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Phylogeographic pattern of the high-alpine plant species *Eritrichium nanum* (Boraginaceae) within the Carpathians

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Abstract

The Carpathians represent not only a European hotspot of plant diversity for both species richness and endemism, but also an important stepping-stone area in historical migrations between the flora of the Asian and European mountain systems and a starting point of postglacial recolonizations for many species. Yet, until recent years, phylogeographical studies for alpine or arctic-alpine plants were focused on the Alps, whereas peripheral mountain ranges, including the Carpathians, were either neglected or insufficiently sampled. In this study, we aimed to complement the Alpine phylogeographic structure of an emblematic high-alpine European endemic taxon, *Eritrichium nanum*, by focusing on the Carpathian range of the species. We sampled nine populations from the South-Eastern Carpathians and performed ITS1 sequencing and AFLP fingerprinting. In case of ITS1 region, all the populations, no matter of their geographic origin, presented the same ribotype. The AFLP analysis indicated that, within the Carpathians, the extant populations of *E. nanum* comprised two major allopatric lineages. One important result of the research was the discovery that the species' sole important genetic break was located in the Southern Carpathians, separating populations of the Retezat Mountains from all the others in the Carpathians.

Keywords: AFLP; European mountains; internal transcribed spacer 1; phylogeographical boundaries

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Introduction

Eritrichium nanum (L.) Gaudin (Boraginaceae family) is a European endemic taxon emblematic for the high-alpine ecosystems, belonging to an elite group of at least 36 species occurring at elevations above 3500 m in the Central Alps (Ellenberg, 1988). The taxon is distributed exclusively in the Alps and Carpathians. Its typical habitats are crevices on cliffs exposed to wind and sun, or fine gravel with sparse vegetation (Lechner-Pock, 1956).

In the Alps, the distribution area is discontinuous, although it spans the entire Alpine range from the Maritime Alps in South-Western France to the Julian Alps in Italy and Slovenia. Along with these geographical discontinuities, the species' bedrock preference also changes. Whereas in the Western and middle Alps it grows exclusively on siliceous bedrock, it occurs sometimes also on calcareous bedrock in the Eastern Alps. The two bedrock ecotypes are morphologically indistinguishable (Lechner-Pock, 1956).

The phylogeographic pattern of *E. nanum* was revealed only partially (the range of the Alps) by the previous studies of Stehlik *et al*. (2001, 2002). Its complete phylogeographic pattern was not yet revealed as the Carpathians were not included in these researches. Phylogeographical studies for alpine or arctic-alpine plants in Europe were initially focused on the Alps, whereas more peripheral mountain ranges were either neglected or insufficiently sampled (Mráz and Ronikier, 2016). Only in last years, this trend has been reversed with the appearance of high-resolution studies focused on other biogeographical units of European Alpine System (*sensu* Ozenda, 1985), including the Carpathians (Skokanová *et al*., 2019; Šrámková *et al*., 2019; Macková *et al*., 2020; Stachurska-Swakoń *et al*., 2020). Still, the state of knowledge on the intraspecific diversity and genetic relationships of high-mountain plant populations of the Carpathians remains far from being exhaustive.

Therefore, for the present study we have investigated the genetic structure of *Eritrichium nanum* throughout the Carpathians, with the aim of revealing the species phylogeographic pattern in the eastern part of its range.

Materials and Methods

Model species

Carpathian distribution of *Eritrichium nanum* is limited to the south-eastern part of this range, the species does not occur in the Western Carpathians (Mirek *et al*., 2020). Here, *Eritrichium nanum* is a rare plant with small populations, occurring exclusively on subalpine to alpine sunny limestone boulders and cliffs. It was mentioned from the **Rodna, Rarău, Giumalău, Ceahlău, Hăşmaş, Ciucaş, Bucegi, Postăvaru, Piatra Mare, Piatra Craiului,** Iezer-Păpuşa, **Făgăraş, Buila-Vânturariţa** and **Retezat** Mts. (Grinţescu, 1960; Oprea, 2005; Bartók *et al*., 2016) - in bold are the mountainous massifs where the presence of the species is certain, documented by herbarium material deposited in CL, BVS, SIB, IAGB, I, IASI or BP (acronyms according to Thiers, 2016).

