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GENETIC STRUCTURE OF THE *ERYTHRONIUM DENS-CANIS* L. AND *SCILLA BIFOLIA* L. POPULATIONS FROM THE EASTERN CARPATHIAN BASIN – PHYLOGEOGRAPHIC AND CONSERVATION APPROACHES

Summary of the PhD thesis

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KEYWORDS

conservation genetics
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phylogeny
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rpl32-trnL
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Scilla bifolia s.l.

INTRODUCTION

This study presents the most important results of the author from the last three years, concerning the phylogeny/phylogeography of two monocotyledonous species - *Erythronium dens canis* L. (LILIACEAE) and *Scilla bifolia* L. (ASPARAGACEAE) from throughout much of their distribution range, with a focus on the Eastern Carpathian Basin.

Plant biology researches, even those with ecological characters are not complete without applying modern technics of molecular biology.

Researchers started to be interested in the biogeography of the Carpathian Basin since the 1970s, (Bănărescu and Boşcaiu, 1973), in times when studies relied on paleontological (fossils) and palinological data, on one hand, and on biogeographical data, on the other. Actual researches complete the former theories and add new ones, using clear arguments, based on results of molecular studies. Actual technics of DNA isolation, amplification and sequencing of targeted fragments allow new data aquisition (based on genealogy), data which can be used in molecular taxonomy studies using computational methods. In this way classical taxonomy (based on morphological characters) is completed, and the taxonomic position of uncertain taxa can be clarified. On a finer scale, these data help on finding the degree of relatedness of different populations belonging to the same species, beeing an important instrument in phylogeographical studies. Moreover, the genetic diversity of

populations (which has an important application in conservational projects, in selection of populations of conservational success - conservation genetics) can be calculated based on these data.

The main question of the researh was if cryptic glacial refugia, where a series of species managed to survive glaciations and expanded afterwards their range of distribution, reaching there actual distribution range existed in the Eastern part of the Carpathian Basin (Transylvania).

The reasons in choosing these two species were:

- former studies concerning *Erythronium dens-canis* found a Transylvanian lineage with high genetic diversity, confined to the Carpathian Basin, and a non-Transylvanian lineage, which is much more uniform and has a wide distribution range. We proposed to study the structuring of this Transylvanian lineage with a much denser sampling as compared with the previous study.

- we searched for a second species which can be sampled together with the former one (another early spring geophyte) and in case of which the same molecular marker can be applied. We decided to choose *Scilla bifolia*, because in pilot testing this turned out to have a high variability of the sequencies.

1. THEORETICAL BACKGROUND

1. 1.General characterization of the species

1.1.1. *Erythronium dens-canis*

Erythronium dens-canis L. belongs to subfamily LILIOIDEAE, family LILIACEAE of the order LILIALES (Bremer and colab., 2009).

The genus *Erytrhonium* appeared approx. 12 million years ago in the eastern part of Asia, from where it spread in Europe, the easter and western parts of North America (Patterson and Givnish, 2002). It has a disjunct distribution: from the 28 species, 17 appear in the western and 6 in the eastern part of North America, and 5 species in Europe (Allen et al., 2003; Bartha et al., 2015b). The genus is morphologic homogenous, easy to recognise. Out of the 5

Eurasian species just one is present in Europe, *Erythronium dens-canis* (Săvulescu, 1966), *E. sajanense, E. sibiricum, E. japonicum* and *E. caucasicum* are Asian species.

In Romania, *Erythronium dens-canis* has been traditionally known as beeing present with two subspecies: *E. dens-canis* ssp. *dens-canis* and *E. dens-canis* ssp. *niveum* (Baumg) Buia et Păun (Ciocârlan, 2009). The taxonomic status of the subspecies has not been studied by molecular methods. According to the Flora Europaea (Richardson, 1980) these taxa appear as varieties. The chromosome numbers of the species are 2n=24 (Moore, 1982; Ciocârlan, 2009), but tetraploid populations are also known (Siljak-Yakovlev et al., 2010).

Erythronium dens-canis is widely distributed in southern Europe (Pirin Mts., France, Italy, Hungary, Czech Repuplic, Slovakia, The northern Balkan Peninsula) (Richardson, 1980).

