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MOLECULAR TAXONOMY STUDIES ON SEVERAL ENDEMIC OR RARE PLANTS SPECIES FROM ROMANIA

- Summary of the PhD thesis -

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The Objectives

The study is focused on selected plant species/subspecies endemic for Romania, or Daco-Balkan endemic species and some rare species. For each plant species one or more objectives were established:

Pulmonaria filarszkyana

Objective 1: Identification of "barcode" molecular markers able to differentiate between the species *Pulmonaria rubra* and *Pulmonaria filarszkyana*;

Objective 2: Investigation of the phylogenetic relationships between *Pulmonaria rubra* and *Pulmonaria filarszkyana*;

Onosma pseudoarenaria

Objective 1: Investigation of the phylogenetic relationships between *Onosma arenaria* and *Onosma pseudoarenaria*;

Objective 2: Genetic diversity assessment of the Romanian populations of *Onosma pseudoarenaria* and *Onosma arenaria*;

Eritrichium jankae

Objective 1: Investigation of the phylogenetic relationships between *Eritrichium nanum* and *Eritrichium jankae*;

Primula leucophylla

Objective 1: Identification of "barcode" molecular markers able to differentiate between the species *Primula elatior* and *Primula leucophylla*;

Objective 2: Investigation of the phylogenetic relationships between *Primula elatior* and *Primula leucophylla*;

Objective 3: Redefining the areal of the species *Primula leucophylla* based on molecular analysis;

Primula intricata

Objective 1: Investigation of the phylogenetic relationships between *Primula elatior* and *Primula intricata*.

Introduction

The development of molecular methods in the last decade has allowed scientists to re-evaluate plant species with questionable taxonomic status. Our study is part of this recent trend, focusing on selected rare or endemic species/subspecies from the Romanian flora which have an uncertain taxonomic status. In the absence of genetic studies, the selected taxa are considered to be distinct species or subspecies or even a variety of other species, based solely on morphological characters. Since morphological differences that distinguish between species/subspecies are minor and can be the result of individual polymorphisms caused by environmental factors and not the result of the interspecific differences, we considered it appropriate to conduct molecular genetics studies to help elucidate their taxonomic status. The problematic taxa selected for the study are: *Pulmonaria filarszkyana*, *Onosma pseudoarenaria*, *Eritrichium jankae* (family Boraginaceae), *Primula leucophylla* and *Primula intricata* (family Primulaceae). The taxonomic status is discussed in terms of relationship with the species whose subspecies or varieties they are considered to be: *Pulmonaria rubra*, *Onosma arenaria*, *Eritrichium nanum* and *Primula elatior*.

Theoretical Background

1. Phylogeny and phylogenetic analyzes (selection)

The principle underlying phylogeny is that members of a group or clade share a common evolutionary history and they are more related to each other than with other group members. Molecular phylogeny involves the use of biological macromolecules (proteins, nucleic acids) to obtain information about the evolutionary history of the taxa and their relationships.

The phylogenetic methods of tree construction involve several steps after the dataset has been obtained: alignment of sequences, choosing the best substitution model, tree construction and tree evaluation. There are two major groups of methods for constructing phylogenetic trees, namely distance-based methods and methods based on character analysis. The most common fenetic methods are UPGMA (“Unweighted Pair Group Method with Arithmetic means), NJ (“Neighbor Joining”) and ME („Minimum Evolution”), while the most popular methods based on the characters analysis are: MP („Maximum Parsimony”), ML („Maximum Likelihood”) and “Bayesian inference”.

2. Molecular markers used in plant phylogeny and taxonomy (selection)

The various types of molecular markers differ in terms of principle, methodology and applications, which creates the problem of selecting the most appropriate marker for solving the proposed research topic. In plant phylogenetics, the highest degree of trust in the generated trees is given by the congruence of information acquired from the three genomes: plastidial, nuclear and mitochondrial (Savolainen and Chase, 2003). However, most studies are based on information from a single genome, a possible explanation for this being the lack of suitable genetic markers. Because intraspecific phylogenies based on several independent data systems are expensive and difficult to obtain, one can use several marker systems, more or less optimal, which represents a compromise between a sufficiently high level of variability of the markers and their utility to resolve clear genealogies (Eidesen *et al.*, 2007).

The most commonly used molecular markers in plant taxonomy and phylogeny are classified into the following two categories: proteins and nucleic acids. The first category includes isozymes and the second cathegory includes microsatellites, SNPs, specific genes, noncoding regions and other regions. The main methods for detection of DNA molecular markers include sequencing, genotyping, RFLP technique, AFLP technique and RAPD technique.

3. Characterization in terms of taxonomy, morphology, ecology and phenotypic variability of the selected plants (selection)

Pulmonaria rubra (common name lungworts) is a Carpathian-Balkan endemic species commonly spread in beech forests or beech and fir forests. On occasions, it can go as high as the lower alpine belt, in habitats with *Juniperus* sp. and *Alnus viridis*, up to about 2000 m (Täuber, 1982).

Regarding the variability of the species, most authors consider that the species *P. rubra* has two subspecies: *P. rubra* ssp. *eurubra* (*P. rubra* ssp. *rubra*) (fig. 1) and *P. rubra* ssp. *filarszkyana* (Sârbu *et al*., 2001; Gușuleac, 1960; Beldie, 1979). However, Borza believes that *P. filarszkyana* is only a variety of *Pulmonaria rubra* (Borza, 1947), while Ciocârlan (2000) considers the species *P. filarszkyana* as a distinct species, taxonomically unrelated with *P. rubra*.

Fig. 1. *Pulmonaria rubra* ssp. *rubra* (photo: Dana Șuteu).

<u>*P. filarszkyana*</u> (*P. rubra* ssp. *filarszkyana)* is a species endemic in the Eastern Carpathians (Beldie, 1979). It differs from *P. rubra* by a much shorter (1 mm) and softer indumentum (Gușuleac, 1960). It is generally considered to be widespread in the spruce forests and juniper bush from the Maramureş, Rodna and Suhard Mountains.

<u>*Onosma arenaria*</u> (common name golden drops) is a hispid, robust, rich foiled plant with a gray-greenish appearence. While in Europe it is very common, excepting the northern regions, in Romania it has a controversial distribution. Some authors consider that it can be found in sandy, arid hills, steppe or dunes from the regions Baia Mare, Cluj, Hunedoara, Timişoara, Bucureşti, Constanţa, Iaşi (Grinţescu and Nyárády, 1960), while others locate it only in the Danube Delta (Teppner, 1996).

Regarding the variability of the species, Ciocârlan (2000) believes that it has two subspecies: *O. arenaria* ssp. *arenaria* and *O. arenaria* ssp. *pseudoarenaria,* the latter considered a species endemic to Transylvania.

<u>*Onosma pseudoarenaria*</u> (*O. arenaria* ssp. *pseudoarenaria*) is a sun loving species that lives in the hilly area (fig. 2) on dry, sandy soils. It is characterized by populations

composed of only a few individuals, mainly because it has difficulties to reproduce, due to the sclerified fruit. These particularities give it the status of vulnerable species (Dihoru and Negrean, 2009). It differs from *O. arenaria* by several characteristics: number of chromosomes (2n=26 and not 2n=20 as for *O. arenaria*), setules 4-5 times shorter (not 8-10 as for *O. arenaria*) than the setae and the space between pads is hairless (not covered by rare and short hairs as for *O. arenaria*).

It is common in the following regions: Cluj, Brasov, Hunedoara (Grinţescu and Nyárády, 1960).

Some authors (Ciocârlan, 2000, 2009) consider *O. pseudoarenaria* as a subordinate species of *O. arenaria*. Other authors (Rauschert, 1976) consider that eight other subspecies from Central Europe and the Balkans subordinate to it, including *Onosma pseudoarenaria* ssp. *pseudoarenaria*, considered a Transylvanian endemic species. Flora Europaea considers *O. pseudoarenaria* as endemic for Romania, with no subordinate subspecies. Rauschert's subspecies are considered to be taxonomically independent (Ball and Riedhl, 1972).

Fig. 2. *Onosma pseudoarenaria* in its natural habitat from Suatu, Cluj County (photo: Liviu Filipaș).

<u>*Eritrichium nanum*</u> (arctic alpine forget-me-not) is a rare plant, with poor populations, which can be met on sunny limestone rocks, in the alpine and subalpine belts (fig. 3). It occurs in Central and Southern Alps and South-Eastern Carpathians (Chater, 1972).

Variability of this species is quite discussed, because some authors consider its subspecies as separate species or varieties. Flora Europaea (Chater, 1972) and Ciocârlan (2009) take into consideration only two subspecies: *E. nanum* ssp. *nanum* and *E. nanum* ssp. *jankae*.

Fig. 3. *Eritrichium nanum* ssp. *nanum* in its natural habitat from Piatra Iorgovanului (left; photo: Dana Șuteu) and Ciucaş Mountains (right; photo: Liviu Filipaș).

Eritrichium jankae (*Eritrichium nanum* ssp. *jankae*) is an endemic species for Romania (Beldie, 1967; Grinţescu, 1960; Borza, 1947; Ciocârlan, 2000; Chater, 1972). It differs morphologically from *E. nanum* ssp. *nanum* by the inflorescence and leaves with hairs silvery white, by stems of 5 to 7.5 cm (not 1-5 cm as for ssp. *nanum*), and also by leaves 3 to 4.5 mm wide (not 1.5-3 mm as for ssp. *nanum*) and the slightly longer calyx. Still, it has the same number of chromosomes. It is found in Rodna, Ciucaş, Bârsei, Bucegi, Iezer, Făgăraş, Căpăţânii and Godeanu Mountains (Ciocârlan, 2009).

Primula elatior (common name oxlip) is a perennial, herbaceous, diploid 2n=22 species (Jacquemyn *et al.*, 2004). It occurs in mixed deciduous forests from temperate areas of Western and Central Europe. In the north it reaches Denmark and southern Sweden and in the east it reaches central Asia (Woodell, 1969). In Romania, it is a common species (Nyárády and Gușuleac, 1960).

The variability of *Primula elatior* has been the subject of much debate in the scientific world and a consensus regarding the forms and varieties of this species has not yet been reached. Most authors assign the subspecies *leucophylla* and *intricata* to *Primula elatior*, aside from many other forms, varieties and subspecies (Ciocârlan, 2000; Valentine and Kress, 1972; Wright Smith and Fletcher, 1947).

Primula leucophylla (*Primula elatior* ssp. *leucophylla*) shows a similar morphology (fig. 4) to *P. elatior*, from which it can hardly be distinguished. Characters which distinguish it are: gray and very hairy leaves on the lower side and calyx of 6-8 mm length (Valentine and Kress, 1972; Ciocârlan, 2000).

