Contribuţii Botanice – 2016, LI: 7-16
Grădina Botanică "Alexandru Borza"
Cluj-Napoca

DETECTION OF BARCODE MARKERS ABLE TO DIFFERENTIATE THE CARPATHIAN ENDEMIC TAXON *PULMONARIA FILARZSKYANA* JÁV. FROM *PULMONARIA RUBRA* SCHOTT.

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Abstract: DNA barcoding represents an accurate, fast and automatable tool that enhanced species identification and species delineation. It relies on the use of standardized gene regions as internal species tags. The barcode markers should meet the followings requirements: they must have short length, conserved flanking sites and display significant genetic variability. Due to the complexity of plant genomes, all attempts to find a universal marker failed. For different plant species, different barcode markers or combinations of markers have been reported. In this study, we aimed to discover barcode markers for an efficient differentiation of two close related species from the genus *Pulmonaria* (Boraginaceae). *Pulmonaria rubra* Schott. and the Carpathian endemic *P. filarszkyana* Jáv. share a strong morphological resemblance and controversial taxonomic relationships, the latter being considered either a subspecies or a variety of the former, or a full ranked independent species. We tested fourteen target regions, of which thirteen belonged to the chloroplast and one was nuclear. Most regions lacked polymorphism or did not have a reliable, reproductible amplification. Only three regions displayed genetic variability: ITS1, *rpL16* and *psbD-trnT*. Along the full length of the three sequences, six SNPs were detected, but they proved to be sufficient for a significant delimitation of the two taxa within subsequent phylogenetic analysis.

Keywords: Boraginaceae, *Pulmonaria*, cpDNA, ITS1, SNP, barcode.

Introduction

The main goal of DNA barcoding is to establish a resource of DNA sequences available to the scientific community [20], that can provide insights into species-level taxonomy and contribute towards the taxonomic process of defining and delimiting species [19, 34], and amend the process of assigning unknown specimens to known species [21].

The selection of barcoding regions involves choosing one or a few standard loci that can be sequenced routinely and reliably in very large and diverse sample sets, resulting in easily comparable data which enable species to be distinguished from one another. Due to advances in molecular genetics, sequencing technology, and bioinformatics, DNA barcoding is allowing users to quickly and accurately recognize known species and retrieve information about them. Moreover, it has the potential to speed the discovery of the thousands of species yet to be named [4, 50].

Technically, when evaluating suitable genetic loci for DNA barcoding, three main criteria must be fully addressed: (i) significant species-level genetic variability and divergence, (ii) an appropriately short sequence length in order to facilitate DNA extraction and amplification, and

(iii) the presence of conserved flanking sites for developing universal primers [27]. In the case of the animal world, the mitochondrial gene cytochrome c oxidase subunit 1 (CO1) fits well these criteria and was selected by the Consortium for the Barcode of Life (CBOL) as the ideal gene for DNA barcoding animal species. In contrast, finding a plant equivalent has proved more difficult [13, 26], some of the reasons being the extensive evolutionary divergence between taxa since they first appeared, genome rearrangements, the widespread appearance of interspecific and intergeneric hybrids, and the huge diversity in plant life history and breeding behavior [14].

For centuries, the genus *Pulmonaria* (Boraginaceae) drew the interest of botanists worldwide [31], primarily because of its ornamental and medicinal properties [3], and lately it has become also known for its complex taxonomy and problematic delimitation of species [5, 39]. Morphologically, *Pulmonaria* species are fairly similar in having pink-blue actinomorphic tube-shaped flowers and hairy leaves, and therefore they were often considered as varieties of the same species rather than as separate species [11].

In a previous study [46], we have tackled by means of PCR-RFLP and chloroplast microsatellites the delimitation of two taxa from the genus *Pulmonaria*: *Pulmonaria rubra* Schott. and *P. filarszkyana* Jáv. They share close, yet controversial, taxonomic relationships and a strong morphological resemblance. *P. filarszkyana*, an endemic taxon for the Eastern Carpathians, has a controversial taxonomic status, being considered either a subspecies or a variety of the Carpatho-Balkanic species, *P. rubra*, or a full ranked independent species [46]. The differentiating morphological characteristics (indumentum size and leaf shape) are rather discrete and prone to misinterpretation, mainly because of the existence of intermediate forms.

For the present study, we aimed to use the barcode concept to identify molecular markers that could distinguish the two taxa in a readily, reliable and accurate manner.