The plant is common in most parts of Romania, growing in decidous forests from the lowlands to the hilly regions (Săvulescu, 1966; Ciocârlan, 2009).

Erythronium dens-canis is an eurasian geophyte, mesophyte, mesotherm, mesotrph species, characteristic of Carpinion (Sanda et al., 1983).

Erythronium dens-canis is a myrmecochorous species, the secondary dispersion of the seeds is made by ants. As a consequence, the species has a limited capacity of seed dispersion.

The phylogeny of the *Erythronium* has been largely studied. Phylogenies based on morphological characters and molecular analyses (*matK*, nrITS, *rps16* and 5′ *trnK*) show *Erythronium*, *Amana* and *Tulipa* in the same, well supported clade, with *Gagea* as sister group. All of the phylogeographic groups of the *Erythronium* (Eurasian, Eastern North American and Western North American) form well supported subclades (Allen et al. , 2003; Clennett et al., 2012)**.**

The biology of *Erythronium dens-canis* is poorly understood, unlike that of non-European species of the genus, about which considerable information exists (Guitián et al., 1999; Guitián et al., 2003).

1.1.2. *Scilla bifolia*

Scilla genus (ASPARAGACEAE, ASPARAGALES) (Bremer and colab., 2009) is an extremely heterogenous genus, very variable as it concerns its caryological characters (Speta, 1979). It has about 100 species, distributed in southern Europe, Central and Western Asia (Ghavami et al., 2009).

The Central- and Western European *Scilla* populations have been included until 1970 in a single, largely defined species: *Scilla bifolia* s.l. Detailed taxonomic studied proove the existance of a group of species – series of taxa with different poliploidy level: x=9 şi 2n=18, 36, 54, associated with some ecological and morphological characters.

According to Ciocârlan (2009) the species has two subspecies in Romania: ssp. *drunensis* Speta and ssp. *bifolia.*

Scilla bifolia L. has a large distributional range in Europe, it is a common species in Romania, it grows in decidous forests from the plain to the mountainous regions. Is an eurasian geophyte, mesophyte, mesotherm and mesotroph, species of Querco-Fagetea, Carpinion, Alno-Padion (Sanda et al. , 1983).

1.2. Phylogenetic and phylogeographic studies

1.2.1. Theoretical considerations

Phylogeny shows the evolutionary relationships between different taxonomic groups, it deals with resolving the evolutionary history of a group of organisms, a taxon. 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. Current researches for resolving the degree of relatedness of taxa use information about macromolecules (DNA, proteins) besides morphological characters.

Understanding how historical events have helped to shape the current geographical dispersion of genes, populations and species is the major goal of phylogeography, a term that was introduced by Avise et al. (1987). Phylogeography can be defined as a 'field of study concerned with the principles and processes governing the geographic distributions of genealogical lineages, especially those within and among closely related species' (Avise, 2000). Advancing of the methodology (DNA technology), as well as the analytical approach of phylogeography allows the assessment of the postglacial chronological and geographical distribution of different genetic lineages.

Phylogeographic studies of alpine plants covering several European mountain ranges are numerous. The Quaternary history of the flora of Alps is well understand (Tribsch and Schönswetter, 2003; Schönswetter et al., 2005; Ehrich et al., 2007; Schneeweiss and Schönswetter, 2011). There are relatively few recent studies on biogeography of the Carpathian Basin (Schmitt et al., 2006; Provan and Bennett, 2008; Varga, 2010; Bálint et al.,

2011; Ronikier, 2011; Ujvárosi and Markó, 2011; Schmitt and Varga, 2012), especially based on the study of angiosperms. Phylogeographic studies from the Carpathians on different plant species are known mostly on alpine species (Schönswetter et al. , 2005; Puşcaş et al., 2008; Ronikier et al., 2008; Csergő et al., 2009; Ronikier et al., 2012).