It is considered an endemic species for the Eastern Carpathians and it is spread in: Maramureş, Rodna, Giurgeu, Suhard, Postăvaru, Ceahlău, Rarău and Obcinele Bucovinene

Mountains (Nyarady and Guşuleac, 1960), while some authors believe it can also be found in the Piatra Craiului, Călimani, Ciucaş, Nemira and Vrancea Mountains.

Fig. 4. *Primula leucophylla* in Ceahlău Mountains (photo: Liviu Filipaș).

Primula intricata (*P. elatior* ssp. *intricata*) is found in wet meadows from the subalpine and alpine belts, in the Pyrenees, the Alps and the Balkan Peninsula (Nyárády and Gușuleac, 1960). In Romania, it is a rare species that lives in the Bucegi and Bârsei Mountains (Beldie, 1979; Nyárády and Gușuleac, 1960).

It differs from *P. elatior* by longer bracts, corolla lobes more deeply emarginate and calyx of similar length with the capsule (10-12 mm, unlike *P. elatior* where the capsule surpasses the calyx) (Nyárády and Gușuleac, 1960).

Its taxonomic status is still debated. Some authors consider it a distinct species (Grenier and Godron, 1853; Nyarady and Guşuleac, 1960; Şuteu *et al*., 2011), others consider it a variety of *P. elatior* (Pax and Knuth, 1889), while others consider it as a subspecies of *P. elatior* (Lüdi, 1927; Ciocârlan, 2000; Valentine and Kress, 1972; Beldie, 1979; Wright Smith and Fletcher, 1947).

Personal Contribution

4. Materials and Methods (selection)

Sampling strategy

The biological material used for analysis was harvested from May to September, 2006-2010, from several locations. A minimum of 5 individuals from each population were sampled. The samples were immediately placed in tubes filled with silica gel. Vouchers were also taken, to verify the species collected. Subsequently, these vouchers were deposited in a herbarium. Additionally, for the analysis of the species *P. elatior - P. leucophylla - P. intricata*, herbarium material was used from the Cluj-Napoca herbarium collection located in the „Alexandru Borza” Botanical Garden.

DNA extraction

DNA was extracted using several methods, namely: a protocol adapted from Doyle and Doyle (1987, 1990), a protocol adapted from Mengoni (Mengoni *et al.*, 2000) and a protocol based on a commercial kit: DNeasy Plant Mini Kit (Qiagen).

Chloroplastic microsatellites analysis

The analysis was performed with the use of six primers described by Weising and Gardner (1999): ccmp 2, ccmp 3, ccmp 4, ccmp 6, ccmp 7 and ccmp 10. This analysis was used only for testing the degree of polymorphism in some populations belonging to the genera *Onosma* and *Pulmonaria*. The results were interpreted by analyzing the presence/absence of amplification bands corresponding to each primer within a polyacrylamide gel.

PCR-RFLP analysis

PCR-RFLP analysis was performed on three regions belonging to the chloroplast genome: trnD-T, trnC-D and psbA-trnS (Demesure *et al.*, 1995). This analysis was used only for testing the degree of polymorphism in some populations belonging to the genera *Onosma* and *Pulmonaria*. The results were interpreted by analyzing the presence/absence of amplification bands corresponding to each primer within a polyacrylamide gel.

AFLP analysis

AFLP analysis was performed on all individuals belonging to selected populations of the genera *Primula*, *Eritrichium* and *Onosma*. For the different genera of plants, different combinations of primers were used, as suggested in the existing literature. Scoring was performed with GeneMapper v.4.0 software (Applied Biosystems) in order to produce a matrix based on presence/absence. The AFLPdat script in R platform (Ehrich, 2006) was used to calculate the proportion of polymorphic loci, Nei's genetic diversity (Nei, 1987) and the rarity index. Based on Nei and Li distance (Nei and Li, 1979) a Neighbor Joining tree was generated using Splistree program v. 4.10 (Huson and Bryant, 2006). Analysis of molecular variance (AMOVA) was performed using the program Arlequin v. 3.5 (Excoffier and Lischer, 2010). Also within the program Arlequin, the F_{ST} value ("fixation index") was calculated. "Principal Coordinate" analysis (PCO) was performed with the program PAST (Hammer et al., 2001) using two indices: Simpson index and Euclidean index.

Sequencing

Sanger sequencing was performed for the plant genera considered in the analysis: *Pulmonaria*, *Onosma*, *Eritrichium* and *Primula*. Sequencing was performed on the ITS1 region belonging to the nuclear genome and on the chloroplastic regions: introns *trnL*, *trnG*, *rpl16*, *rps16*, intergenic spacers *rpl32-ndhF*, *rps16-trnK*, *trnL-trnF*, *trnD-trnE*, *trnS-trnfM*, *psbD-trnT*, *trnH-psbA*, *trnS-trnG*, *trnT-trnL*. The obtained sequences were assembled and edited manually using the program BioEdit v.7.0.9.0 (Hall, 1999). Subsequently, sequence analysis was performed with the program Mega 4.1 (Tamura et al., 2007). Tree construction was performed by NJ, ML, ME, UPGMA methods, using the Kimura 2P model (1980).

5. Results and discussion (selection)

5.1. Results on *Pulmonaria* genus

Chloroplastic microsatellites analysis did not reveal polymorphism for any primer combination between the populations of *Pulmonaria rubra* and *Pulmonaria filarszkyana*.

PCR-RFLP analysis did not reveal any polymorphism between the populations of *Pulmonaria rubra* and *Pulmonaria filarszkyana* based on the DT and AS primers, but it shows a polymorphism for primer CD in the bands 2 and 5 due to an insertion/deletion.

Sequencing analysis

From the 13 selected regions we succeeded in amplifying and sequencing only 8 regions: ITS1, *rpL16*, *trnG*, *trnL*, *rpl32-ndhF*, *psbD-trnT*, *trnD-E* and *trnH-psbA*. A concatenated sequence of these markers, with a length of about 5000 nucleotides, was used to build the trees using the ME (fig. 5), ML, NJ, UPGMA methods.

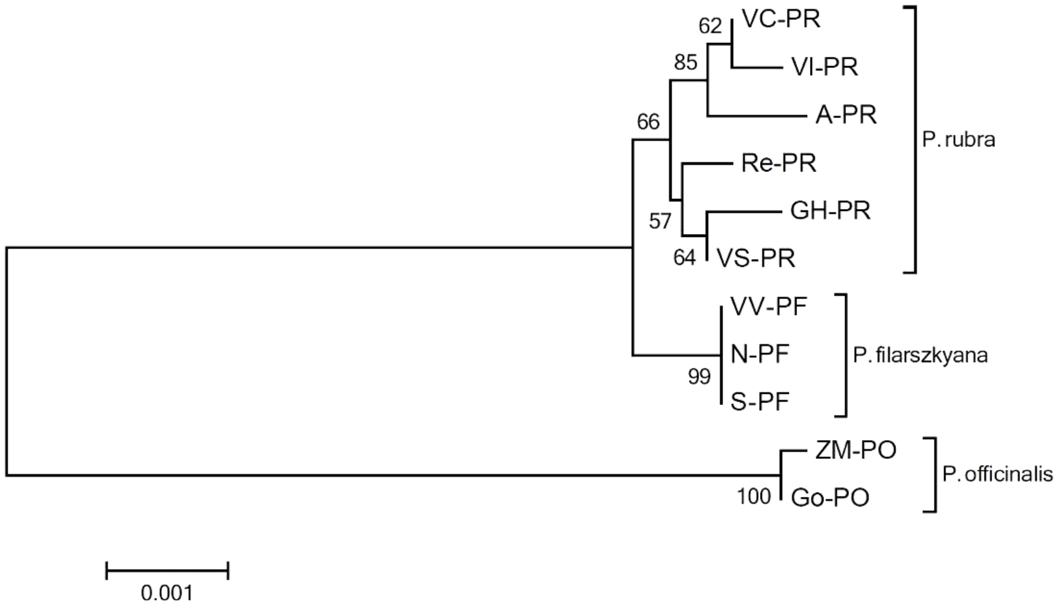

Fig. 5. ME dendrogram of the *Pulmonaria* genus, based on ITS1, *rpL16, trnG, trnL*, *rpl32-ndhF*, *psbD-trnT*, *trnD-E* and *trnH-psbA* sequences.

5.2. Discussion on *Pulmonaria* genus

Investigation of the phylogenetic relationships between *Pulmonaria rubra* and *Pulmonaria filarszkyana*

Trees generated through ME (fig. 5), ML, NJ and UPGMA methods are congruent and show two groups: the first consists of *P. officinalis* populations and the second consists of *P. rubra* and *P. filarszkyana* populations. The group formed by *P. officinalis* populations is separated with a maximum bootstrap value, showing that the taxonomic status of this species is clearly defined by the morphological characters. In the second group, the *P. filarszkyana* subgroup is unitary. All three populations exhibit the same haplotype regardless of the markers. This is understandable if we take into account the proximity between the collection points, all belonging to Maramureş County. This taxon is restricted to a few locations in the Eastern Carpathians, thus allowing a continuous gene flow between locations. The taxon *P. rubra* is separated into two subgroups. The first subgroup (consisting of populations from Cepelor Valley, Arieşeni and Ierii Valley) might be formed due to the proximity of the collection points, but despite this proximity all three populations have slightly different haplotypes. A second subgroup (consisting of populations from Retezat, Giurgeu-Hăşmaş and Pârâul Văii Seci) gathers populations from distant areas of the country, which can be explained by the fact that the gene flow path of *P. rubra* populations is along the Carpathian arc. *P. rubra* is an important species of the association *Fagetum dacicum*, association characterized by pure or almost pure beech forests. This association continues almost uninterrupted along the Carpathian arc, which facilitates the gene flow. An exception is represented by the Western Carpathian populations, which are separated from those in the Eastern and the Southern Carpathians by valleys such as the Someş Valley and Mureş Valley. These valleys are could represent possible geographic barriers for the gene flow. Moreover, there is no continuity between the beech forests from the Western Carpathians group and the rest of the Carpathian groups.

The group of *P. filarszkyana* populations is well defined within the *P. rubra* group. However, the placement of this taxon within the group, supports the theory that *P. filarszkyana* is a subspecies of *Pulmonaria rubra* and not a distinct species such as *P. officinalis*.

5.3. Conclusions on *Pulmonaria* genus

In this case the resolution of chloroplastic microsatellites is not sufficient to differentiate within the *Pulmonaria* genus.