Material and Methods

Taxon sampling

Six, and respectively three, populations of *P. rubra* and *P. filarszkyana* were sampled from the South-Eastern Carpathians (Table 1). In addition, two populations of *Pulmonaria officinalis* were collected as an outgroup. Young, green leaves of random individuals were gathered for each population. Plant material was dried in tubes with silica gel and stored at room temperature until DNA extraction. Voucher specimens were collected and deposited in the collection of the Institute of Biological Research, Cluj-Napoca.

DNA isolation and DNA sequencing

Total DNA was extracted from approximately 13 mg of dried plant material following the protocol of Mengoni et al. [32], with some minor modifications. DNA quality was estimated on a 1% agarose gel stained with ethidium bromide.

Thirteen plastidial regions (introns *rpL16*, *rps16*, *trnG*, *trnL*, and intergenic spacers *rpl32-ndhF*, *rps16-trnK*, *trnD-E*, *trnS-fM*, *psbD-trnT*, *trnH-psbA*, *trnS-G*, *trnT-L*, *trnL-F*) and one ribosomal nuclear - internal transcribed spacer 1 (ITS1) - were tested for polymorphism.

The 50 μL PCR mix was identical for all the reactions and was composed of 1x Taq Buffer; 2.5 mmol·L^{-1} $MgCl_2$; 0.2 mmol·L^{-1} of each dNTP; 0.12 μmol·L^{-1} of each primer; 0.16 mg·mL^{-1} of BSA; 1 U of TaqPolymerase (Fermentas) and 10 μL of diluted genomic DNA. The primers and the PCR parameters are shown in Table 2 for each of the target regions. The same primers were used for both the PCR and cycle sequencing.

Table 1: Sampled populations of *P. rubra* and *P. filarszkyana*: numbering, acronym, geographic origin, coordinates.

No.	Species	Population code	Location	Coordinates
1	*Pulmonaria filarszkyana*	VV-PF	Valea Vaserului, Maramureş County	47°44'18.6"N 24°50'30.3"E
2	*Pulmonaria filarszkyana*	N-PF	Negoescu, Rodna Mts, Maramureş County	47°36'35.84"N 24°44'06.69"E
3	*Pulmonaria filarszkyana*	S-PF	Şesuri, Maramureş County	47°36'42.56"N 24°57'18.18"E
4	*Pulmonaria rubra*	VC-PR	Valea Cepelor, Alba County	46°27'55.33"N 22°44'19.31"E
5	*Pulmonaria rubra*	A-PR	Arieşeni, Alba County	46°28'30.46"N 22°44'26.75"E
6	*Pulmonaria rubra*	VI-PR	Valea Ierii, Cluj County	46°33'45.85"N 23°15'50.04"E
7	*Pulmonaria rubra*	VS-PR	Pârâul Văii Seci, Bistriţa-Năsăud County	47°28'19"N 24°49'18"E
8	*Pulmonaria rubra*	GH-PR	Giurgeu-Hăşmaş Mts, Harghita County	46°40'46.41"N 25°49'21.37"E
9	*Pulmonaria rubra*	Re-PR	Cascada Lolaia, Retezat Mts, Hunedoara County	45°25'36.88"N 22°53'48.73"E

Table 2: The PCR primers and parameters used for amplifying ITS1 and thirteen cpDNA regions in *Pulmonaria* sp.

