AcademicPres

Available online at www.notulaebotanicae.ro

Print ISSN 0255-965X; Electronic 1842-4309

Not Bot Hort Agrobot Cluj, 2011, 39(1):24-29

Notulae Botanicae Horti Agrobotanici Cluj-Napoca

Does *Primula intricata* Gren. et Godr. Merit Species Rank? A Taxonomic Revision Based on nrDNA, cpDNA and AFLP Data

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Abstract

Primula intricata Gren. et Godr. has an unclear taxonomic status: it was originally described as a distinct species but subsequently was considered a subspecies (*Primula elatior* subsp. *intricata)* or even a variety (*P. elatior* var. *intricata*) of *Primula elatior* (L.) Hill. No prior genetic studies were performed on this group of *Primulaceae*, therefore we considered useful to investigate taxonomies boundaries within the *P. elatior-intricata* group. We explored genetic differences between *Primula intricata* and *Primula elatior* group by applying three different types of molecular markers: nuclear ribosomal DNA (ITS1), chloroplast DNA (spacer *trnH-psbA* and intron *trnL*) and Amplified Fragment Length Polymorphisms (AFLP). We found a solid differentiation between *P. intricata* and *P. elatior* group, differentiation that was confirmed by all the employed molecular markers. This finding enabled us to propose a valid species rank for *Primula intricata*, as a separate taxon from the *P. elatior* group.

Keywords: AFLP, cpDNA, ITS, *Primulaceae*, taxonomy

Introduction

Primula intricata Gren. et Godr. belongs to subgenus *Primula* L. which is characterized by rosettes shoots, leaves hairy and revolute vernation (Valentine and Kress, 1972). The species is confine to upper mountain-subalpine moist grassland and it has a distribution restricted to South and Central part of Europe (Smith and Fletcher, 1946; Valentine and Kress, 1972).

P. intricata was originally described by Grenier and Godron (1853) from the Eastern Pyrenees as a distinct species with the following morphological features: leaves finely pubescent on both sides, oblong, obtuse, gradually narrowed into a long petiole and with a broad wing becoming obsolete below; scape, pedicels and the calyx with a fine tomentum. Subsequently Pax and Knuth (1889) reconsidered the taxonomic rank of *P. intricata* and ascribed it to *P. elatior* group (*P. elatior* var. *intricata* (Gren. et Godr.) Pax. Lüdi (1927) considered *P. intricata* as a distinct subspecies within the *P. elatior* group (*Primula elatior* (L.) Hill subsp. *intricata* (Gren. et Godr.) Lüdi) and this opinion is maintained by Valentine and Kress (1972) in *Flora Europaea*. According to *Flora Euroapaea*, *P. intricata* can be differentiated from *P. elatior* subsp. *elatior* by the leaves gradually narrowing into petiole and the capsule tapering markedly (Valentine and Kress, 1972).

Many taxonomical issues, especially where the morphological boundaries between very close taxa are scarce, were elucidated with the development of molecular markers (Foggi *et al.*, 2006; Ishida *et al.*, 2003; Lihová *et al.*, 2004; Manoko *et al.*, 2007; Muratović *et al.*, 2005; Schönswetter *et al.*, 2004; Schönswetter *et al.*, 2009).

The main goal of this study was to investigate whether there is a genetically sound basis for supporting *P. intricata* as a distinct species from *P. elatior* s. str. and to investigate the taxonomic boundaries between *P. intricata* and *P. elatior* group.

Materials and methods

Sampling strategy

In order to investigate the relationship between *Primula intricata* and *Primula elatior* group, we sampled seven populations from the Carpathians and the Alps (Fig. 1, Tab. 1) as follows: five populations of *Primula elatior*, one population of *Primula elatior* subsp. *leucophylla* and one population of *Primula elatior* subsp. *intricata*. Young, green leaves of five random individuals were collected for each population and stored in silica gel. Voucher specimens of all populations were deposited in CL Herbarium (Babeş-Bolyai University, Cluj-Napoca). Only for cpDNA and ITS sequences analysis we added one herbarium sample of *Primula elatior* subsp. *intricata* from Carpathians (Piatra Craiului Massif, CL no 510241) and two samples of *Primula veris* and *P. acaulis* as outgroups.