Taxon sampling

Nine populations of *Eritrichium nanum* were sampled in the Carpathians (Figure 1, Table 1). Young, green leaves of five random individuals were collected for each population. The two populations from Retezat Mountains were very scarce, therefore only three or four individuals were collected. Plant material was dried in tubes with silica gel and stored at room temperature until DNA extraction. Voucher specimens for all populations were collected and deposited in the herbarium of Babeş-Bolyai University, Cluj-Napoca (CL Herbarium).

Table 1. Sampled populations of *Eritrichium nanum*: numbering, acronym, geographic origin, coordinates

Pop. No.	Population code	No. of ind.	Location	Geographic coordinates
1	ReI-EN	3	Retezat Mts., Piatra Iorgovanului Peak	45°16'58.04"N 22°50'54.23"E
2	ReP-EN	4	Retezat Mts., Piule Peak	45°18'04.21"N 22°54'29.99"E
3	PC-EN	5	Piatra Craiului Mts., Curmătura Saddle	45°33'13.00"N 25°15'40.00"E
4	BO-EN	5	Bucegi Mts., Omu Peak	45°26'28.57"N 25°27'02.83"E
5	BB-EN	5	Bucegi Mts., Baba Mare Peak	45°24'47.32"N 25°28'09.98"E
6	CG-EN	5	Ciucaș Mts., Gropșoarele Peak	45°29'21.68"N 25°58'34.56"E
7	CZ-EN	5	Ciucaș Mts., Zăganul Peak	45°29'01.61"N 25°58'46.34"E
8	GH-EN	5	Hășmaș Mts, Piatra Singuratică	46°41'04.42"N 25°49'34.60"E
9	Ce-EN	5	Ceahlău Massif, Cușma Dorobanțului	46°59'06.64"N 25°57'25.73"E

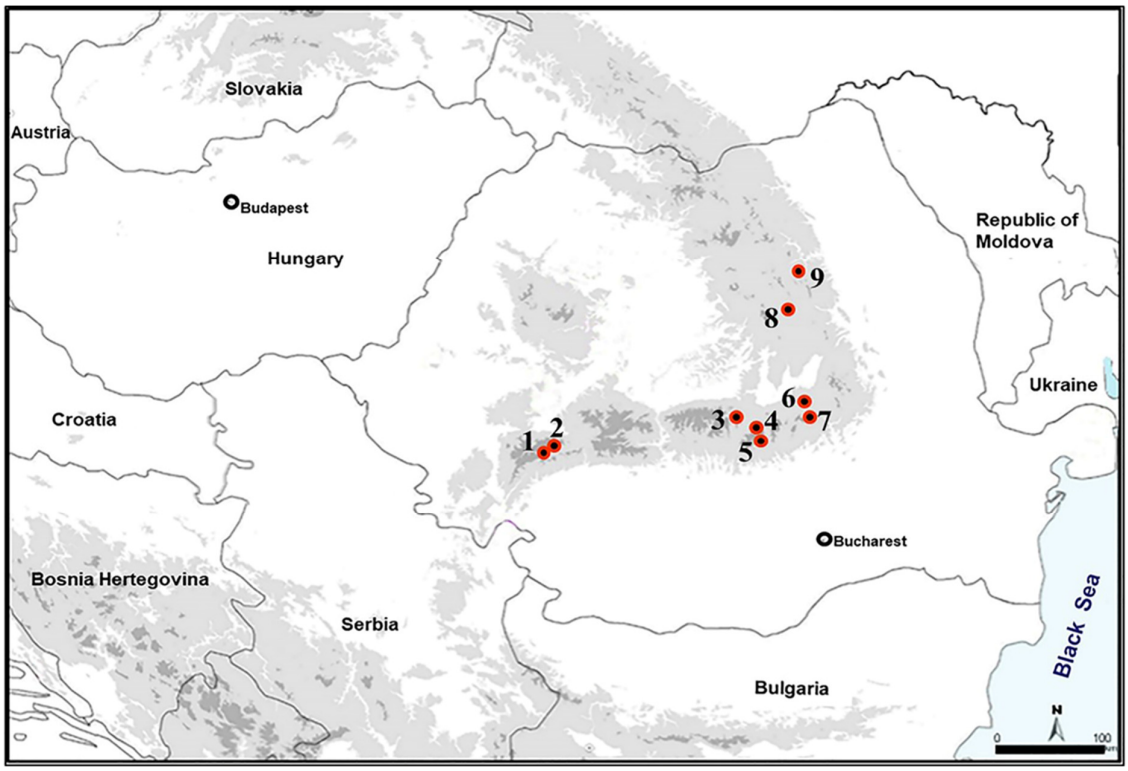

Figure 1. Map showing the sampled populations of *Eritrichium nanum* from Romanian South-Eastern Carpathians
Details of populations are given in Table 1

DNA isolation

Total DNA was extracted from approximately 13 mg of dried plant material using the CTAB protocol of Mengoni *et al*., 2000. DNA quality was estimated on 1% agarose gel stained with ethidium bromide, and the concentration was quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA).