Region	Primers	PCR parameters
ITS1	ITS 2 and ITS 5 [54]	94ºC 5 min; 35 X (94ºC 1 min, 52ºC 45 sec, 72ºC 2 min); 72ºC 10 min
rpL16	rpL16F71 and rpL16R1516 [45]	80ºC 5 min; 35 X (95ºC 1 min, 50ºC, ramp 0.3ºC/sec 1 min, 65ºC 5 min); 65ºC 4 min
rps16	rpS16F and rpS16R2 [33]	80ºC 5 min; 35 X (94ºC 30 sec, 55ºC 30 sec, 72ºC 1 min); 72ºC 5 min
trnG	trnG and trnG2G [43]	80ºC 5 min; 30 X (95ºC 1 min, 66ºC 4 min); 66ºC 10 min
trnL	trnLc and trnLd [47]	94ºC 3 min; 35 X (94ºC 45 sec, 56ºC 45 sec, 72ºC 1 min); 72ºC 10 min
rpl32-ndhF	rpL32-R and ndhF [44]	80ºC 5 min; 30 X (95ºC 1 min, 50ºC, ramp 0.3ºC/sec→65ºC 1 min, 65ºC 4 min); 65ºC 5 min
rps16-trnK	rpS16x2F2 and trnK$^{(UUU)}$x1 [44]	80ºC 5 min; 30 X (95ºC 1 min, 52ºC, ramp 0.3ºC/sec→60ºC 1 min, 65ºC 4 min); 65ºC 5 min
trnD-E	trnD^{GUC}F [12] and trnE^{UUC} [43]	80ºC 5 min; 30 X (95ºC 45 sec, 56ºC 30 sec, 72ºC 4 min); 72ºC 5 min
trnS-trnfM	trnS^{UGA} and trnfM^{CAU} [12]	80ºC 5 min; 30 X (95ºC 30 sec, 55ºC 30 sec, 72ºC 2 min); 72ºC 5 min
psbD-trnT	psbD and trnT$^{(GGU)}$R [44]	80ºC 5 min; 30 X (95ºC 1 min, 50ºC, ramp 0.3ºC/sec→60ºC 1 min, 65ºC 4 min); 65ºC 5 min
trnH-psbA	trnH^{GUG} [49] and psbA [38]	80ºC 5 min; 35 X (94ºC 30 sec, 55ºC 30 sec, 72ºC 1 min); 72ºC 10 min
trnS-trnG	trnS^{GCU}F and trnG^{UUC} [43]	80ºC 5 min; 30 X (95ºC 1 min, 66ºC 4 min); 66ºC 10 min
trnT-trnL	trnT^{UGU}F and trnL^{UAA}R [47]	96ºC 5 min; 35 X (96ºC 1 min, 55ºC 2 min, 72ºC 2 min 30 sec); 72ºC 5 min
trnL-trnF	trnLc and trnL-Ff [47]	80ºC 5 min; 35 X (94ºC 1 min, 50ºC 1 min, 72ºC 2 min); 72ºC 5 min

Prior to sequencing, the PCR products were purified by gel purification using WizardR SV Gel and PCR Clean-Up System, according to the manufacturer's protocol (Promega).

Sequencing (of both strands) was performed using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) with 5x sequencing buffer. Excess primers and labeled dideoxynucleotide triphosphates were removed by purification with Sephadex and Sephacryl (1:1) (GE Healthcare Bio-Sciences AB). The samples (total volume of 20 μL) were prepared prior to sequencing by adding 10 μL of HiDi formamide and then migrated onto an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) using a 36 cm capillary and POP7^{TM} polymer.

Data analysis

Sequences were assembled, edited, and manually aligned using BioEdit version 7.0.9.0 [18]. Subsequently, phylogenetic analyses were performed using the programme MEGA version 4.1 [48]. Trees construction was achieved by different methods: Maximum Likelihood (ML), Maximum Parsimony (MP), and Neighbour Joining (NJ), with the use of Kimura's two parameter [23]. Bootstrap values were calculated from 1000 replicates.

Results

While some chloroplastic regions could not be amplified for the entire set of populations (*trnS-fM* and *trnL-F*) and other regions had trouble sequencing (*rps16-trnK*, *trnS-G*, *trnT-L*, *rps16*), successful amplification and sequencing for the entire set of populations were obtained only for the following eight regions: ITS1, *trnG*, *rpL16*, *trnL*, *rpl32-ndhF*, *psbD-trnT*, *trnD-E*, and *trnH-psbA* (GenBank accession numbers: JX196756-JX196843).

Five regions revealed no polymorphism (*rpl32-ndhF*, *trnD-E*, *trnG*, *trnL* and *trnH-psbA*) and produced identical sequences for *P. rubra* and *P. filarszkyana*.

Only three regions (ITS1, *psbD-trnT* and *rpL16)* produced consistent, though few, polymorphic changes between the two taxa. Within the 343 nucleotides of ITS1, three base changes were identified between *P. rubra* and *P. filarszkyana*: two transversions and one deletion (Table 3). The intergenic spacer *psbD-trnT* had 1225 nucleotides in case of *P. rubra* and *P. filarszkyana* but exposed only one modification (Table 3). The intron *rpL16* had 1070 bases and displayed two relevant modifications (transversions) (Table 3).