Classical theories of the quaternary history of species in temperate Europe place glacial refuges in peninsulas of southern Europe (the Iberian Peninsula, the Balkans and the Apennine). According to this theory a number of species have survived glaciation during the Pleistocene periods in these areas, where diversity has increased (Ibrahim et al., 1996; Taberlet et al., 1998; Hewitt, 1999; Schmitt, 2007). With the retreat of glaciers to the North , the species expanded to the North colonizing new areas released by the action of glaciers: the expansion - contraction model - EC. Recently, studies of phylogeography based on molecular analyses have provided new data on the history of species during glaciations. In the 2000s a new theory appeared, that a number of species have survived in so-called cryptic refugia located in different areas in the North (Stewart and Lister, 2001; Bhagwat and Willis, 2008; Provan and Bennett, 2008). These refugia are located in different parts of Europe: Western Europe (Valtueña şi colab., 2012), Central Europe (Michl et al., 2010) and in the Carpathians.

Extra -Mediterranean refugia were confirmed in Europe for a number of species (Stewart and Lister, 2001; Kotlík et al., 2006; Sommer and Nadachowski, 2006; Schmitt, 2007; Bhagwat and Willis, 2008; Birks and Willis, 2008; Provan and Bennett, 2008; Svenning et al., 2008; Stewart et al., 2010; Varga, 2010). Most studies focus on terrestrial taxa , but there are data for the aquatic and even marine fauna, prooving the existence of cryptic refugia in Europe in these environments, too (Provan and al., 2005; Sedivá et al., 2008; Pauls et al., 2009; Bálint et al. , 2011).

Phylogeographic studies besides theoretical importance have an important part as applied science, as used in conservation:

1. identifying populations with high genetic diversity , identifying possible refugia of this diversity: Regional genetic diversity can be important for long-term survival of many species, so it is relevant to the conservation of species and habitats (Frankham, 1995). Knowledge of changes in the distribution of genetic diversity over time helps in identifying conservation units (Hampe and Petit, 2005).

2. In the current context of climate change: Knowing the history of different species in the past climate conditions, we can give predictions on their future survival and distribution (Provan and Bennett, 2008).

1.2.2. Molecular technics and markers used in plant phylogeny and phylogeography

The molecular markers used in phylogenic and phylogeographic studies belong to two categories: proteins (isozyme studies) and DNA. The DNA markers are SNPs (Single Nucleotide Polymorphism), SSR – Simple Sequence Repeats (or STR – Short Tandem Repeats), specific genes, non-codic regions of different genes, intergenic spacers. Plant phylogeny and phylogeography studies use informations provided by three genomes: nrDNA, cpDNA and mtDNA.

Two main groups of markers used in phylogeography and phylogeography can be distinguished: nuclear and non-nuclear markers. The main difference between them is in the way of their inheritance. Nuclear genome presents a biparental inheritance, while those from organelles (mitochondria and chloroplasts) present unipaternal mode of inheritance in case of organisms with sexual reproduction.

Nr ITS is the most widely used nuclear marker in phylogenetic studies. In the recent years single-copy or low-copy nuclear genes are being increasingly used.

After the selection and application of the markers and therefore obtaining the data set, the next steps are sequence alignment, recontstruction and evaluation of phylogenetic trees.

The methods, techniques used are based on sequencing, genotyping, RFLP – Restriction Fragment Length Polymorphisms, AFLP – Amplified Fragment Length Polymorphisms and RAPD – Random Amplified Polymorphic DNA.

Methods by which phylogeny can be reconstructed based on genetic data are multiple, most of them can be grouped into one of the following categories: distance methods, methods based on the principle of parsimony and methods based on probability.

Testing phylogenetic trees also can be made in several ways. The most widely used of these are statistical tests, bootsrap test (Felsenstein, 1985) is most often used.

1.3. Conservation genetics – an applied science

Conservation of genetic diversity, one of the aspects of biodiversity is a fundamental issue of conservation biology, as it provides the basic material for evolutionary processes, ie. adaptative potential in changing environment.

Conservation genetics uses theoretical knowledge and genetic techniques to reduce the risk of extinction of endangered species. Its long-term aim is the conservation of species as dynamic entities capable to cope with environmental changes. Currently the focus is on the

consequences arising from the reducing of once large populations (with outcrossing individuals) in small populations where inbreeding effects and stochastic factors dominate.