Analysis of PCR-RFLP markers showed polymorphism between the taxa *P. rubra* and *P. filarszkyana* when using region trnC-trnD.

Studies based on sequences analysis indicate that the taxon *P. filarszkyana* should be considered a subspecies of the species *Pulmonaria rubra*.

5.4. Results on *Onosma* genus

Chloroplastic microsatellites analysis did not reveal polymorphism for any primer combination between the populations of *Onosma* genus.

PCR-RFLP analysis did not reveal any polymorphism between the populations of *Onosma* genus based on the DT primer, but it showed polymorphism for CD and AS primers. The polymorphism revealed by the CD primer is difficult to distinguish because of small differences between the bands. The definition of the haplotypes is therefore based only on the AS primer. The polymorphism generated by the AS primer is visible at band number 5 and it represents an insertion/deletion of a nucleotide fragment.

Sequencing analysis

Based on ITS1 sequence of 350 bp, trees were constructed using the ME (fig. 6), ML, NJ, UPGMA methods.

From the 13 selected regions we succeeded in amplifying and sequencing only 9 regions: *rpL16*, *trnG*, *trnL*, *rpl32-ndhF*, *psbD-trnT*, *trnD-E*, *trnT-L*, *rps16-trnK* and *trnH-psbA*, with a concatenated length of about 6000 nucleotides, which have been used for construction of phylogenetic trees. Trees were built through the ME (fig. 7), ML, NJ, UPGMA methods.

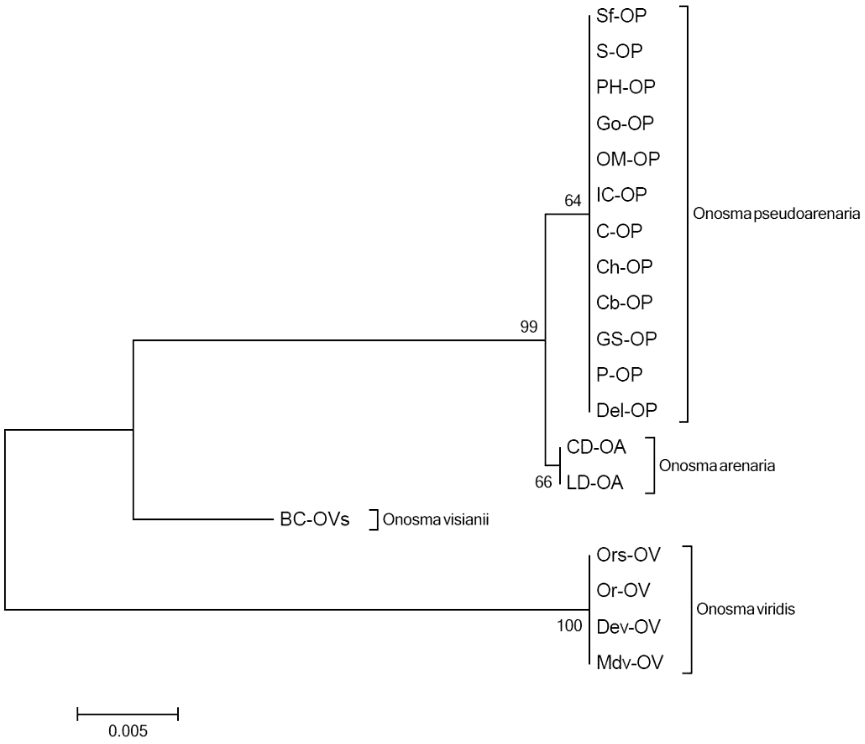

Fig. 6. ME dendrogram of the *Onosma* genus, based on ITS1 sequence.

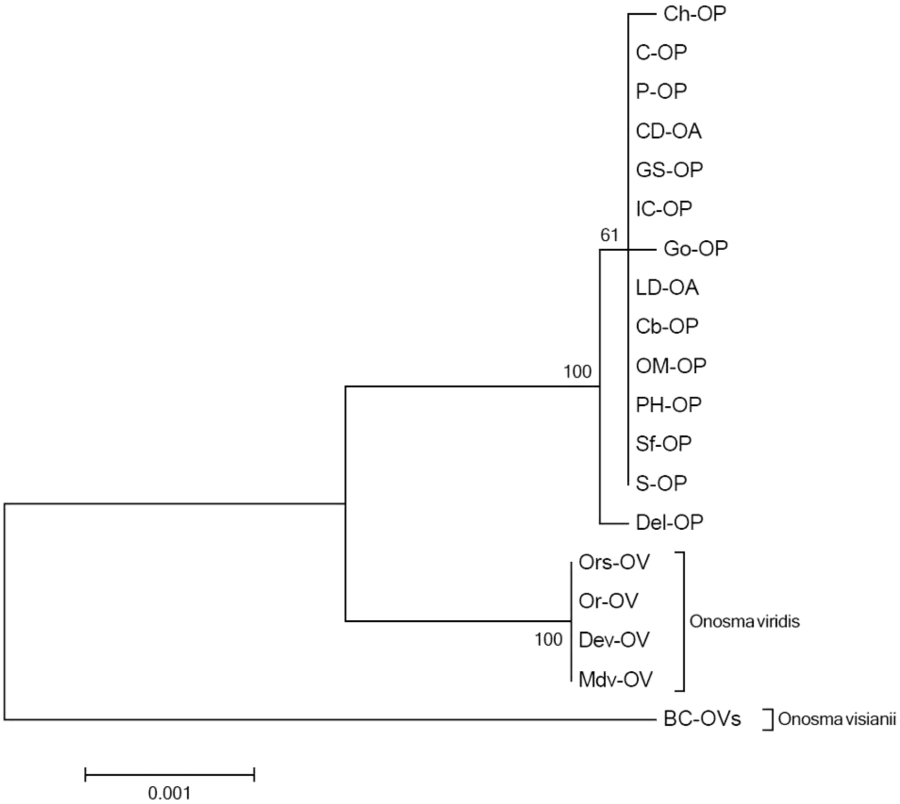

Fig. 7. ME dendrogram of the *Onosma* genus, based on *rpL16, trnG, trnL, rpl32-ndhF, psbD-trnT, trnD-E, trnT-L*, *rps16-trnK* and *trnH-psbA* sequences.

AFLP analysis

Using the AFLPdat script on the platform R we determined the polymorphic loci proportion, Nei's genetic diversity and the rarity index (Table 1).

Table 1. The proportion of polymorphic loci, genetic diversity and rarity index for the *Onosma* populations.

Species	Population code	The proportion of variable markers	Genetic diversity	Index rarity (DW)
Onosma viridis	Ors-OV	0.0660	0.0440	21.1819
Onosma visianii	BC-OVs	0.1718	0.0845	192.8069
Onosma arenaria	CD-OA	0.1762	0.0819	28.2933
	LD-OA	0.0748	0.0352	59.9596
Onosma pseudoarenaria	Cb-OP	0.2334	0.1002	17.7642
	C-OP	0.2951	0.1353	29.4634
	Del-OP	0.2070	0.0845	19.3396
	GS-OP	0.2290	0.1027	30.9598
	Go-OP	0.1277	0.0851	5.3400
	IC-OP	0.1938	0.0886	31.9461
	OM-OP	0.1806	0.0809	21.0883
	PH-OP	0.1806	0.0771	44.7643
	Sf-OP	0.2731	0.1490	12.1602
	S-OP	0.1497	0.0722	16.4296

A Neighbor Joining tree was generated based on the AFLP matrix (fig. 8).

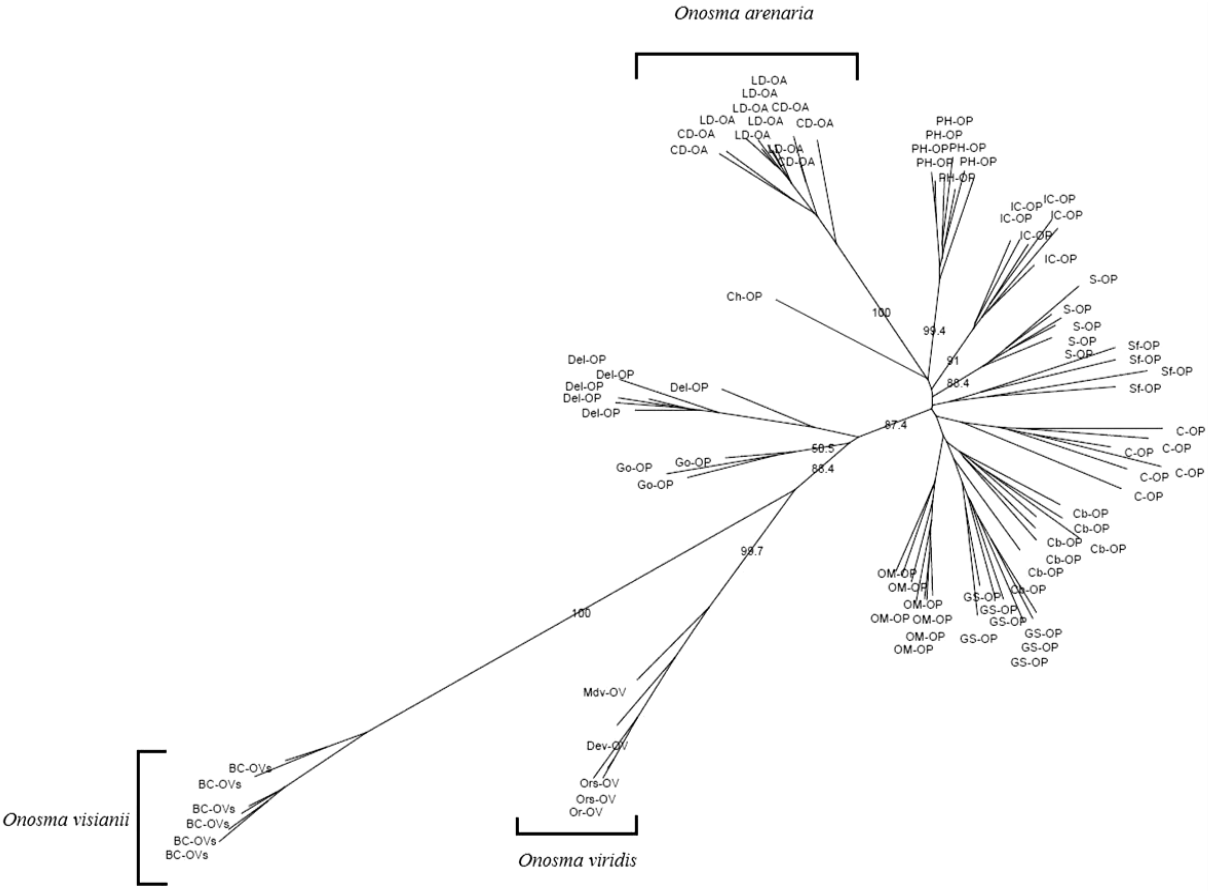

Fig. 8. NJ tree of the *Onosma* genus based on the AFLP matrix.