Table 3: Type and position of the revealed polymorphisms in *Pulmonaria rubra* and *P. filarszkyana*. The dots stand for identity, while the line stands for missing character.

Population code	Polymorphism					
	ITS1			*psbD-trnT*	*rpL16*	
	74	164	226	712	379	986
VV-PF	A	T	G	G	T	G
N-PF	.	.	.	.	.	.
S-PF	.	.	.	.	.	.
VC-PR	C	A	-	A	A	T
A-PR	C	A	-	A	A	T
VI-PR	C	A	-	A	A	T
VS-PR	C	A	-	A	A	T
GH-PR	C	A	-	A	A	T
Re-PR	C	A	-	A	A	T

The ML analysis (Fig. 1) based on the concatenated sequences of the eight successfully sequenced markers revealed two main groups: one group comprised all the *P. rubra* populations, whereas the second group clustered the three *P. filarszkyana* populations. Furthermore, both the MP and NJ analyses exhibit the same pattern (data not shown).

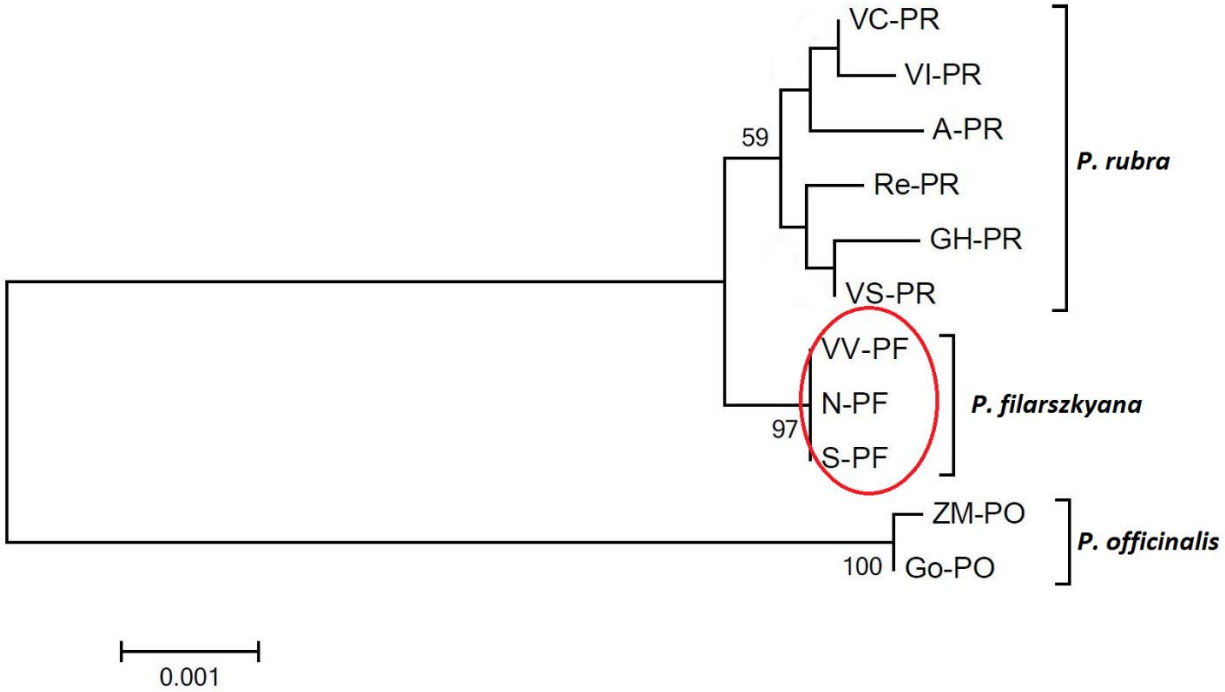

Fig. 1: The ML tree based on the concatenated sequences of ITS1, *rpL16*, *trnG*, *trnL*, *rpl32-ndhF*, *psbD-trnT*, *trnD-E* and *trnH-psbA*. Names of populations as in Table 1.

Discussion

Barcode markers selection

An ideal DNA barcode should be universal, reliable, cost effective and show good discriminatory power [6]. As none of the previously proposed barcodes perfectly meets all these criteria, the general trend is to consider necessary the use of more than one marker to barcode plants [13, 26, 27, 37]. In addition, one should take into account the fact that closely related plants are difficult to barcode, because it has been shown that recent histories of hybridization can homogenize or even uncouple plastid genome phylogenies from species phylogenies [42]. So, bringing input from nuclear markers was also suggested that it might improve the phylogenetic confidence. These are the reasons that determined us to test in our study more regions, both nuclear and plastidial.