The molecular data provides information on the evolutionary history and genetic differentiation of species, which helps in making the right decisions on conservation priorities. As a result, conservation genetics constitutes a very important part of molecular biology (Freeland, 2005).

Along with rare species common species require conservation activities, as well. Conservation of diversity of common species has other objectives than those of rare species. Since the existence of the species is not endangered, the evolutionary fitness of the species should be kept.

This draws attention to the need of conservation actions before a taxon reach critical points. Perhaps this is the biggest long-term challenge of conservation genetics: not only to save endangered species, but prevent actually common taxa ever becoming endangered. The purpose of conservation actions for common species is to preserve genetic variability of the species. To designate the target populations, population genetic studies are required. As a first step in common species conservation, the study of the genetic variability of the target species is important (Millar and Libby, 1991).

2. THE OBJECTIVES

Erythronium dens-canis:

- Study of phylogeography of *Erythronium dens-canis* in the Eastern Carpathian Basin, with denser sampling than Bartha et al. (2015a), and with a focus on the surroundings of the Apuseni Mountains.
- Assessing the role of partitioning of the Transylvanian lineage by the Apuseni Mountains.
- Finding a suitable nuclear marker and use of it in intrapopulational genetic diversity studies.

Scilla bifolia:

- Phylogeographic study comprising as much as possible the whole range of the species in Europe.
- Population structure studies in the Carpathian Basin.

Both species:

- Determination of phylogenetic conservation units (unique lineages, subclades and their geographic distribution).
- Determination of populations/geographic ranges with high genetic diversity (with high conservation values).
- Understanding the origins of the lineages, identification of the putative glacial microrefugia for a better understanding of the biogegography of Carpathian Basin.

3. MATERIALS AND METHODS

3.1. Sampling

The vegetal material used was collected in March-May 2014 and 2015. In addition, data of the collections from 2013 from Bartha et al. (2015a) have been used, as well as specimens from different collectors/suppliers from herbaria (in case of *Scilla bifolia*). Leaf samples collected were dried and stored in silica gel.

According to the objectives, in case of *Erythronium dens-canis* samples were collected mainly from the surroundings of the Apuseni Mountains. Samples were collected from 107 populations (Fig.1) and on other 27 samples from the study of Bartha et al. (2015a) were used. As outgroups *Erythronium sibiricum* (Fisch. & C.A.Mey.) Krylov, *Erythronium japonicum* Decne. şi *Amana edulis* (Miq.) Honda were selected.

