The fragments were viewed using silver staining [23].

RESULTS AND DISCUSSION

The PCR-RFLP at *Pulmonaria* sp.

As a result of the gel analysis for the three chloroplastic primers DT, CD, AS (Figure 1), we found out that the DNA fragments obtained through amplification and digestion have different sizes but present polymorphism only in the case of the CD primer. The polymorphism is more obvious in the 2nd and 5th band. The polymorphism between the two bands is linked, therefore only two different haplotypes can be distinguished. For *Pulmonaria rubra* we can identify only one haplotype, arbitrarily named P1, while for *Pulmonaria filarszkyana* we can identify another haplotype arbitrarily named P2.

From the point of view of genetic studies, the two species belonging to *Boraginaceae* family differentiate at the CD primer by the insertion/deletion of a sequence at the 2nd and 5th fragment level.

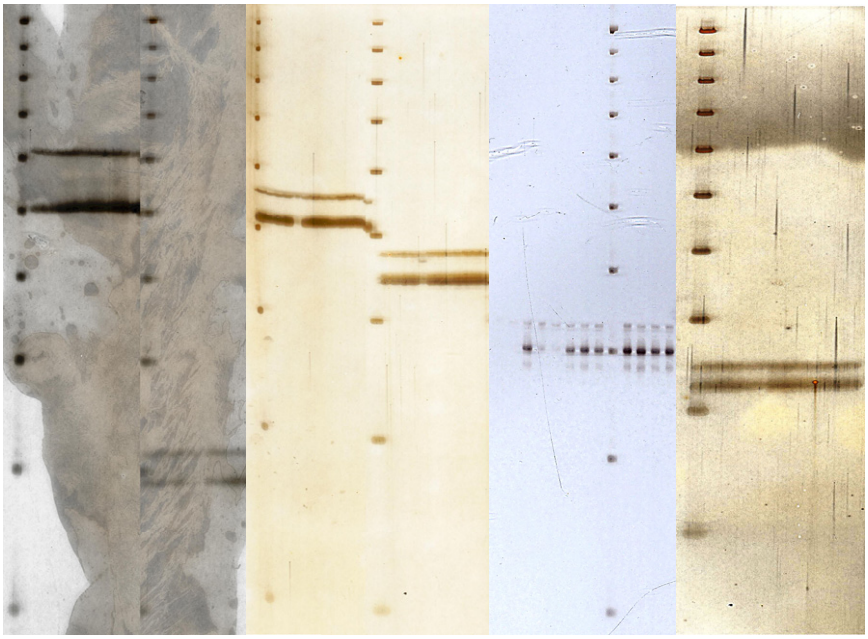


AS **CD** **DT**

Figure 1. Polyacrylamide gel electrophoresis of PCR products, using primers AS, CD and DT for Pulmonaria rubra and Pulmonaria filarszkyana after endonuclease digestion (RFLP)

The polymorphism of SSR sequences at *Pulmonaria* sp.

All six primers (ccmp2, ccmp3, ccmp4, ccmp6, ccmp7 and ccmp10) were tested for the two *Pulmonaria* species. Sequences analysis did not distinguish variability for the simple repetitive sequences neither at the intraspecific nor at the interspecific level (Figure 2).



ccmp2 ccmp3 ccmp4 ccmp6 ccmp7 ccmp10

Figure 2. SSR electrophoresis using primers cccmp2, ccmp3, ccmp4, ccmp6, ccmp7,ccmp10 for Pulmonaria rubra and Pulmonaria filarszkyana

CONCLUSION

In order to distinguish the two *Pulmonaria* species at the populational, subspecies or species level, the use of chloroplastic microsatellites is not sufficient.

The results derrived from RFLP analysis suggest that the two *Pulmonaria* species have a different genetic structure, haplotype P1 being characteristic to *Pulmonaria rubra* and the haplotype P2 being specific to *Pulmonaria filarszkyana.* The data we obtain denote that the two presumptive species are indeed independent species without relations of taxonomic subordination. It is possible that any of the two haplotypes detected to be characteristic to one species could appear in the other species due to the fact that the two species were collected from different geographical regions. This could be possible even more because of the high state of conservation of the cpDNA, and this polymorphism can be the effect of a phylogeographical structure.

Therefore it is absolutely necessary to continue this study using markers for the nuclear and mitochondrial plant genomes in order to confirm the results obtained from the analysis of the chloroplastic genome.

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