"Principal Coordinate" analysis results based on the AFLP matrix when using the Euclidean index are shown in figure 9.

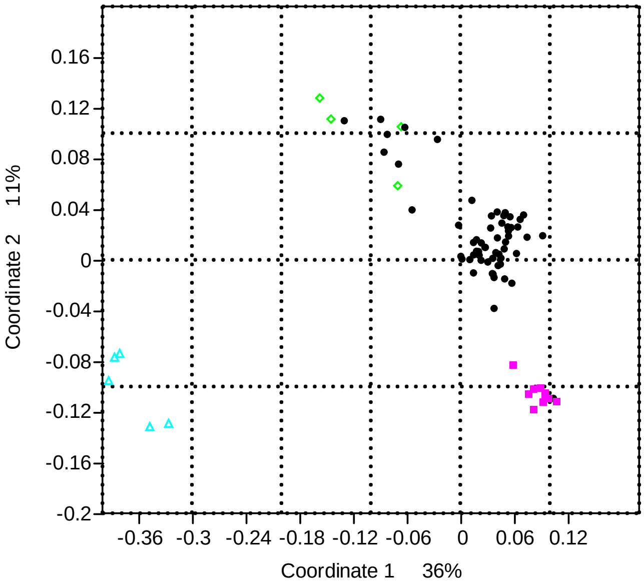

Fig. 9. PCO results using the Euclidean index for *Onosma* genus (black – *O. pseudoarenaria*, green – *O. viridis*, purple – *O. arenaria*, blue – *O. visianii*).

The analysis of molecular variance (AMOVA) was performed. The resulting total genetic variance was split between the different levels (within populations, between populations and between population groups). Several ways of defining groups were tested, and for each of them the molecular variance has been calculated at different levels. The results obtained without defining any group are listed in Table 2.

Table 2. The AMOVA results without defining any group for the genus *Onosma*.

Source of variation	**Percentage of the variation**	**F_{ST}**
Between populations	63.68083	0.63681
Within populations	36.31917	

When defining three groups, first corresponding to the species *O. visianii*, second corresponding to the species *O. viridis* (all four populations) and third representing a common group for *O. arenaria* and *O. pseudoarenaria* populations, the results shown in Table 3 were obtained.

Table 3. The AMOVA results with three groups for *Onosma* genus.

Source of variation	**Percentage of the variation**	**F_{ST}**
Between groups	51.95108	0.77651
Between populations within groups	25.69971	
Within populations	22.34921	

When defining four groups, first corresponding to the species *O. visianii*, second corresponding to the species *O. viridis* (all four populations), third corresponding to the species *O. arenaria* (two populations) and fourth corresponding to the species *O. pseudoarenaria* (all 12 populations), the results shown in Table 4 were obtained.

Table 4. The AMOVA results with four groups for *Onosma* genus.

Source of variation	**Percentage of the variation**	**F_{ST}**
Between groups	48.31	0.72692
Between populations within groups	24.38	
Within populations	27.30	

5.5. Discussion on *Onosma* genus

Investigation of the phylogenetic relationships between *Onosma arenaria* and *Onosma pseudoarenaria*

Trees generated based on ITS1 sequence constructed through the ME (fig. 6), ML, NJ and UPGMA methods, show the same structure irrelevant of the method employed. In the sub-subgroup consisting from populations of *O. arenaria* and *O. pseudoarenaria*, a weak separation of the two taxa based on a single transversion can be noticed. ITS1 sequences were successfull in solving intrageneric taxonomic problems for genera closely related with the genus *Onosma* (Weigend *et al.*, 2009; Cohen and Davis, 2009; Mansion *et al.*, 2009; Selvi *et al.*, 2009). However, in this case, their resolution is very low, similar to the findings of Kolarčik *et al.* (2010): “the two taxa are grouped in one clade with low internal resolution due to a lack of variation”.

Trees constructed through the ME (fig. 7), ML, NJ and UPGMA methods, based on concatenated sequences of nine chloroplastic regions show a different situation compared with the trees based on ITS1 sequence. The populations belonging to *O. arenaria* and *O. pseudoarenaria* are no longer separated as in the case of the ITS1 sequence. The only separating population of this subgroup is the population of *O. pseudoarenaria* subsp. *tridentina* collected from Serbia. This difference between the resolutions of the two markers can be explained by their nature. Chloroplastic DNA has a nonrecombinant nature, a low mutation rate and maternal inheritage. Thus, it would maintain genetic models and illustrate ancient historical processes for longer periods of time than the biparental inherited nuclear markers (Comes and Kadereit, 1998). Nuclear DNA, due to its high rate of recombination and mutation, would illustrate more recent genetic processes. Returning to the taxa in question, some authors (Teppner, 1971) state that the two species are relatively new and were formed by hybridization. Thus, *O. pseudoarenaria* is probably a result of alotetraploidization of a

Haplotricha taxon with 12 chromosomes (exp. *O. echioides*) with an Asterotricha taxon with 14 chromosomes (exp. *O. fastigiata*), and *O. arenaria* is a result of backcrossing of *O. pseudoarenaria* with a Haplotricha taxon with 12 chromosomes. Given the closely related history of the two taxa, this lack of variation at chloroplastic DNA level is explicable, while at the nuclear DNA level the two species diverge faster from each other. The differentiation of the *O. pseudoarenaria* subsp. *tridentina* population from the other populations is explicable by the fact that it is a subspecies (some authors even consider it as valid species - Ball and Riedhl, 1972), and by the biogeographic signal, due to the geographical distance between populations.

The estimation of genetic diversity parameters, the proportion of variable markers and rarity index based on AFLP matrix (Table 1), did not generate conclusive results, because the values are relatively equal for all the *Onosma* populations. The only species that is clearly distinguishable based on its rarity is the species *O. visianii*, differentiation which may be due both to the taxonomic signal and biogeographic signal, the population being collected from Croatia.

The NJ tree based on the AFLP matrix (fig. 8) reveals the following situation: the populations of *O. viridis* and *O. visianii* are separated from the main group, and subsequently separate from each other. The length of the branches in the case of these two species shows more clearly their separation from the *O. arenaria – O. pseudoarenaria* group. Within this group, the two populations of *O. arenaria* are separating with maximum bootstrap value from the *O. pseudoarenaria* group. The length of this branch suggests again that the separation of these two species is relatively recent, but at the nuclear genome level they are clearly differentiated. In the same group the differentiation of two populations can be observed: the population from Serbia which is obviously genetically isolated from the rest and the population from Govăjdia (Hunedoara County) which is located in a wooded area, in the Southern Carpathians, and therefore it is also clearly genetically isolated. The remaining populations of *O. pseudoarenaria* form distinct subgroups. However, in the case of the populations from Păuliş (Hunedoara County), Izvorul Crişului (Sălaj County) and Suatu (Cluj County) the bootstrap values are high and show the recent genetic isolation of these populations.

AMOVA analysis based on the AFLP matrix when no groups are defined shows an interpopulational variation of 63.68% (Table 2), suggesting relatively genetically distinct populations which can be grouped based on taxonomic and/or biogeographic criteria. The same fact is also supported by the high value of F_{ST} (0.63681). When defining three groups

(Table 3), the first composed from the populations of *O. visianii*, the second consisting of the *O. viridis* populations and the third consisting from the *O. arenaria* and *O. pseudoarenaria* populations, a percentage of variation between groups of 51.95% is obtained, which shows a good separation and definition of the groups. The increased F_{ST} index value (0.77651) suggests the same clear differentiation of the groups. When defining four groups (Table 4), the first composed from the populations of *O. visianii*, the second consisting of the *O. viridis* populations, the third consisting from the populations of *O. arenaria* populations and the fourth consisting from the *O. pseudoarenaria* populations, the percentage of variation between groups is slightly lower than in the previous case (48.31%), suggesting that *O. arenaria* is delimited from *O. pseudoarenaria*, but this separation is relatively recent compared with the delimitation of the species *O. visianii* and *O. viridis* from the group *O. arenaria – O. pseudoarenaria*. Simultaneously, a decrease of F_{ST} (0.72692) is observed, which shows the same less obvious delimitation of the species *O. pseudoarenaria* from *O. arenaria*, compared with the clear differentiation of other species such as *O. visianii* and *O. viridis*.

PCO analysis based on AFLP matrix (fig. 9) shows a strong delimitation of the species *O. visianii* from the rest of the species, a clear separation but not so strong of the species *O. arenaria*, a delimitation of the species *O. viridis* along with the two *O. pseudoarenaria* populations that were most divergent (one from Serbia and one from Govăjdia) and a common group composed of the remaining *O. pseudoarenaria* populations.

Therefore, most analysis indicate that the two species, *Onosma arenaria* and *Onosma pseudoarenaria*, represent relatively recent distinct species. This result confirms the palynology and cariology analysis.

5.6. Conclusions on *Onosma* genus

nrADN sequence, ITS1, shows a slight separation between *O. pseudoarenaria* and *O. arenaria*, based on a single mutation.

Analysis of sequences from nine chloroplastic regions does not reveal any separation of the two taxa.

AFLP analysis results clearly separate the two taxa.

These results, added to the cariology and palynology differentiation, show that the two taxa represent distinct units recently detached from each other.

5.7. Results on *Eritrichium* genus

Sequencing analysis

Based on ITS1 sequence of 350 bp, trees were constructed through the ME (fig. 10), ML, NJ, UPGMA methods.