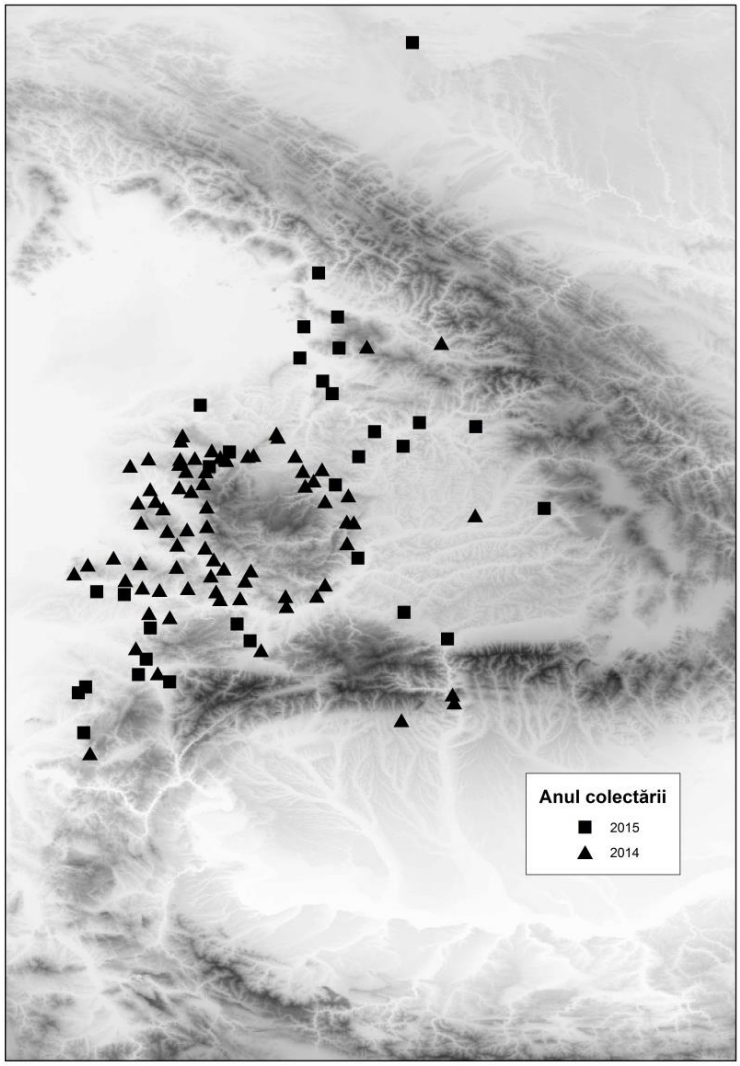

Fig.1. Geographical position of the studied *Erythronium dens-canis* populations

In case of *Scilla bifolia* samples were collected as far as possible throughout of the whole distribution range of the species, with a denser sampling in the Carpathian Basin.

A total of 128 populations of *Scilla bifolia* were sampled (Fig.2.) – and samples of additional putative closely related (mostly Anatolian) taxa were kindly provided by Hasan Yildrim: *Scilla ingridae, S. vardaria, S. sardensis, S. siehei, S.luciliae, S. sibirica ssp. armena, Puschkinia scilloides, S. × allenii*. As outgrups *Hyacinthoides italica, Hyacinthella heldreichii, Scilla leepii, Bellevalia koyuncui* and *Muscari mirum* were used.

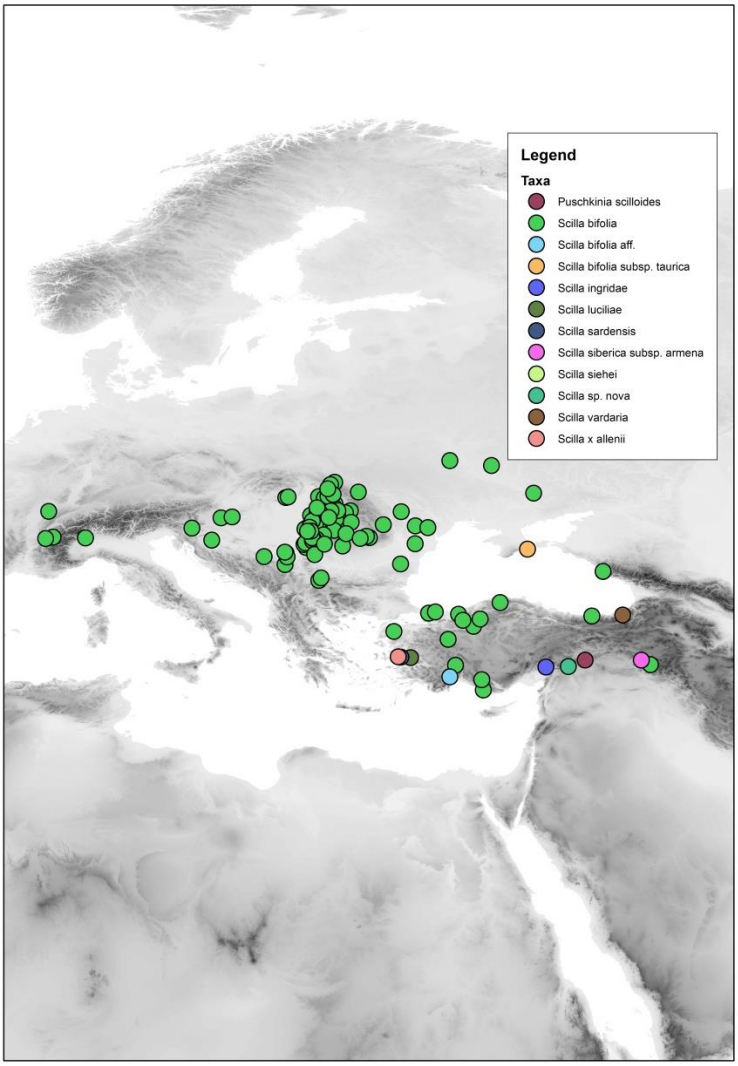

Fig. 2. Geographical origin of the samples used for the *Scilla bifolia* s.l. study

3.2. DNA extraction

Total genomic DNA was extracted according to protocol from dried leaf fragments from one sample per population, using the Analytik Jena kit.