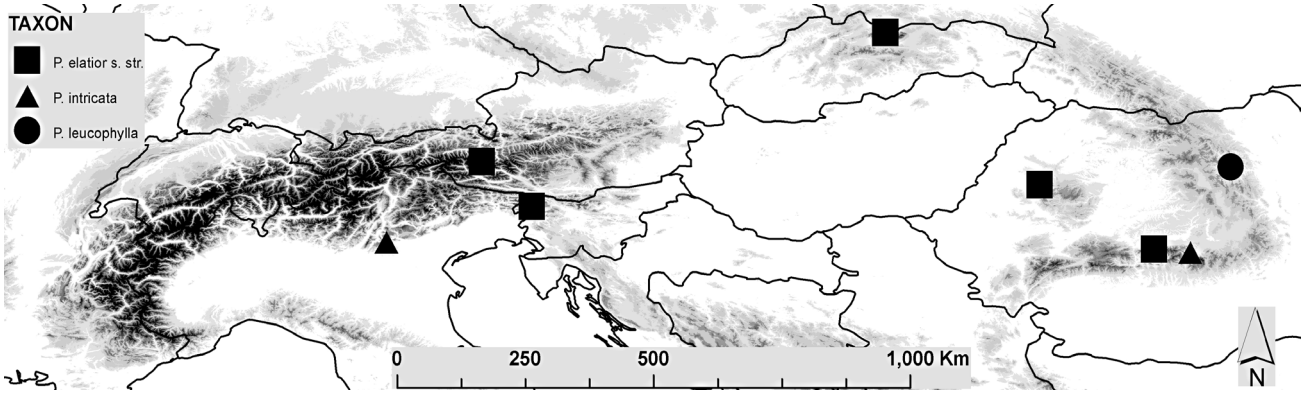

Fig. 1. Location of the populations of *Primula* sp. sampled for molecular analysis

DNA extraction and AFLP protocol

Total DNA was extracted from roughly 13 mg of silica gel dried leaf tissue using DNeasy 96 Plant Mini Kit (Qiagen) according to the manufacturer's protocol, excepting the final elution which took place in 80 µl in order to increase the DNA concentration. Three random individuals from the total sample set were extracted twice as blind samples (Bonin *et al.*, 2004). The herbarium sample was extracted using a modified CTAB method of Doyle and Doyle (1987).

The AFLP protocol (based on Vos *et al.*, 1995) followed the basic lines of Puşcaş *et al.* (2008) with minor modifications. Double digestion of genomic DNA was performed for two hours at 37°C in a 20 µl mix using 2 U of *Mse*I and 5 U of *Eco*RI (Fermentas). Specific adapters were ligated to DNA in a 40 µl volume for 2 h at 37°C using 1 U of T4 DNA Ligase (Fermentas). Diluted 10 X, digested and ligated DNA was subjected to a preselective amplification using primers EA (5′-GACTGCGTACCAATTCA-3′) and MC (5′-GATGAGTCCTGAGTAAC-3′) in a 25 µl volume containing 1X Buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.2 µM of each primer and 0.5 U of AmpliTaq DNA Polymerase (Applied Biosystems). Diluted 20 X, preselective products underwent selective amplification with the following primer combinations: EACC-MCAA, EACC-MCAT and EACT-MCAT (Jacquemyn *et al.*, 2009). Selective amplifications took place in a 12.5 µl volume containing 1X Buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 0.2 µM of each primer and 0.5 U of AmpliTaq Gold® DNA Polymerase (Applied Biosystems). Following this step, excess labelled primers were removed by purification with Sephadex and Sephacryl (1:1) (GE Healthcare Bio-Sciences AB). Finally, 1.5 µl of diluted (50 X) purification was added to a 10 µl mixture (10:0.1) of HiDi

Tab. 1. Location (mountain system, Massif), altitude and genetic diversity parameters (revealed by AFLP analyses) of the sampled populations of *Primula* sp.