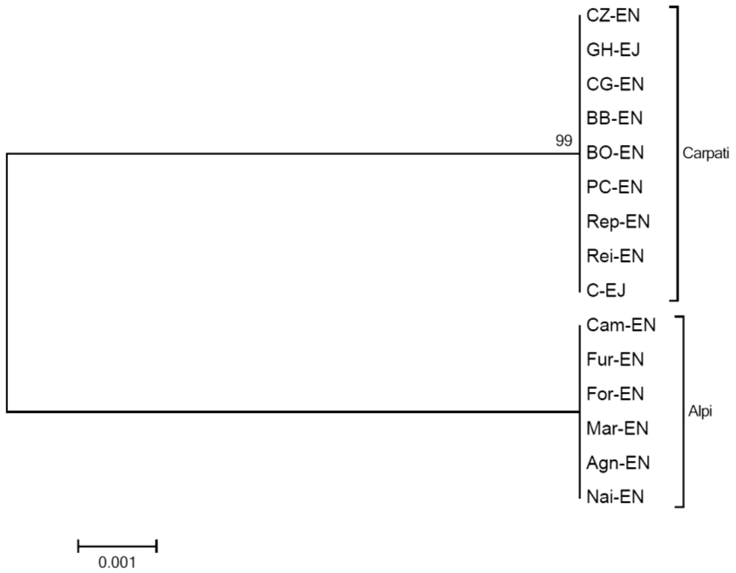

Fig. 10. ME dendrogram of the *Eritrichium* genus, based on ITS1 sequence.

From the 13 selected regions we succeeded in amplifying and sequencing only 9 regions: *rpL16*, *trnG*, *trnL*, *rpl32-ndhF*, *psbD-trnT*, *trnD-E*, *trnT-L*, *rps16* and *trnH-psbA*. Their concatenated length of about 5800 nucleotides was used for construction of phylogenetic trees. Trees were constructed through the ME (fig. 11), ML, NJ, UPGMA methods.

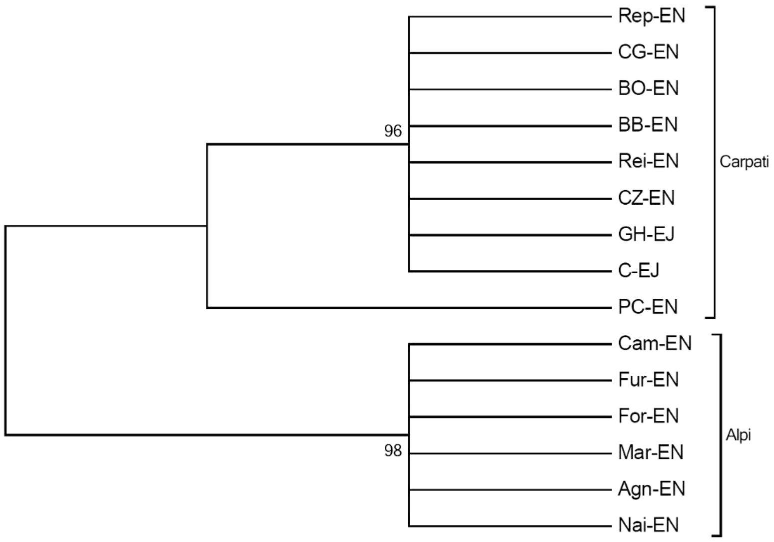

Fig. 11. 50% ME consensus tree for the *Eritrichium* genus, based on *rpL16*, *trnG*, *trnL*, *rps16*, *rpl32-ndhF*, *psbD-trnT*, *trnD-E*, *trnT-L* and *trnH-psbA* sequences.

AFLP analysis

The parameters determined using the AFLPdat script are: polymorphic loci, Nei's genetic diversity and rarity index (Table 5).

Table 5. The proportion of polymorphic loci, genetic diversity and rarity index for the *Eritrichium* genus.

Species	Populations	The proportion of variable markers	Genetic diversity	Rarity index (DW)
Eritrichium jankae	GH-EJ	0.3957	0.2228	25.7616
	C-EJ	0.5294	0.2852	24.0836
Eritrichium nanum	ALPI-EN	0.2459	0.1345	94.9871
	PC-EN	0.3636	0.1903	42.2113
	CZ-EN	0.4759	0.3172	18.4224
	CG-EN	0.3957	0.1803	62.1173
	BO-EN	0.3689	0.1786	39.5904
	Rei-EN	0.2566	0.1381	24.4530

The Neighbor Joining tree was generated based on the AFLP matrix (fig. 12).

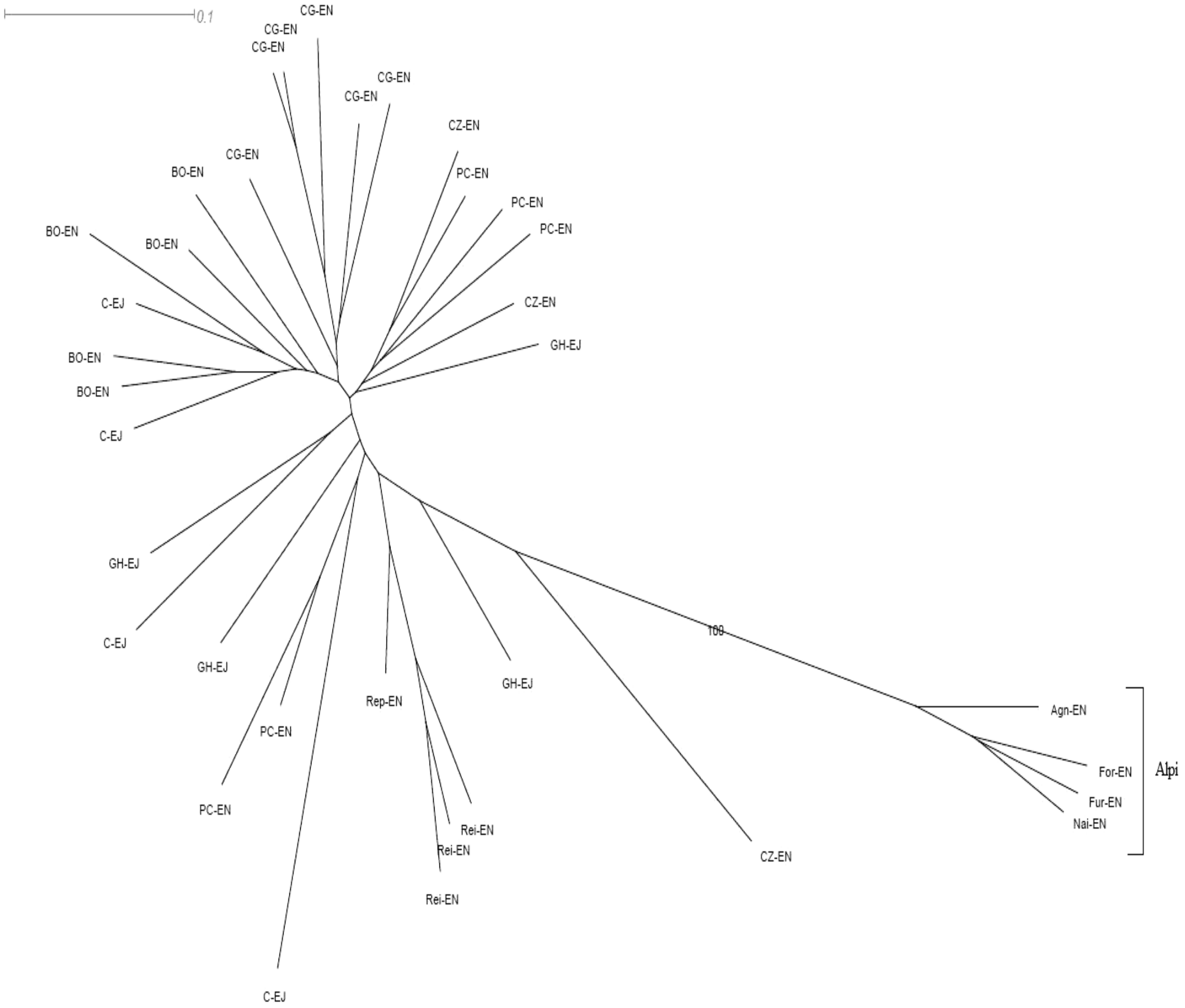

Fig. 12. NJ tree of the *Eritrichium* genus based on the AFLP matrix.

The AMOVA results when two groups were defined, the first corresponding to the *Eritrichium nanum* populations from the Alps and the second corresponding to the Carpathian populations of *E. nanum* and *E. jankae,* are shown in Table 6.

Table 6. The AMOVA results with two groups for *Eritrichium* genus.

Source of variation	**Percentage of variation**	**F_{ST}**
Between groups	53.48	0.63086
Between populations within groups	9.60	
Within populations	36.91	

When defining three groups on both the taxonomic and biogeographic criteria, the first group containing the populations of *E. nanum* from the Alps, the second group containing the populations of *E. nanum* from the Carpathians and the third group containing the populations of *E. jankae,* we obtained the results shown in Table 7.

Table 7. The AMOVA results with three groups for *Eritrichium* genus.

Source of variation	**Percentage of variation**	**F_{ST}**
Between groups	29.9	0.42897
Between populations within groups	15.4	
Within populations	54.7	

The results of "Principal Coordinate" analysis based on the AFLP matrix are shown in figure 13.

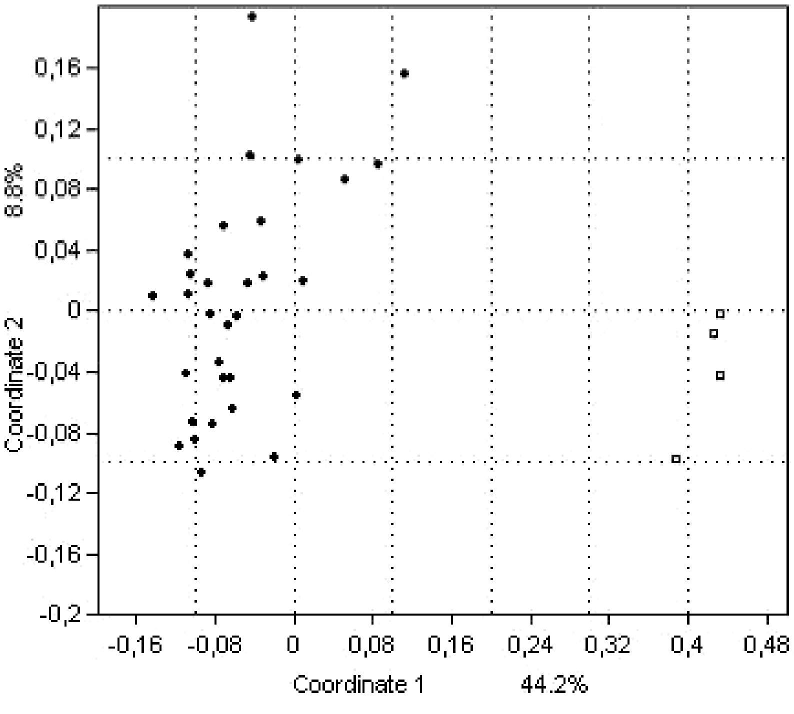

Fig. 13. PCO results for *Eritrichium* genus based on the AFLP matrix (black – populations of *E. nanum* and *E. jankae* from the Carpathians, blue square - the populations of *E. nanum* from the Alps).