3.3. Molecular markers used

In case of *Erythronium dens-canis rpl32-trnL* and *rps15-ycf1* intergenic spacers were used. According to test screenings of different plastid regions, these appear to be some of the most variable loci of the Small Single Copy (SSC) region. For intrapopulational variability studies

low copy nuclear genes from the litearature were tested (Denton et al., 1998; Roncal et al., 2005; Day et al., 2014), as well as primers designed in the lab.

In case of *Scilla bifolia* the *rpl32-trnL* intergenic spacer was used.

3.4. PCR amplification

PCR amplification of two intergenic spacer regions (*rpl32-trnL* and *rps15-ycf1*) was made in case of *Erythronium dens canis*, and of *rpl32-trnL* in case of *Scilla bifolia.*

PCR was performed in 25µl reaction volumes containing 12.5 µl 2x MyTaq Red Mix (Bioline, Luckenwalde, Germany), 9.5 µl dd water, 1 µl of each preimers (10 µM, Macrogen, The Netherlands) and 1 µl DNA template solution of unknown concentration. The primers used were: *rpl32-F – trnL*$^{(UAG)}$ (for the *rpl32-trnL* region) and M1F - M1R (for the *rps15-ycf1* region).

DNA amplification was performed in a Gradient Palm-CyclerTM (Corbett Research, Sidney, Australia), using the following parameters: 94^{0}C/4 min., 40 x (94^{0}C/45 sec., 61^{0}C/45 sec., 72^{0}C /45 sec) 72^{0}C/7 min. Succes of PCR was checked by agarose-gel electrophoresis.

PCR products were column purified using a PCR purification kit (Jena Bioscience, Jena, Germany) in case of sequencing in tubes.

In case of *Erythronium dens-canis* amplification of some low copy nuclear genes was tried for testing them as nuclear markers. Volume and composition of reactions was the same as in case of plastid markers.

3.5. Sequencing

Sequencing was performed by Sanger Method at Macrogen Inc. (The Netherlands), using the reverse primer for *rpl32-trnL* region and the forward one for *rps15-ycf1i* region respectively.

3.6. Phylogenetic and phylogeographic analysis

3.6.1. DNA sequence alignment

rpl32-trnL and *rps15-ycf1* sequences were directly exported from chromatograms in FASTA format using ChromasLite v.2.01 (Technelysium Pty).

Sequences were aligned manually in MEGA5 (Tamura *et al.*, 2011).

3.6.2. Haplotype network reconstruction

Haplotype calling and the simultaneous parsimony network reconstruction was performed in TCS (Clement et al. 2000) at 95 % connection limit without taking into consideration gaps and structural mutations due to their putative homoplaseous nature.

3.6.3. Reconstruction of the phylogenetic trees based on plastid DNA sequencies

Maximum likelihood analysis based on RAxML (Stamatakis, 2006) was made, using RaxMLGUI v. 1.2 (Silvestro and Michalak, 2012), under the GTR + Γ model of sequence evolution (as recommenden by the RAxML manual). Support for the nodes of the ML topology was assessed via the rapid bootstrap algorithm implemented in RAxML employing 500 replicates (Stamatakis et al. 2008).

Visualisation and editing of phylogenetic trees was made in TrieeView (Page, 1996), FigTree ver. 1.3.1 (A. Rambaut; http://tree.bio.ed.ac.uk /software/figtree/), MrEnt (Zuccon and Zuccon, 2006) and CorelDRAW X3.

3.6.4. Calculation of the genetic diversity indices of the clades in case of *Erythronium dens-canis*

In case of *Erythronium dens-canis* indices of genetic diversity of the different clades were calculated, using Dna SP v.5.(Librado and Rozas, 2009).