Loc. Nr.	Designate taxa	Mountain System	Massif	Location name	Altitude (m)	Proportion of variable markers	Gene Diversity	Rarity
1	*Primula elatior* (L.) Hill subsp. *elatior*	Alps	Glocknergruppe Alpen	Grossglockner Hochalpenstrasse	2000	0.286	0.146	3.766
2	*Primula elatior* (L.) Hill subsp. *elatior*	Alps	Julian Alps	Soca Valley	1750	0.133	0.061	2.804
3	*Primula elatior* (L.) Hill subsp. *elatior*	Western Carpathians	West Tatra	Kobylarzowy Żleb	1650	0.267	0.132	5.503
4	*Primula elatior* (L.) Hill subsp. *elatior*	South-Eastern Carpathians	Vlădeasa	Stâna de Vale	1096	0.235	0.113	3.279
5	*Primula elatior* (L.) Hill subsp. *elatior*	South-Eastern Carpathians	Făgăraş Mts.	Netedu	2250	0.21	0.103	5.155
6	*Primula elatior* (L.) Hill subsp. *leucophylla* (Pax) Hesl.-Harr.f. ex W.W.Sm. et H.R.Fletcher	South-Eastern Carpathians	Ceahlău	Toaca	1812	0.146	0.072	2.885
7	*Primula elatior* (L.) Hill subsp. *intricata* (Gren. et Godr.) Lüdi	Alps	Alpi dell'Adamello e della Presanella	Passo di Campo	1470	0.133	0.068	8.005
8	*Primula elatior* (L.) Hill subsp. *intricata* (Gren. et Godr.) Lüdi*	South-Eastern Carpathians	Piatra Craiului	Turnu	1900	-	-	-

* this sample was used only for cpDNA and ITS analyses

formamide and GeneScan 500 ROX (Applied Biosystems). The purified PCR products were migrated on ABI PRISM® 3130 Genetic Analyzer (Applied Biosystems) using a 36 cm capillary and POP 7™ polymer. The size-calibrated genescan files were imported into GeneMapper v.4.0 (Applied Biosystems) for scoring. Fragments within the 50–500 bp range were scored to produce a present/absence matrix.

cpDNA and nrDNA protocol

One nuclear region (ITS1) and two chloroplast DNA regions (the intergenic spacer *trnH-psbA* and the intron *trnL*) were used in the present study. The nuclear ribosomal DNA was amplify using primers ITS2 and ITS5 (White *et al.*, 1990). The chloroplast region *trnH-psbA* was amplified using the primers trnH^{GUG} and psbA (described by Shaw *et al.*, 2005). For amplifying the *trnL* region the following primers were used: trnLc and trnLd (Taberlet *et al.*, 1991).

Amplifications were performed in a 50 µl total reaction volume with 1X Taq Buffer, 2.5 mM $MgCl_2$, 0.5 mM of each dNTP, 0.12 µM of each primer, 0.16 mg/ml BSA, 2 U of Dream Taq Polimerase (Fermentas) and 10 µl of diluted genomic DNA. The amplification conditions for *trnH-psbA* were the ones mentioned in Shaw *et al.* (2005), with the annealing temperature of 55°C. For *trnL* the amplification conditions were the following: 94°C, 3 min; 35 x (94°C, 45 s; 56°C, 45 s; 72°C, 1 min); 72°C, 10 min. The nrDNA ITS1 had the following parameters: 94°C, 5 min; 35 x (94°C, 1 min; 52°C, 45 s; 72°C, 2 min); 72°C, 10 min.

PCR products were purified by gel purification using Wizard® SV Gel and PCR Clean-Up System, according to the manufacturer's protocol (Promega). Sequencing was performed in a 20 µl volume using BigDye Terminator Cycle Sequencing Ready Reaction Kit, v. 3.1 according to the manufacturer's suggestions (Applied Biosystems), using the following thermal cycle parameters 96°C, 10 s; 35 x (96°C, 10 s; 60°C for ITS1, *trnL* and 50°C for *trnH-psbA*, 10 s; 66°C, 4 min. Both DNA strands were sequenced. Excess primers and labeled ddNTPs were removed by purification with Sephadex and Sephacryl (1:1) (GE Healthcare Bio-Sciences AB). The samples were prepared prior sequencing by adding 10 µl of HiDi formamide. The samples were run on an ABI PRISM® 3130 Genetic Analyzer, Applied Biosystems. Sequences were assembled and edited using BioEdit v.7.0.9.0 (Hall, 1999).