DNA sequencing

Two individuals from each population were sequenced for the nuclear region of ITS1 (Internal Transcribed Spacer 1). *ITS2* and *ITS5* primers (White *et al*., 1990) were used for both the PCR and cycle sequencing.

The 50 µL volume of PCR mix was composed of 1× polymerase buffer (Fermentas, Thermo Fisher Scientific, USA); 0.8 mmol·L^{-1} dNTPs; 2 mmol·L^{-1} $MgCl_2$; 0.12 µmol·L^{-1} of each primer; 8 µg·mL^{-1} of BSA; 1 U of TaqPolymerase (Fermentas, Thermo Fisher Scientific, USA); and 10 µL of diluted genomic DNA. The following parameters were applied: initial denaturation for 5 min at 94 °C; followed by 35 cycles of 1 min at 94 °C, 45 sec at 52 °C and 2 min at 72 °C; and a final extension of 10 min at 72 °C.

The PCR products were purified on 1% agarose gel using the commercial kit Wizard SV Gel and PCR Clean Up System (Promega, USA) according to the manufacturer's protocol.

Sequencing (of both strands) was performed using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Scientific, USA) with 5× sequencing buffer. Excess primers and labeled dideoxynucleotide triphosphates were removed by purification with Sephadex and Sephacryl (1:1) (GE Healthcare Bio-Sciences AB, USA). The samples (total volume 10 µL) were prepared prior to sequencing by adding 10 µL of HiDi formamide and then loaded onto an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, USA).

AFLP fingerprinting

The amplified fragment length polymorphism (AFLP) analysis followed Vos *et al*. (1995) with minor modifications (Şuteu *et al*., 2011). Three pairs of selective primers were selected from Stehlik *et al*. (2001): EcoRI-AGA/MseI-GAG, EcoRI-AGC/MseI-GAT, EcoRI-AGG/MseI-GCA, and EcoRI-ATG/MseI-CAG. The AFLP reproducibility tests (Bonin *et al*., 2004) included three random samples from the total sample set, which were extracted twice as within-plate replicates. The AFLP analysis was performed on all the individuals collected from each population.

Data analysis

ITS1 sequence

Sequences were assembled, edited, and manually aligned using BioEdit version 7.0.9.0 (Hall, 1999). The relationships between populations were analysed using the program MEGA version 4.1 (Tamura *et al*., 2007). Three more accessions were added from GenBank: *Eritrichium villosum* (GenBank: JQ388502.1), *Eritrichium splendens* (GenBank: JQ388501.1), and *Eritrichium sericeum* subsp. *sericeum* (GenBank: JQ388500.1). The tree was constructed using Kimura 2-parameter model implemented within the Maximum likelihood (ML) method, and bootstrap values were calculated from 1000 replicates.

AFLP

AFLP fragments were manually scored within the size range of 50-500 bp using Gene Mapper version 4.0 (Applied Biosystems, Thermo Fisher Scientific, USA). Data reliability was tested by comparing duplicates, and only fragments that separated unambiguously were used. The distribution of these fragments was coded into a presence/absence binary matrix. The AFLP error rate was calculated as the number of mismatches (i.e., 0/1 or 1/0) divided by the number of matches (i.e., 0/0 and 1/1) in each pair of replicates (Bonin *et al*., 2004) and fragments with mismatches in more than one replicate pair were omitted from the analysis. The proportion

of polymorphic loci within each population, Nei's gene diversity (Nei, 1987), and the frequency downweighted marker values (DW; Schönswetter and Tribsch, 2005) were estimated using the AFLPdat R-script (Ehrich, 2006). A neighbor-joining network was constructed with SplitsTree version 4 (Huson and Bryant, 2006), using the Neighbor-net method. Bootstrap values were calculated from 1000 replicates. The relationships among individuals were analysed using PCoA based on the Bray-Curtis model computed with the PAST version 2.17 software (Hammer *et al*., 2001). Furthermore, a hierarchical analysis of molecular variance (AMOVA) partitioning was performed using ARLEQUIN version 3.5 (Excoffier and Lischer, 2010) at three different levels: within populations, among populations, and among geographical groups of populations.