For long time, the multicopy nuclear internal transcribed spacer (ITS) was a leading candidate for barcoding [26], though some authors [1] considered that ITS should be used with caution due to its complex and unpredictable evolutionary behavior. Some studies [9] showed that, when compared against plastidial markers, ITS has the highest overall discriminating power, this finding being consistent with numerous previous studies showing that this nrDNA region evolves rapidly, leading to genetic changes that can differentiate closely related, congeneric species. A systematic survey across the major eukaryote kingdoms (Fungi, Plantae and Animalia) that took into consideration the following criteria: the presence of DNA barcoding

gaps, species discrimination efficiency, sequence length distribution, GC content distribution and primer universality, showed that ITS1 has higher discrimination power and more conserved flanking regions than ITS2 in more taxonomic groups, and, overall, represents a better DNA barcode than ITS2 in eukaryotes [52]. Ensuing the use of ITS1, our results were in agreement with those reports. The full length of ITS1 in *Pulmonaria* was only 343 bases, but it revealed three potentially informative characters (PICs). These point mutations (two transversions and one deletion) were consistent between all the populations of *P. rubra* and *P. filarszkyana* (Table 3).

There are some current studies that recommend the use of a barcoding system consisting of two plastidial genes, *rbcL* and *matK*, but a unique identification to species level was acquired in only 72% of cases and the remainder until 100% was matched to groups of congeneric species [6]. It was also suggested that the proposed core barcode, *rbcL* + *matK*, or these together with plastid regions, produced lower levels of discrimination than ITS alone or the combination of ITS with any plastid DNA markers. Furthermore, earlier studies [8, 16] indicated that noncoding sequences have a much more complicated evolution pattern and more frequent insertion and deletion events as they are under less functional constraint than the coding regions and, thus, providing greater levels of variation that should be exploit within low level taxonomies.

Therefore, for our study of closely related taxa, we chose to use, in addition to ITS, different chloroplastic non-coding regions, both intergenic spacers and introns. The selected regions (*trnS-fM*, *trnL-F*, *rps16-trnK*, *trnS-G*, *trnT-L*, *rpl32-ndhF*, *psbD-trnT*, *trnD-E*, *trnH-psbA*, *rps16*, *trnG*, *rpL16*, and *trnL*) were already noticed by other authors for their discriminatory power [7, 15, 17, 25, 27, 29, 30, 35, 36, 40, 53, 56, 58].

Nevertheless, our results pointed out only two polymorphic chloroplastic markers: the intergenic spacer *psbD-trnT* which exposed only one point modification, while the intron *rpL16* had two relevant modifications (Table 3). The two cloroplast markers combined revealed an equal number of SNPs as ITS1, but the sequence length was almost seven time larger (2295 bases for *rpL16* + *psbD-trnT*, whereas only 343 bases for ITS1). Moreover, if we refer to the whole length of the seven successfully sequenced chloroplastic markers we obtained only three modifications in 4659 bases. This fact might have derived from the highly conservative nature and slow evolutionary rate of the chloroplast genome comparative with the nuclear genome. However, *psbD-trnT* was marked for its informative potential [10, 22, 51), while [44] reported it as a region that offers levels of variability previously unseen in the chloroplast genome. The *rpL16* intron was equally reported for polymorphism at intraspecific level [2, 24, 28, 41, 55], although it was used mainly for resolving upper taxonomic levels [45, 57].

Delimitation of Pulmonaria rubra and P. filarszkyana

One of the intended purposes of DNA barcode is to effective delimit closely related species. The taxonomic status of *P. filarszkyana* is questionable and still represents a debate within the taxonomy of *Pulmonaria* genus. This bias is maintained and also amplified by the appreciable morphologic resemblance of the two taxa.

There has been only one previous attempt to resolve their delimitation by means of molecular biology [46]. Here we used microsatellite genotyping and PCR-RFLP, both techniques targeting chloroplast regions. The six microsatellites (ccmp2, ccmp3, ccmp4, ccmp6, ccmp7, and ccmp10) revealed no polymorphic variation between *P. rubra* and *P. filarszkyana*. The three chloroplast regions investigated by PCR-RFLP were *trnD-T*, *trnC-D* and *psbA-trnS*, but only *trnC-D* showed a consistent differentiating pattern between the two taxa.