Data analysis

For the AFLP dataset, the total number of AFLP bands, the proportion of polymorphic loci within each population, Nei's gene diversity (Nei, 1987) and the rarity index (corresponds to the frequency down weighed marker value proposed by Schönswetter and Tribsch, 2005) were estimated using AFLPdat R-script (Ehrich, 2006). A neighbor joining tree using Nei and Li's distance (Nei and Li, 1979) measure was constructed with Splistree v. 4.10 (Huson and Bryant, 2006). Bootstrap values were obtained with 1000 replicates.

Analysis of molecular variance (AMOVA), with partition of the total genetic variance into different levels (within populations, among populations, among groups of populations), were done with ARLEQUIN version 3.5 (Excoffier and Lischer, 2010).

Principal Coordinate Analysis (PCoAs) based on between-individual Jaccard similarities (Jaccard, 1901) were carried out by using the ADE-4 software package (Thioulouse *et al.*, 1997).

The relationships among detected cpDNA haplotypes were analyzed using the program Mega 4.1 (Tamura *et al.*, 2007). The tree construction was made using Neighbor Joining method, based on Kimura's (1980) genetic distance.

Results and discussions

AFLP data

Using three pairs of primers, 236 scorable fragments have been generated, of which 212 (89.8 %) were polymorphic. The length of fragments ranged from 54 to 456 bp. The repeatability of AFLP results was high (97.92 % for the overall test). The final matrix consisted of 35 individuals and 157 unambiguous polymorphic markers.

Analysis of molecular variance based on AFLP data was initially performed on *Primula* sp. populations without defining any groups. The percentage of variation was very high among populations (67.70, data not shown). A second AMOVA was performed after defining three groups corresponding to the three taxa: *P. elatior*, *P. leucophylla* and *P. intricata* (Tab. 2).

The highest amount of genetic variation in the sampled populations was found among groups (Tab. 2, AMOVA 43.96 % overall populations) and suggests that the groups are well differentiated, with relative low gene flow within. The variation among populations within groups is also relatively high. This result might be due to an inner structure

Tab. 2. Results of analysis of molecular variance (AMOVA) based on the AFLP matrix with three defined groups

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation
Among groups	2	320.937	13.47673 Va	43.96
Among populations within groups	4	218.320	9.35029 Vb	30.50
Within populations	28	219.200	7.82857 Vc	25.54
Total	34	758.457	30.65558	

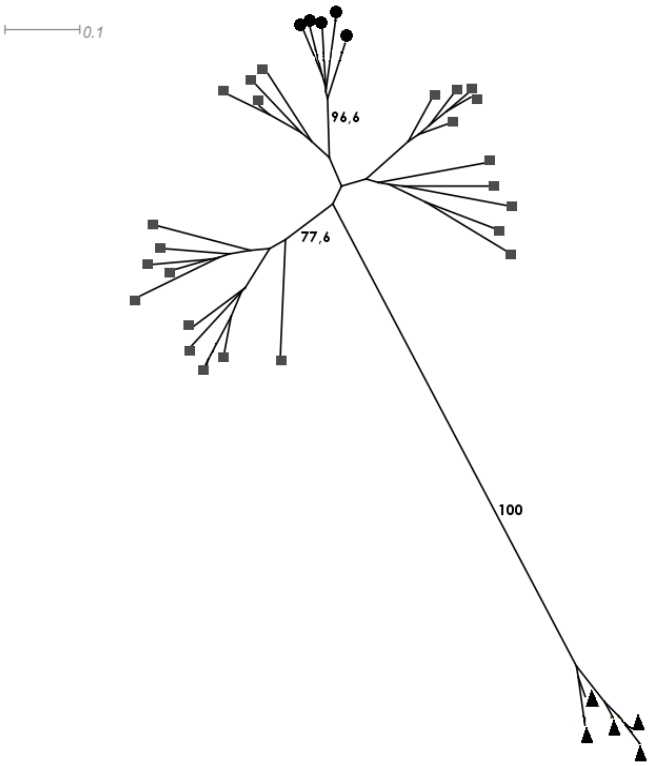

Fig. 2. Neighbor Joining analysis based on the AFLP data. Symbols are the same as in Fig. 1

within groups based on geographical criteria (the three populations of *P. elatior* were collected from distant localities from South-Eastern Carpathians, Western Carpathians and Alps).