4. RESULTS

4.1. Results of DNA extraction, sequence amplification, sequencing and alignment of DNA fragments

Extraction of DNA was performed successfully from all of the samples. Concentrations of the genomial DNA obtained varied between 100-400 ng / µl.

4.1.1. *Erythronium dens-canis*

4.1.1.1. Plastid DNA

The amplification and sequencing of plastid regions *rpl32* - *trnL* and *rps15* - *ycf1* was performed successfully for each specimen of *Erythronium dens-canis*. A total of 156 *rpl32* - *trnL* and *rps15* - *ycf1* sequences were used in this study, 107 of which were newly generated.

4.1.1.2. Nuclear DNA

The amplification of chosen nuclear regions failed. As a consequence, we quit using nuclear markers for testing signal phylogeography of populations in lack of nuclear sequences.

4.1.2. *Scilla bifolia*

Amplification of *rpl32* - *trnL* region failed in 10 samples. Most of them are herbarium specimens collected several years ago. In 4 cases sequencing failed and in 6 cases we received only partial sequences. As a consequence, these samples were excluded from analysis.

4.2. Number, structure, distribution and haplotype network for the *Erythronium dens-canis*

TCS analysis identified 63 haplotypes of *Erythronium dens-canis* and 3 haplotypes of *E. caucasicum* (Fig.3.). Haplotype network presents a clear structure. Combining information on the geographical origin of haplotypes and network topology, seven main groups can be identified:

– a Caucasian group – containing 3 samples of *E. caucasicum* from Bartha et al. (2015a) study

– a non-Transylvanian group (colored in yellow) – containing nearly all haplotypes of the species distributional range from the Atlantic coast to the southern slopes of the Carpathians, except most of the samples from Transylvania. (Group comprises mainly samples from Bartha et al. (2015a)).

– a Transylvanian group comprising the subgroups: four subgroups containing samples from Transylvania, corresponding to the 4 clades identified by phylogenetic tree reconstruction (see below). These are t1 (group colored in blue), t2 (group colored in red), t3 (group colored in green), and t5 (group colored in orange) and a subset (t4) containing two samples of a population from Croatia from Bartha et al. (2015a).

The non-Transylvanian group is star shaped, its center being the most common haplotype, totalling 22 samples. It presents a wide distribution area. A number of 16 rare haplotypes (most of them represented by a single sample) are connected to this central haplotype with one to several mutational steps.

Samples from Transylvania, although coming from a smaller geographic area presents a more complex structure.

Of the 4 Transylvanian groups, t1 is the largest. It is divided into two subgroups : one has in central a widespread haplotype, totalling 23 samples. To this central haplotype 8 rare haplotypes are connected by single mutational steps, and other 6 haplotypes by several mutational steps. The other subgroup is less numerous, it is connected to the prior subgroup with a central haplotype consisting of 8 samples, to which another 7 haplotypes are connected.

Group t2 presents a less obvious structure , with a total of 13 haplotypes. One of these is larger (7 samples), one consists of three and one of two samples, respectively, the remaining haplotypes being formed only by a single sample.

Group t3 has two bigger haplotypes, (8 and 7 samples, respectively), as well as two haplotypes represented by a single sample. To one of them a haplotype with 2 samples is connected by one mutational step, which is connected with an other haplotype (2 samples).

Group t5 is composed of a single haplotype (3 samples).