5.8. Discussion on *Eritrichium* genus

Investigation of the phylogenetic relationships between *Eritrichium nanum* and *Eritrichium jankae*

Nuclear ITS1 sequence analysis indicates no separation between the taxa *Eritrichium nanum* and *Eritrichium jankae*. All the populations belonging to the *Eritrichium* genus collected from Romania show the same ribotype. However, a definite biogeographical separation can be noticed between the populations collected from the Carpathians and populations collected from the Swiss Alps. All methods of tree construction - ME (fig. 10), NJ, ML, UPGMA - generate the same pattern of separation with a bootstrap value of 99 between the populations from these two geographical areas. Moreover this separation based on biogeographic criteria is maintained at the level of AFLP markers. The NJ tree generated based on the AFLP matrix (fig. 12) shows no separation between the populations of *Eritrichium* from Romania, but separates with a maximum bootstrap value these populations from the Swiss populations.

Chloroplastic sequences analysis presents a somehow different situation. The tree generated from these regions (fig. 11) shows the same separation between the populations of *Eritrichium* collected from the Carpathians and those collected from the Swiss Alps, observed in the case of ITS1 sequence, but another separation is observed within the populations of *Eritrichium* collected from Romania. The *Eritrichium* population collected from the Piatra Craiului Massif is separating from the rest of the Romanian populations. This population has an intermediate haplotype, between the one exhibited by the populations from the Alps and the one existing in the Carpathians populations.

The proportion of variable markers and genetic diversity estimates based on the AFLP matrix (Table 5) show no significant differences between the *Eritrichium nanum* and *E. jankae* populations. In the case of the rarity index a high value (94.98) is observed for the populations from the Alps, which indicates their divergence from the Carpathian populations, based on biogeographical criteria, but this index makes no separation between the *E. jankae* and *E. nanum* populations.

AMOVA analysis reveals a clear separation of the group with Alpine populations from the group with Carpathian populations. More than half of the variation (53.48%) is due to the intergroup component (Table 6). Identically, the F_{ST} index increases from 0.37392 when no groups are defined to 0.63086 when defining two groups (one in the Alps and one in the Carpathians), which shows that the two groups are well defined. When performing AMOVA analysis with the inclusion of a group composed of the two populations of *E. jankae* (table 7), we obtain a much smaller variance between groups (29.9%) than in the previous case, which indicates that the groups are not so well defined. The same thing is supported by the decrease of the F_{ST} value from 0.63086 at 0.42897.

PCO analysis (fig. 13) based on the AFLP matrix shows the same separation between the *Eritrichium* populations from the Alps and the *Eritrichium* populations from the Carpathians. *E. jankae* populations are not separating from the rest of the Romanian populations in this analysis either.

All the performed analyzes indicate that the taxon *E. jankae* does not deserve the rank of species. Moreover, it doesn't even deserve the rank of subspecies, as the two taxa are undifferentiated at the genetic level. Since morphological differentiation between the two taxa still exists, the taxon *E. jankae* might be considered as an ecotype, genetically undifferentiated from the taxon *E. nanum*. The development of silver-white indumentum in the inflorescence and leaves can slow down water evaporation and might be a change that occurs in response to environmental factors, namely the dry climate where *E. jankae* is found. So, the taxon *E. jankae* can only be a morphologically different ecotype of the *E. nanum* taxon.

5.9. Conclusions on *Eritrichium* genus

Results of analysis based on ITS1, cpADN and AFLP do not separate the taxon *E. jankae* from the taxon *E. nanum*, but separate biogeographically the populations of *E. nanum* from the Alps from the populations of *E. nanum* from the Carpathians, with one exception, represented by the population of *E. nanum* from Piatra Craiului Mountains, which based on cpADN shows an intermediate haplotype between the two groups.

None of the performed analysis indicates the separation of the taxon *E. jankae* from the taxon *E. nanum*, but they reveal that the two taxa are genetically identical.

Based on the obtained results, *E. jankae* is most likely a genetically undifferentiated ecotype of the taxon *E. nanum*.

5.10. Results on *Primula leucophylla*

Sequencing analysis

Sequences from three molecular markers: *rpL16*, *psbD-trnT* and *trnH-psbA*, with a concatenated length of about 2700 nucleotides, were used for tree construction using the ME (fig. 14), ML, NJ, UPGMA methods.

AFLP analysis

The parameters determined by the AFLPdat script (polymorphic loci, Nei's genetic diversity and rarity index) are presented in Table 8.

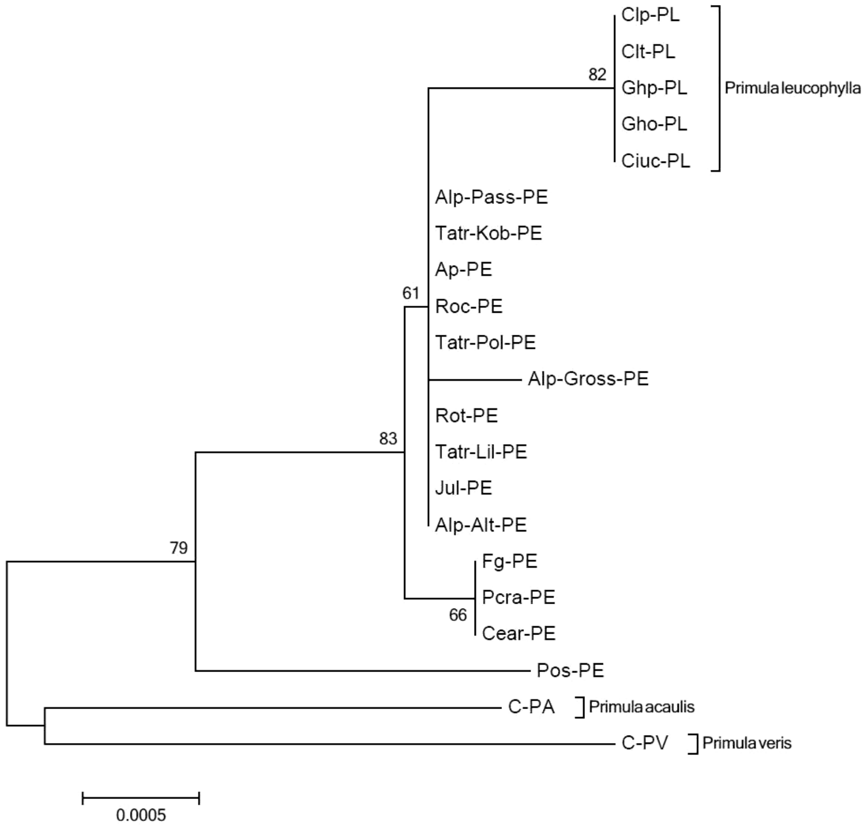

Fig. 14. ME dendrogram for the *Primula* genus, based on *rpL16*, *psbD-trnT* and *trnH-psbA* sequences.

Table 8. The proportion of polymorphic loci, genetic diversity and rarity index for the *Primula* genus.

Species	Populations	The proportion of variable markers	Genetic diversity	Rarity index (DW)
Primula elatior	Ap-PE	0.1630	0.0781	18.4976
	Fg-PE	0.1545	0.0746	25.3190
	Pos-PE	0.2489	0.1270	58.5491
	Pcra-PE	0.1888	0.0892	20.7056
	Roc-PE	0.2060	0.0987	17.1952
	Rot-PE	0.1673	0.0815	14.0721
	Cear-PE	0.1931	0.0969	11.6968
	Tatr-Kob-PE	0.2017	0.0978	21.5133
	Tatr-Pol-PE	0.2060	0.0995	20.0620
	Tatr-Lil-PE	0.2017	0.1101	21.0011
	Alp-Pass-PE	0.1416	0.0695	19.3688
	Jul-PE	0.0944	0.0429	17.8315
	Alp-Alt-PE	0.1373	0.0660	16.3522
	Alp-Gross-PE	0.2017	0.1021	17.0290
Primula leucophylla	Ciuc-PL	0.1759	0.0866	21.8210
	Gho-PL	0.1330	0.0652	14.7325
	Clp-PL	0.0472	0.0472	3.7617
	Clt-PL	0.1201	0.0575	15.6667

Based on the AFLP matrix a Neighbor Joining tree was generated (fig. 15).

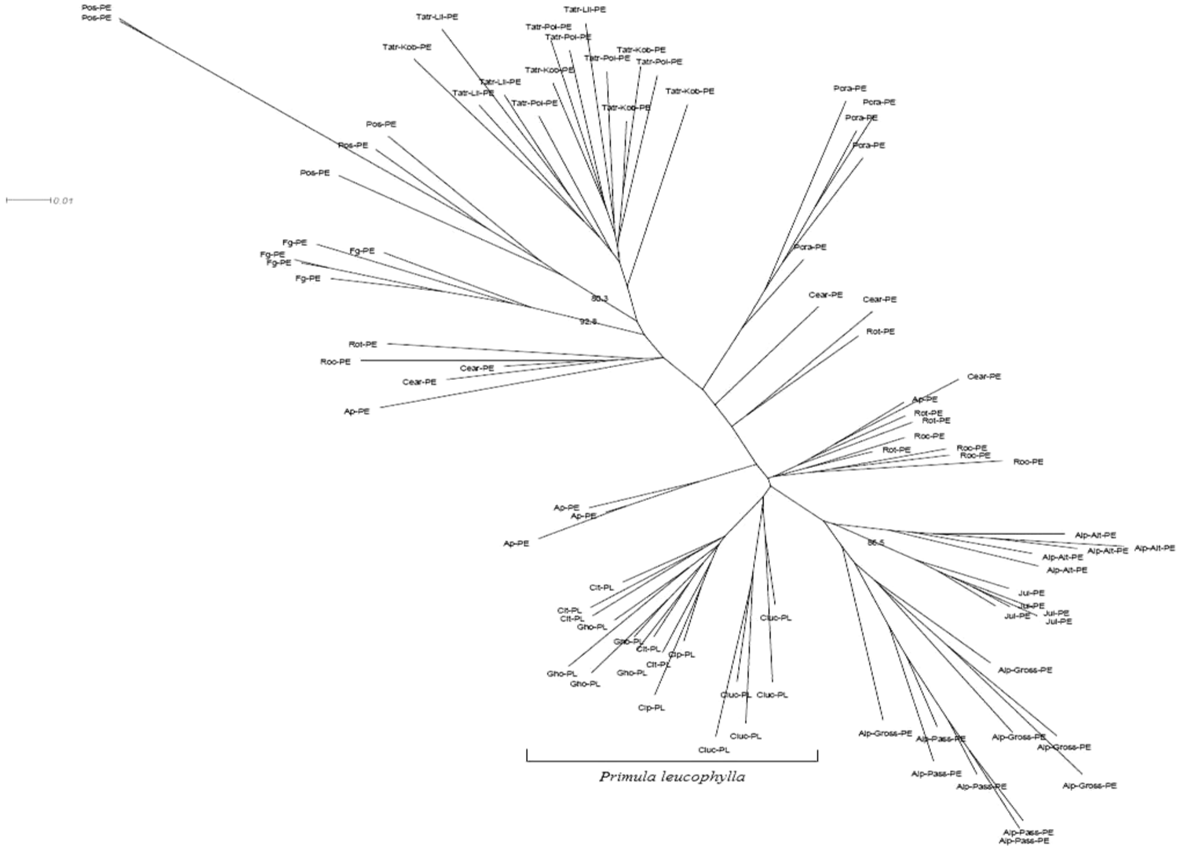

Fig. 15. NJ tree of the *Primula* genus based on the AFLP matrix.