When referring to gene diversity, proportion of variable markers and rarity, the highest genetic diversity and the highest proportion of variable markers were found in one population from the Alps (Glocknergruppe Alpen) while the population of *Primula intricata* was among the poorest. However, the population of *Primula intricata* harbors the highest number of private markers, once again showing its high differentiation from the *P. elatior* group.

In the Neighbor-Joining analysis, two major phylogroups were separated with maximum bootstrap support. One group is represented by the population of *Primula intricata* from the Alps and the second group is composed by the populations of *Primula elatior* and *Primula leucophylla* (Fig. 2). The Alpine population of *Primula intricata* falls into a clearly divergent group, this is most straightforwardly observed in branch lengths and bootstrap support values of the NJ tree.

The PCoA of the AFLP matrix of analyzed individuals confirms approximately the same phylogeographical pattern with two main groups: the population of *Primula intricata* from the Alps clearly differentiated from the second group containing the populations of *P. elatior* and *P. leucophylla* (Fig. 3).

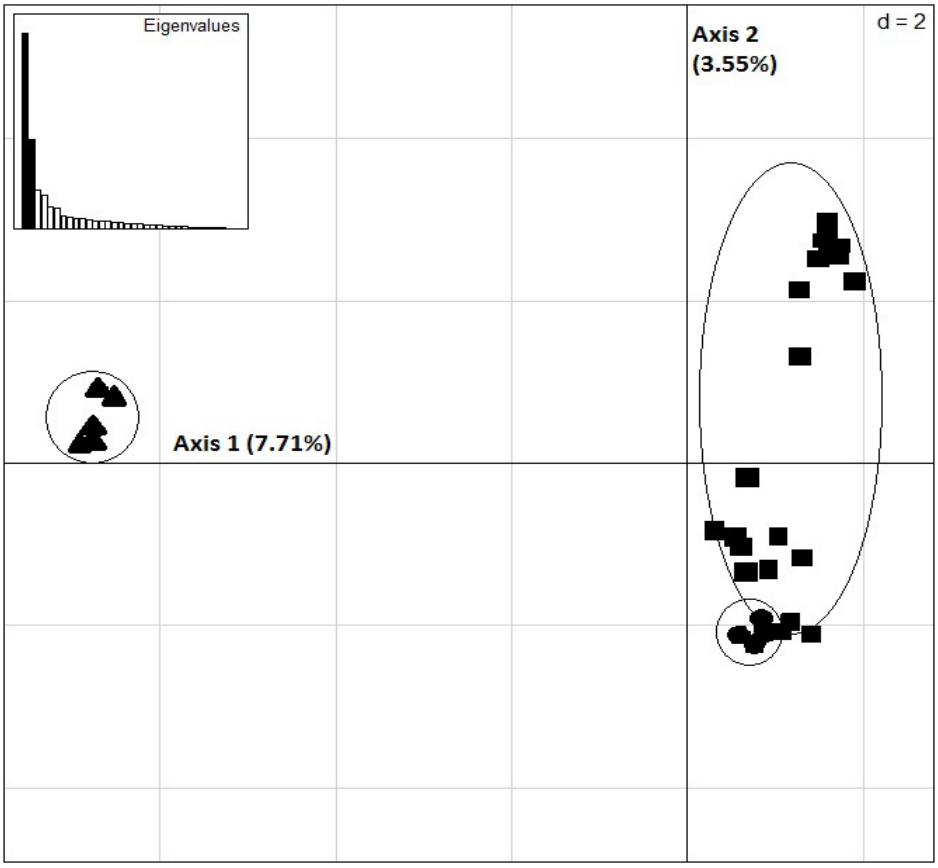

Fig. 3. Results of the Principal Coordinate Analysis (PCoA) based on AFLP data, symbols are the same as in Fig. 1