Results and Discussion

DNA sequences

Internal transcribed spacer 1 (ITS1) belonging to ribosomal DNA (rDNA) is a popular target for examination of phylogenetic relationships, for study of genetic variability and divergence within and between species and even for identification of population groups on a regional scale (Forough *et al*., 2018; Skubic *et al*., 2018). Though it is known that mutations occur at a relatively rapid rate in internal transcribed spacers, in case of *E. nanum* all the populations, no matter of their geographic origin, presented the same ribotype. The trimmed ITS1 sequence had 328 bp (GenBank accession Nos. JX161481 - JX161489). The ML analysis (Figure 2) suggested that *E. nanum* grouped with *E. sericeum*, while *E. villosum* and *E. splendens* formed a separate cluster.

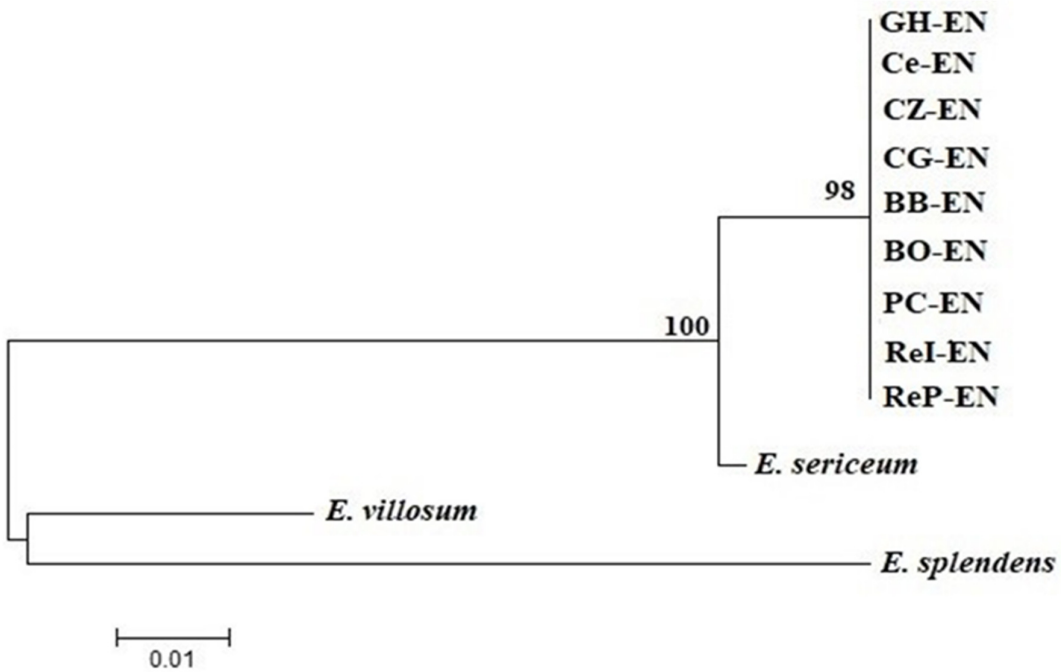

Figure 2. ML tree based on the ITS1 sequence for the *Eritrichium nanum* populations

AFLP fingerprinting

Altogether, 198 fragments were scored, and 81.31% of the fragments were polymorphic across the data set. The repeatability of the AFLP results was very high (100% for the overall test). The lengths of the fragments ranged from 52 to 499 bp.

In terms of **polymorphic loci**, the highest value occurred in Ceahlău population (Ce-EN - 0.5; Table 2). The least number of polymorphic loci occurred in Piule Peak (ReP-EN, mean 0.1) and Piatra Iorgovanului Peak (ReI-EN, mean 0.17) populations. The **gene diversity** varied from 0.05 to 0.27, with a mean of 0.17. The highest gene diversity (0.27) was detected in the *E. nanum* population from Gropșoarele Peak (CIG-EN) and the lowest in the two pauper populations from the Retezat Mountains. Furthermore, **the rarity** estimated by the frequency down-weighted marker value (DW) revealed a lower number of rare markers in the Retezat Mountains (10.75 and 11.92), while the other populations presented higher close values, ranging from 17.48 to 22.53 (Table 2).

The overall lower genetic diversity detected in the populations from the Retezat Mountains should be interpreted with caution, since the sampling in these populations was not to the same level as in the rest of the dataset (three individuals in ReP-EN, respectively four individuals in ReI-EN).