The AMOVA results when no groups are defined are listed in Table 9.

Table 9. The AMOVA results without defining groups for *Primula* genus.

Source of variation	**Percentage of variation**	**F_{ST}**
Between populations	44.67	0.44669
Within populations	55.33	

If two groups are defined based on taxonomic criteria, the first corresponding to the populations of *Primula elatior* and the second corresponding to the populations of *Primula leucophylla*, the results are shown in Table 10.

Table 10. The AMOVA results with the definition of two taxonomic groups for *Primula* genus.

Source of variation	**Percentage of variation**	**F_{ST}**
Between groups	14.94951	0.50159
Between populations within groups	35.20986	
Within populations	49.84062	

Where three groups are defined based on biogeographical criteria, the first corresponding to the populations of *Primula elatior* from the Alps, the second corresponding to the populations of *P. elatior* from Tatras and third corresponding to the rest of the *Primula* populations from the S-E Carpathians, the results are shown in Table 11.

Table 11. The AMOVA results with the definition of three groups based on biogeographical criteria for *Primula* genus.

Source of variation	Percentage of variation	F_{ST}
Between groups	18.73280	0.48949
Between populations within groups	30.21576	
Within populations	51.05144	

When four groups are defined, on both the taxonomic and biogeographical criteria, the first corresponding to the populations of *Primula elatior* from the Alps, the second corresponding to the populations of *P. elatior* from the Tatras, the third corresponding to the populations of *P. elatior* from the S-E Carpathians and the fourth corresponding to the populations of *Primula leucophylla*, the results are shown in Table 12.

Table 12. The AMOVA results with the definition of four groups based on biogeographic and taxonomic criteria for the *Primula* genus.

Source of variation	Percentage of variation	F_{ST}
Between groups	21.79270	0.47591
Between populations within groups	25.79792	
Within populations	52.40938	

The "Principal Coordinate" analysis is based on the AFLP matrix. When using the Euclidean index the results are shown in Figure 16 - left, and when using the Simpson index the results are shown in Figure 16 - right.

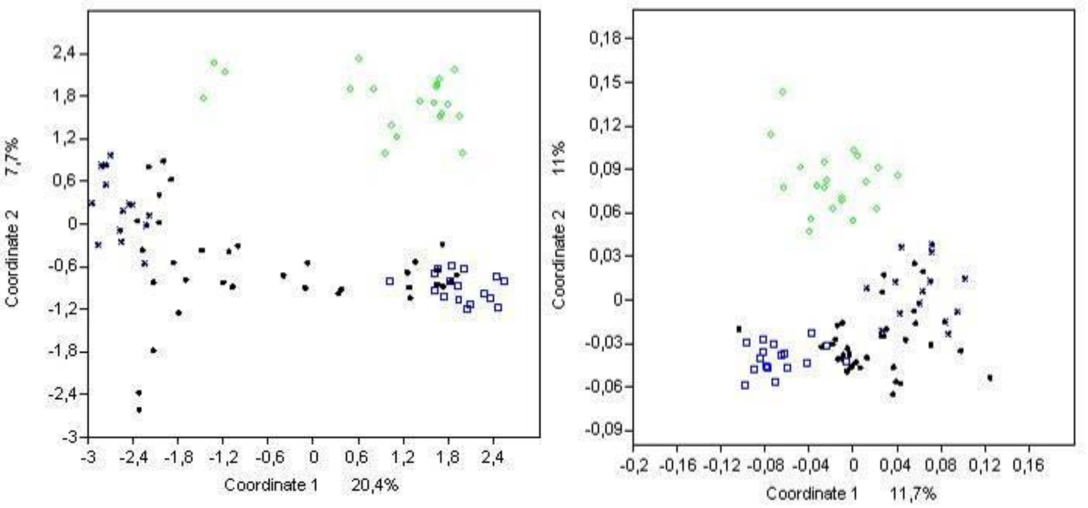

Fig. 16. The PCO results based on the Euclidean index (left) and the Simpson index (right) for *Primula* genus based on the AFLP matrix (black - *P. elatior* from the S-E Carpathians, blue cross - *P. elatior* from Tatras, blue square - *P. leucophylla*, green diamonds - *P. elatior* from the Alps).

5.11. Discussion on *Primula leucophylla*

Investigation of the phylogenetic relationships between *Primula elatior* and *Primula leucophylla*

The proportion of polymorphic loci and the genetic diversity (Table 8) show no separation of *P. leucophylla* from *P. elatior*. The values obtained for *P. leucophylla* are quite low. However, there are populations of *P. elatior* such as that in the Alps, Soca Valley, with even lower values. Rarity index has average values and it does not show the differentiation of the *P. leucophylla* populations from those of the species *P. elatior*.

Neighbor-Joining analysis (Fig. 15) shows a population structuring for *P. leucophylla*, but this clustering is not supported by valid bootstrap values.

PCO analysis reveals a grouping based on biogeographical rather than taxonomic criteria. Thus, the populations of *P. elatior* from the Alps are separated from the rest of the populations from the Carpathians (fig. 16). When using the Simpson index (fig. 16 - right) there is a slight separation of the taxon *P. leucophylla* within the Carpathians group.

The AMOVA analysis without defining any groups (Table 9) shows a wide intrapopulational variation (55.33%) and a small interpopulational variation, which indicates that most or even all the populations are separated rather poorly from each other and they are genetically uniform. This moderate separation of the populations is supported by a F_{ST} value of 0.44669. When assembly is performed based on taxonomic criteria (*P. elatior* vs *P. leucophylla* - table 10), the variation between the two groups is low (14.94%), indicating that the groups are not well defined. The highest percentage of variation (49.84%) is due to the intrapopulational component. When assembly is performed based on geographical criteria (Table 11), the percentage of variation between groups increases slightly (18.73%) suggesting that the groups are better defined, but the largest variation percentage is still due to the intrapopulational component (51.05%). The best contoured groups occur when assembly is performed on both geographic and taxonomic criteria (Table 12). In this case, the increase of the variation percentage between groups at 21.79% indicates a weak separation of *P. leucophylla* from *P. elatior*.

The trees generated through the ME (fig. 14), ML, NJ and UPGMA methods, based on analysis of the chloroplastic sequences, show about the same clustering. Within the group of *P. elatior* and *P. leucophylla* populations, certain *P. elatior* populations are clearly distinguished: Postăvaru, Făgăraş, Cearcănu and Piatra Craiului. In the subgroup consisting of the remaining populations, the sub-subgroup *P. leucophylla* stands out, comprising all the populations belonging to this taxon. This sub-subgroup is even less separated from the majority of the *P. elatior* populations than some subalpine populations of *P. elatior* from limestone rocks of the S-E Carpathians.

Based on the AFLP analysis and cpADN data, the genetic differentiation between *P. elatior* and *P. leucophylla* is weak. The delimitation of the *P. leucophylla* taxon as a distinct taxonomic unit is not supported by any results. The results indicate the possibility that *P. leucophylla* is an ecotype of *P. elatior*. Indumentum development that slows water evaporation from leaves might be a change that occurs in response to environmental factors, namely the dry climate where *P. leucophylla* is found.

P. leucophylla, found in the high mountain and subalpine belts, is ecologically separated based on altitude from *P. elatior s. str.*, which is widely distributed in the lower mountain belt of the S-E Carpathians (Ciocârlan, 2009). Since it is a marginal ecological taxon, *P. leucophylla* is phenologically separated from *P. elatior s. str.*. This phenological separation may lead to a limitation of the gene flow between the two taxa, leading to a difference in the genetic structure.

5.12. Conclusions on *Primula leucophylla*

AFLP analysis and cpADN analysis do not support the delimitation of *P. leucophylla* as a distinct taxonomic unit from *P. elatior*.

The results suggest that *P. leucophylla* might be an ecotype of the species *P. elatior,* which is adapted to the dry climate.

Due to the ecological and phenological isolation, the *P. leucophylla* ecotype is genetically differentiating from *P. elatior*.

5.13. Results on *Primula intricata*

Sequencing analysis

The sequences from three molecular markers: ITS1, *trnL* and *trnH-psbA*, with a concatenated length of about 1200 nucleotides, were used for tree construction through the ME (fig. 17), ML, NJ, UPGMA methods.

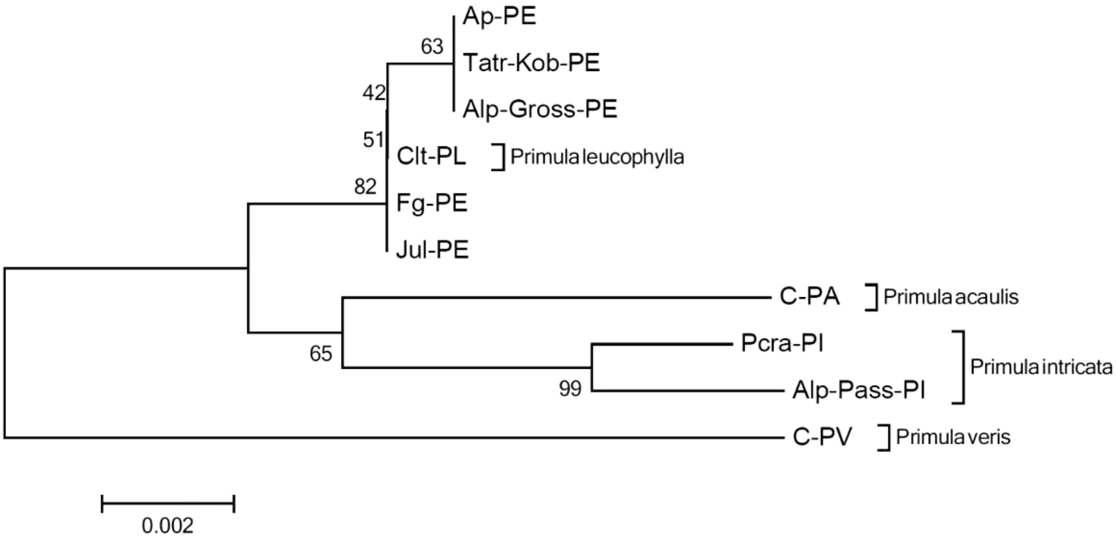

Fig. 17. ME dendrogram for *Primula intricata*, based on ITS1, *trnL* and *trnH-psbA* sequences.