Nevertheless, because PCR-RFLP technique has its drawbacks (time-consuming, low reproductibility, lack of data accessibility), we needed a modern tool that could generate reliable and easily comparable data. This 'wish list' added to the relative low cost, indicated barcode markers as the best choice. Through barcoding of fourteen regions, we were able to discover three regions with variability for the study species. Altogether the polymorphic regions summed up six SNPs that belong solely to the two taxa and were shared by all the populations. Though few, the identified point mutations were capable to clearly delimit the taxa within the subsequent phylogenetic analyses. All the generated trees, regardless of the applied method, delimited the taxa into two groups with a solid bootstrap value (over 97). The *P. filarszkyana* group was homogenous perhaps due to a close geographical sampling (all the samples originated from Maramureş County), while the *P. rubra* group was split into a few subgroups. These subgroups were weekly supported by the bootstrap values (only one above 80, the rest below) and predictably displayed a geographic pattern.

Conclusions

We successfully applied the barcode technique for two close related taxa: *Pulmonaria rubra* and *P. filarszkyana*. We discovered three polymorphic regions, one nuclear (ITS1) and two plastidial (*psbD-trnT* and *rpL16*). These variable regions exposed six point mutations (two transversions and one deletion for ITS1; one transition in *psbD-trnT*; and two transversions for the intron *rpL16*), particular to the two taxa and shared by all the sampled populations. The six SNPs were sufficient to discriminate between the taxa with a strong bootstrap support.

Acknowledgements: The authors would like to thank Liviu Filipaş, Tudor Ursu, Bogdan Hurdu and Adrian Stoica for their valuable help in gathering the sampling material. The authors were financially supported by the BIODIVERS financing programme (PN 16 19).

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DETECTAREA UNOR MARKERI BARCODE CAPABILI SĂ DIFERENȚIEZE TAXONUL CARPATO-ENDEMIC *PULMONARIA FILARZSKYANA* JÁV. DE SPECIA *PULMONARIA RUBRA* SCHOTT.

(Rezumat)

Tehnica de ADN barcoding reprezintă o metodă rapidă și precisă care a îmbunătățit identificarea și delimitarea speciilor. Ea are la bază utilizarea unor regiuni standardizate din genom care servesc drept etichete interne. Markerii utilizați în cadrul acestei metode trebuie să îndeplinească mai multe condiții simultane: să fie reduși ca lungime ceea ce duce la o creștere a ratei de succes în cadrul reacției de amplificare și a secvențializării; să prezinte la capete regiuni conservate care pot servi la atașarea unor amorse universale; și să prezinte un grad optim de variabilitate genetică. Datorită complexității și diversității existente în cadrul genomurilor plantelor, până în prezent nu s-a descoperit un marker universal care să îndeplinească condițiile și care să funcționeze cu succes la toate speciile. Așadar, prezentul studiu are ca scop descoperirea unor markeri barcode care să diferențieze eficient doi taxoni strâns înrudiți din genul *Pulmonaria* (Boraginaceae): *Pulmonaria rubra* Schott. și endemitul est carpatic, *P. filarszkyana* Jáv. Taxonii sunt asemănători din punct de vedere morfologic și prezintă relații taxonomice incerte, controversate, *P. filarszkyana* fiind considerată de către diferiți autori fie o specie distinctă, fie o subspecie sau chiar o varietate a speciei carpato-balcanice *Pulmonaria rubra*. În acest scop au fost testate 13 regiuni cloroplastice: intronii *rpL16*, *rps16*, *trnG*, *trnL*; regiunile intergenice *rpl32-ndhF*, *rps16-trnK*, *trnD-E*, *trnS-fM*, *psbD-trnT*, *trnH-psbA*, *trnS-G*, *trnT-L*, *trnL-F*; precum și regiunea nucleară ribozomală ITS1. S-au reușit amplificarea reproductibilă și secvențializarea a opt regiuni, dar, dintre acestea, doar trei au evidențiat modificări polimorfice consistente între cei doi taxoni. În cadrul secvențelor concatenate ale celor trei markeri, s-au identificat doar șase SNP-uri, dar acestea reușesc să delimiteze semnificativ cei doi taxoni în cadrul analizelor filogenetice.

Received: 8.11.2016; Accepted: 7.12.2016