Table 2. Genetic parameters of populations: proportion of variable markers; gene diversity; frequency-down-weighted marker values (DW), mean of the genetic parameters, and standard deviation (SD)

Population code	Proportion of variable markers	Gene diversity	Rarity (DW)
ReI-EN	0.17	0.11	11.92
ReP-EN	0.10	0.05	10.75
PC-EN	0.34	0.18	19.44
BO-EN	0.33	0.17	17.63
BB-EN	0.35	0.17	19.75
CG-EN	0.48	0.27	17.48
CZ-EN	0.33	0.17	22.53
GH-EN	0.37	0.20	19.77
Ce-EN	0.5	0.25	20.73
Mean	***0.33***	***0.17***	***17.78***
SD	***0.13***	***0.07***	***3.96***

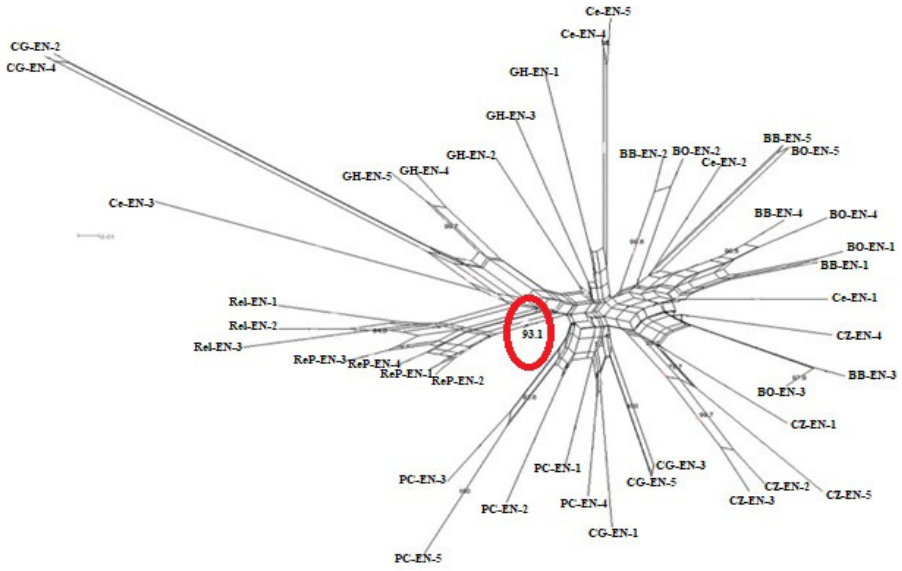

Figure 3. Neighbor-net diagram of the *Eritrichium nanum* populations
Bootstrap values above 70% are shown on major branches. Red oval indicates the cluster formed by ReI-EN and ReP-EN. Names of populations as in Table 1

The Neighbor-net diagram (Figure 3) of the nine populations of *E. nanum* revealed a patchy distribution: the individuals belonging to different populations mixed together or forming groups without bootstrap support. The only exception was a well-supported cluster, formed by the two Retezat populations (ReI-EN and ReP-EN), which separated with a boostrap value of 93.1. This pattern was also confirmed by the PCoA based on the AFLP data for all of the individuals (Figure 4).

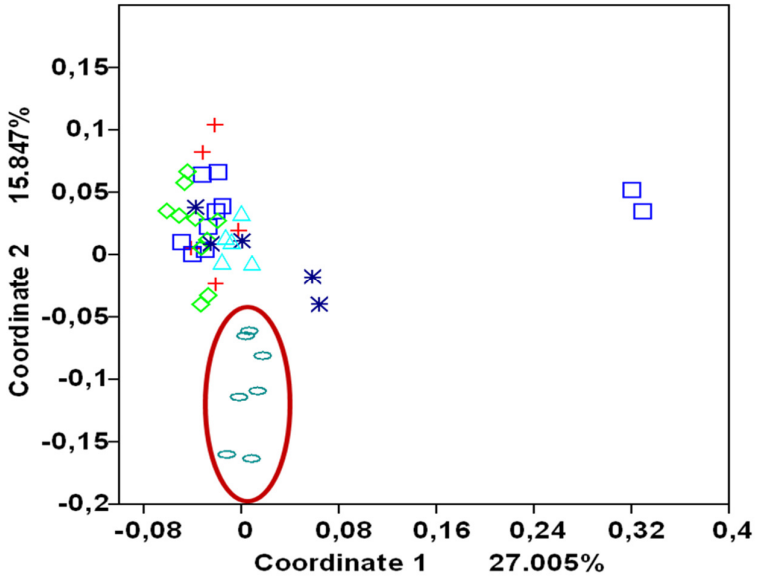

Figure 4. Principal coordinates analysis (PCoA) of *Eritrichium nanum* individuals based on amplified fragment length polymorphism phenotypes
The colours and symbols: red cross - Ce-EN; green diamond - BO-EN and BB-EN; blue triangle - PC-EN; blue square - CG-EN and CZ-EN; blue star - GH-EN; blue oval - ReI-EN and ReP-EN. Red oval comprised ReI-EN and ReP-EN populations