AFLP analysis

Based on the AFLP matrix, a Neighbor Joining tree was generated (fig. 18).

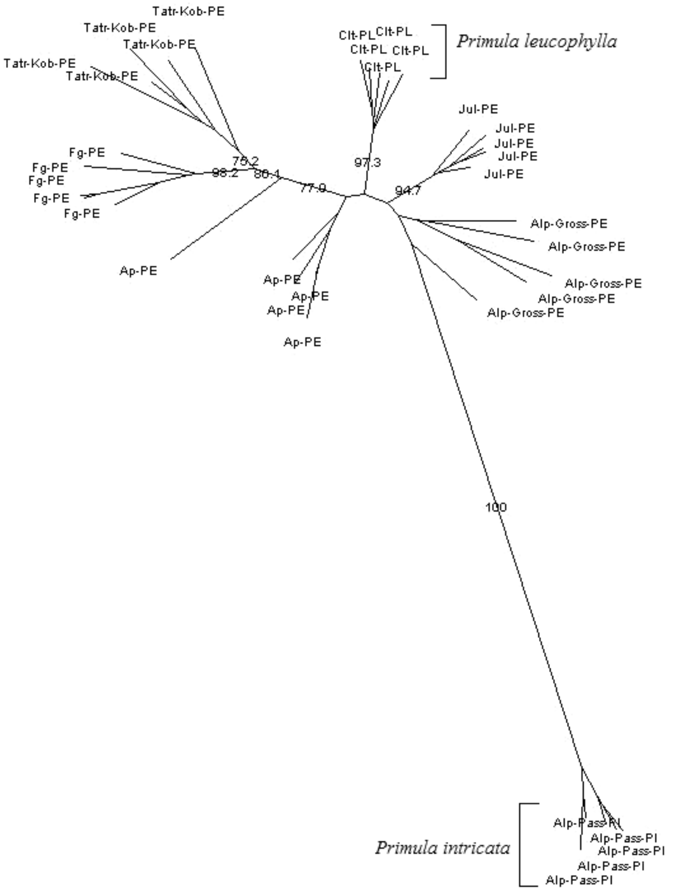

Fig. 18. NJ tree for *Primula intricata* based on the AFLP matrix.

The parameters determined by the AFLPdat script (polymorphic loci, Nei's genetic diversity and rarity index) are presented in Table 13.

Table 13. The proportion of polymorphic loci, genetic diversity and rarity index for the populations of *Primula* genus, used in the study of the species *Primula intricata*.

Species	Populations	The proportion of variable markers	Genetic diversity	Rarity index (DW)
Primula elatior	Ap-PE	0.2356	0.1133	32.6641
	Fg-PE	0.2101	0.1031	54.4281
	Tatr-Kob-PE	0.2675	0.1324	63.3665
	Jul-PE	0.1337	0.0611	27.9473
	Alp-Gross-PE	0.29298	0.1490	32.3495
Primula leucophylla	Clt-PL	0.1401	0.0700	26.6714
Primula intricata	Alp-Pass-PI	0.1337	0.0687	167.1520

The AMOVA results without defining any groups are presented in Table 14.

Table 14. The AMOVA results without defining groups for the *Primula* populations used in the study of the species *Primula intricata*.

Source of variation	Percentage of variation	F_{ST}
Between populations	67.7	0.67701
Within populations	32.3	

If two groups are defined based on taxonomic criteria, the first corresponding to the *Primula elatior* and *Primula leucophylla* populations and the second corresponding to the population of *Primula intricata*, the results are shown in Table 15.

Table 15. The AMOVA results with the definition of two groups based on taxonomic criteria for the *Primula* populations used in the study of the species *Primula intricata*.

Source of variation	Percentage of variation	F_{ST}
Between groups	58.93	0.81298
Between populations within groups	22.36	
Within populations	18.71	

When defining three groups based on taxonomic criteria, the first corresponding to the populations of *Primula elatior*, the second corresponding to the population of *Primula intricata* and the third corresponding to the population of *Primula leucophylla*, the results are shown in Table 16.

Table 16. The AMOVA results with the definition of three groups based on taxonomic criteria for the *Primula* populations used in the study of the species *Primula intricata*.

Source of variation	Percentage of variation	F_{ST}
Between groups	43.92	0.74457
Between populations within groups	30.53	
Within populations	25.55	

The results of the "Principal Coordinate" analysis when using the Simpson index are shown in fig. 19.

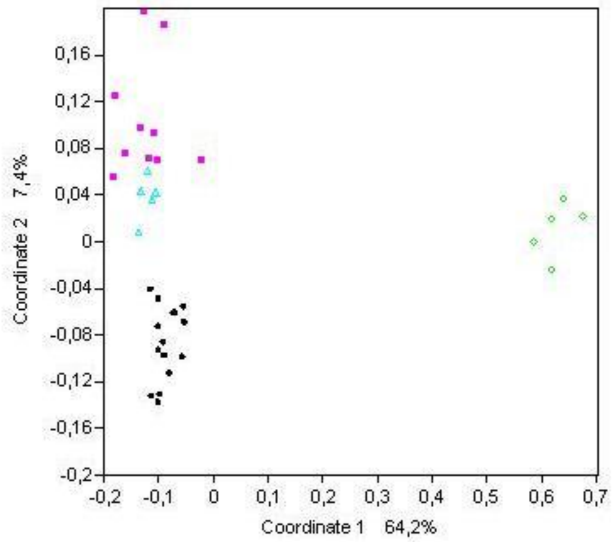

Fig. 19. The PCO results based on the Simpson index for the populations of *Primula* genus, used in the study of *Primula intricata* (black - *P. elatior* from the Carpathians, purple square - *P. elatior* from the Alps, blue triangle - *P. leucophylla*, green diamond - *P. intricata* from the Alps).

5.14. Discussion on *Primula intricata*

Investigation of the phylogenetic relationships between *Primula elatior* and *Primula intricata*

The results of AMOVA analysis when there are no defined groups (Table 14) indicate that the biggest variation is found among populations (67.7%). This suggests the existence of diverse populations which can easily be separated into groups. The high value of the F_{ST} index (0.67701) confirms the same fact. The intrapopulational variation of 32.3% indicates a relatively uniform genetic diversity within the populations. When defining three groups: *P. elatior*, *P. leucophylla* and *P. intricata* (table 16), the largest amount of genetic variation is due to the intergroup component (43.92%) suggesting that the groups are well differentiated from each other, with low gene flow between them. Variation between the populations within groups is also increased, indicating the same fact. Moreover the increase of the F_{ST} index at 0.81298 reconfirms the same pattern. This result might be due to the large distance between the collection points of the populations, so in order to be certain about the differentiation of the groups, another AMOVA analysis has been made with two groups: *P. elatior* together with *P. leucophylla* and separately *P. intricata* (table 15). The result clearly shows that more than half of the genetic variation (58.93%) is explained by the variation component between the groups. This result clearly supports the separation of *P. intricata* from *P. elatior*, even more than the separation of *P. leucophylla* from *P. elatior*, because the result of genetic variation between groups when defining three groups is smaller than in this case. Simultaneously, the decrease of the F_{ST} value at 0.74457 is recorded, which confirms the same fact. So, the *P. intricata* group is compact and well-formed, suggesting the existence of a distinct species.

The PCO analysis (fig. 19) clearly shows the separation of *P. intricata* from the group consisting of *P. elatior* and *P. leucophylla*. This separation is not a result of a geographic separation because the populations of *P. elatior* from the Alps do not group together with the population of *P. intricata* from the Alps, but rather they are grouped with *P. leucophylla* within a *P. elatior* - *P. leucophylla* group.

The tree generated through the NJ method (fig. 18) shows a clear separation with a maximum bootstrap value of two phylogroups. The first group consists of the *P. intricata* population from the Alps and the second group consists of *P. elatior* and *P. leucophylla* populations. The population of *P. intricata* from the Alps is clearly differentiated from the rest of the *Primula* populations by both the maximum value of bootstrap but especially by the NJ tree branch length.

Results of the genetic diversity analysis and the proportion of variable markers (Table 13) indicate the population of *P. intricata* as being among the most parsimonious. Generally, the genetic diversity is low in small and isolated populations as a result of processes such as genetic drift, bootleneck, inbreeding and founder effects (Wright, 1931; Nei, 1975; Young *et al*., 1996). Index rarity (DW) is a measure of divergence and expresses the number of private markers. Its value for *P. intricata* is very high (167.1520), distinguishing it once again from the *P. elatior* group.

Trees generated by the sequences analysis (fig. 17) indicate that the two populations of *P. intricata* are not a part of the *P. elatior* group. Depending on the method, the species *P. intricata* is grouped sometimes in a common group with the species *P. acaulis*. Other results place it closer to *P. elatior,* than to *P. acaulis* or *P. veris*. However, *P. intricata* was never included in the group consisting of *P. elatior* and *P. leucophylla*. The sequences belonging to the *P. intricata* populations are highly divergent from those of the *P. elatior* group. From the concatenated length of 1200 nucleotides of the three markers, over 200 informative sites are found between *P. intricata* and *P. elatior* group. The two populations of *P. intricata*, from the Alps and from the Carpathians, differ by nine changes, which are explained by the geographical distance between the collection points of the two populations, in addition to the fact that the collection points belong to two mountain ranges with different geological structure and different glacial history.

5.15. Conclusions on *Primula intricata*

The results of all the methods used converge and support the separation between *P. intricata* and *P. elatior* group.

Based on the results, the present study considers it necessary to grant an independent species status for *P. intricata*, without taxonomic subordination relations towards *P. elatior*.

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TABLE OF CONTENTS OF THE THESIS

Keywords

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