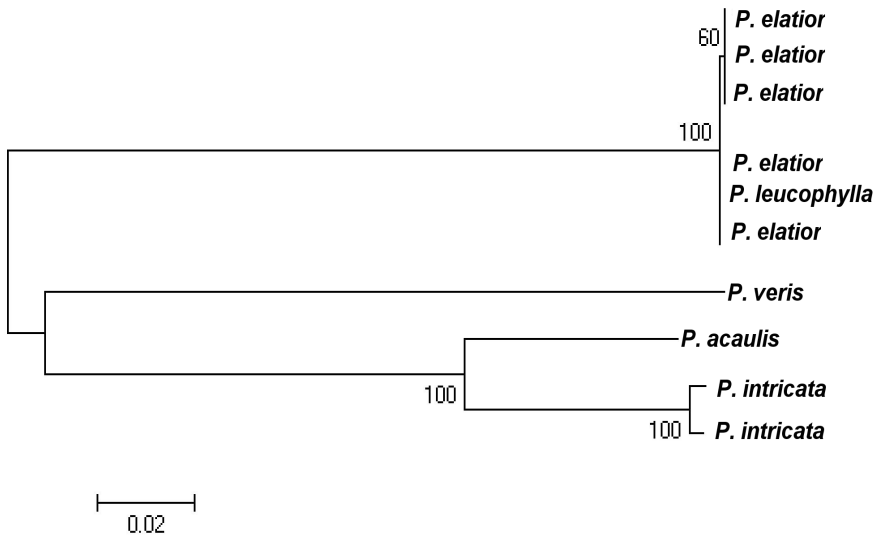

Fig. 4. The Neighbor Joining tree based on the concatenated sequence of ITS-*trnH-psbA-trnL*

cpDNA and nrDNA analysis

The concatenated sequences of ITS, *trnH-psbA* and *trnL* comprised approximatively 1058 nucleotides for populations of *P. elatior* and *P. leucophylla* and 1218 nucleotides for *P. intricata*.

The Neighbor Joining analysis of ITS - cpDNA concatenated sequences differentiates within the sampled populations two major groups: one group composed of *P. intricata* (toghether with *Primula veris* and *P. acaulis*) and the other composed of *P. elatior* and *P. leucophylla*.

The sequences belonging to *Primula intricata* were highly divergent from the ones shared by *Primula elatior* group and displayed over 200 variable sites. Between the 2 populations of *P. intricata*, there were found only 6 transitions, something to be expected when we are dealing with two remote populations from two mountain chains with geological structure and glaciation history different from each other (Ronikier *et al.*, 2008; Puşcaş *et al.*, 2008).

Despite the split of *Primula elatior* group into two, the NJ tree generated from the cumulative data grouped apart the populations of *Primula intricata*, thus once again confirming the separation of *Primula intricata* from the *Primula elatior* group (Fig. 4).

Conclusions

This study is the first to use molecular markers such as DNA sequence data from the plastid and nuclear genomes together with AFLP fingerprinting data to determine the phylogenetic position of *Primula intricata* within the *Primula elatior* group. To date, *Primula intricata* was ascribed to the mentioned group, based on the resemblance of morphological characters, but no genetic studies have been performed so far. The genetic analyses (the high number of private fragments, the branch lengths and the high bootstrap support of both the NJ tree, the PCoA differentiation) stand for a clear separation of *Primula intricata* out of the *Primula elatior* group.

Acknowledgments

The authors are thankful to the Ministry of Education, Research and Innovation, which supported the present study through the program IDEAS (NP–II-ID_PCE_2007-1), as a part of the National Research, Development and Innovation Plan for the period 2007-2013 referred to as the National Plan II (NP II). The number and the title of the project are: 405/1.10.2007/ "Clarifying the taxonomical position of certain Carpathians-Balkan plant species through morpho-ecological and molecular biology methods". The authors also wish to thank for the partial financial support provided from programs co-financed by The Sectoral Operational Programme Human Resources Development, Contract POSDRU 6/1.5/S/3 – „Doctoral studies: through science towards society". We are grateful to Andreas Tribsch, Peter Schönswetter and Božo Frajman for their valuable help in collecting the samples from the Alps. Also we would like to thank to Michał Ronikier for his help in sampling the population from Western Carpathians.

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