Table 3. Analysis of molecular variance of *Eritrichium nanum* populations for different groupings

	Source of variation	df	Sum of squares	Percentage of variance	*FST*/ *FCT*
No groups defined	Among populations	8	357.850	24.22	0.24
	Within populations	33	593.317	75.78	
Ce-EN+GH-EN **vs.** BO-EN+BB-EN+PC-EN+ CZ-EN+CG-EN+ReP-EN+ReI-EN	Among groups	1	37.985	2.69	0.23/ -0.027
	Among populations within groups	7	319.865	25.71	
	Within populations	33	593.317	76.98	
Ce-EN+GH-EN + BO-EN+BB-EN+PC-EN+CZ-EN+CG-EN **vs.** ReP-EN+ReI-EN	Among groups	1	91.110	18.06	0.34/ 0.18
	Among populations Within groups	7	266.740	15.56	
	Within populations	33	593.317	66.38	
Ce-EN+GH-EN **vs.** BO-EN+BB-EN+PC-EN+ CZ-EN+CG-EN **vs.** ReP-EN+ReI-EN	Among groups	2	129.041	9.59	0.27/ 0.10
	Among populations Within groups	6	228.809	17.30	
	Within populations	33	593.317	73.11	

The genetic variance, assessed by AMOVA, varied according to the composed groups (Table 3). The highest amount of genetic variance among groups (18.06%) was found when the grouping referred to the

Retezat populations versus the remaining populations. The division between the Eastern and the Southern Carpathians (as a single group or split into two subgroups) resulted in lower values reflecting the high differentiation of the Retezat populations, as highlighted before by the net. Nevertheless, the highest percentage of variance (66.38% to 76.98%) was due to within populations component, attesting once again the homogeneity and the lack of a solid differentiation between these Carpathian populations.

The results indicated that, within the Carpathians, the analysed populations of *E. nanum* comprised two major lineages. The major genetic break clearly separated a group from the south-western edge of the mountains (Retezat Mountains). All of the present data (Neighbor-net diagram, Figure 3; PCoA, Figure 4; AMOVA, Table 3) might suggest the vicariance of *E. nanum*, followed by survival in a glacial refugium in the south-western part of the Carpathians. This scenario is not surprising, given that the presence of a distinct refugium and evolutionary centre in the western part of the Southern Carpathians was already postulated by Mráz and Ronikier (2016), based on genetic structure of the Carpathian populations of *Campanula alpina* (Ronikier *et al*., 2008; Ronikier and Zalewska-Gałosz, 2014) and *Onobrychis transsilvanica* (Băcilă *et al*., 2015). Furthermore, a very similar phylogeographic pattern was also observed in *Carex curvula* (Pușcaș *et al*. 2008), where the populations from Retezat Mountains were clearly distinct from all those in the Carpathians.

Conclusions

The ITS1 region presented for all of the nine populations, no matter of their geographic origin, the same ribotype. The AFLPs indicated that, within the Carpathians, the analysed populations of *E. nanum* comprised two major lineages. Most of the populations revealed a patchy distribution: individuals belonging to different populations were mixed together or formed not well supported groups. The only exception was a well-supported cluster, formed by the two Retezat populations. One major result of the research was the reconfirmation of the existence of a glacial refugium in the south-western part of the Carpathians, coupled with an important phylogeographic boundary within the southern part of this range.

Authors' Contributions

The contributions of authors to the manuscript are as follows: conceptualization: DȘ, MP, GC; field work: AIS, DȘ; analytical investigation: DȘ; data curation: DȘ, IB; formal analysis: AIS, DȘ, IB, ZRB, MP; funding acquisition: DȘ; investigation: DȘ, IB, ZRB; methodology: DȘ, MP; project administration: DȘ; writing - original draft: DȘ; writing - review and editing: AIS, DȘ, IB, ZRB, MP and GC. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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