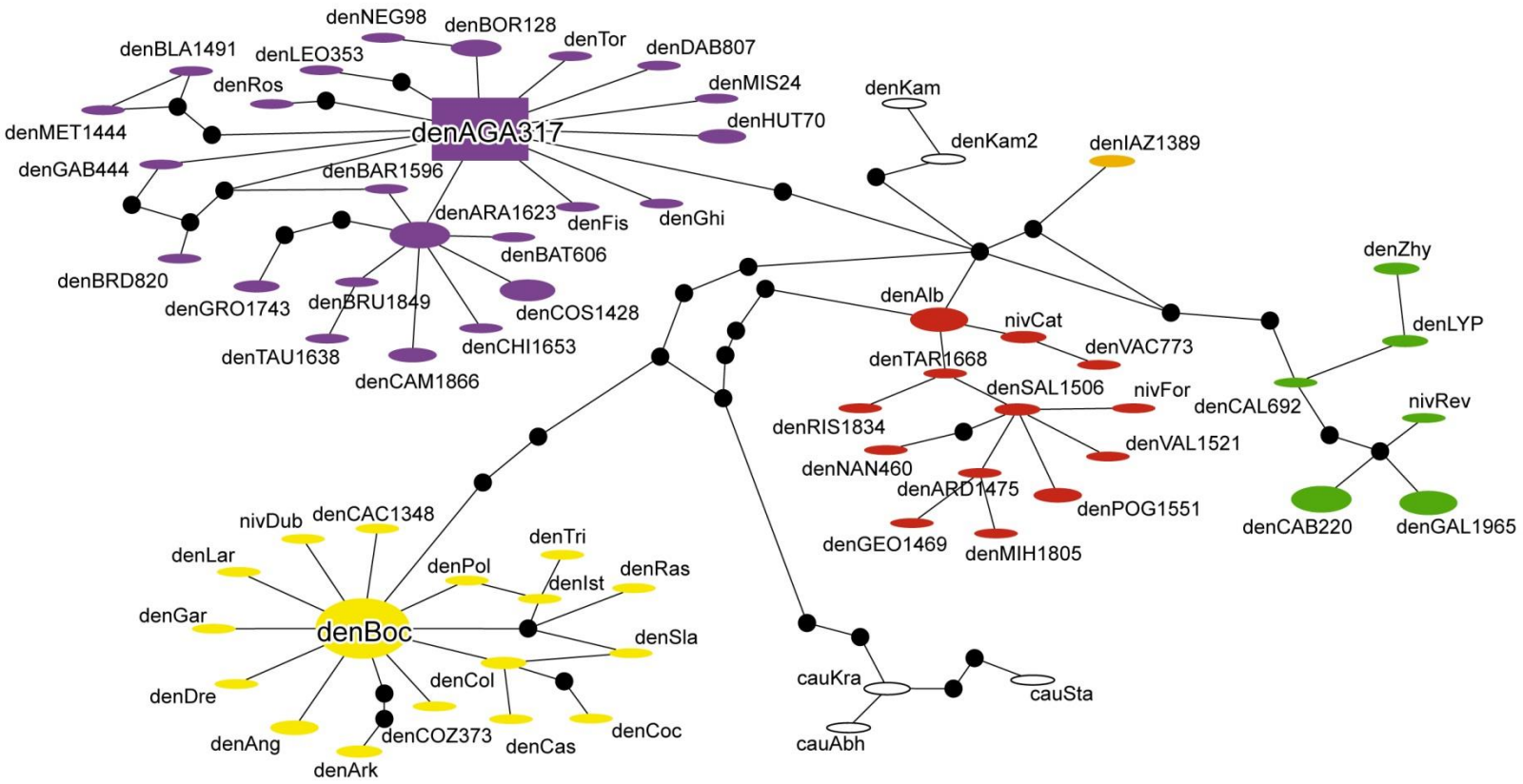

Fig. 3. Haplotype network of *Erythronium dens-canis.* Colours follow those from Fig. 5. Size of circles is proprtional with the number of samples they contain. Small black circles represent unsampled haplotypes.

4.3. Reconstruction of the phylogenetic trees based on plastid DNA sequences

4.3.1. *Erythronium dens-canis*

The analysis of phylogenetic tree generated by ML method (Fig. 4.) shows the existence of three major lineages: a non-Transylvanian lineage (nT – 18 samples from Romania), a Caucasian lineage and a Transylvanian lineage with several clades: t1, t2, t3, t4 and t5.

Erythronium sibiricum used as outgroup appears as sister group with a well supported trichotomy (bs 90 %), consisting of three well-supported clades (Caucasian, non - Transylvanian, and Transylvanian: bs 99 , 91 and 89, respectively). The Transylvanian clade presents a politomy, composed of 5 moderate to well supported clades: bs values 53-96.

Fig. 4. ML phylogram based on *rpl32-trnL* and *rps15-ycf1* plastid sequences of the *Erythronium dens-canis*

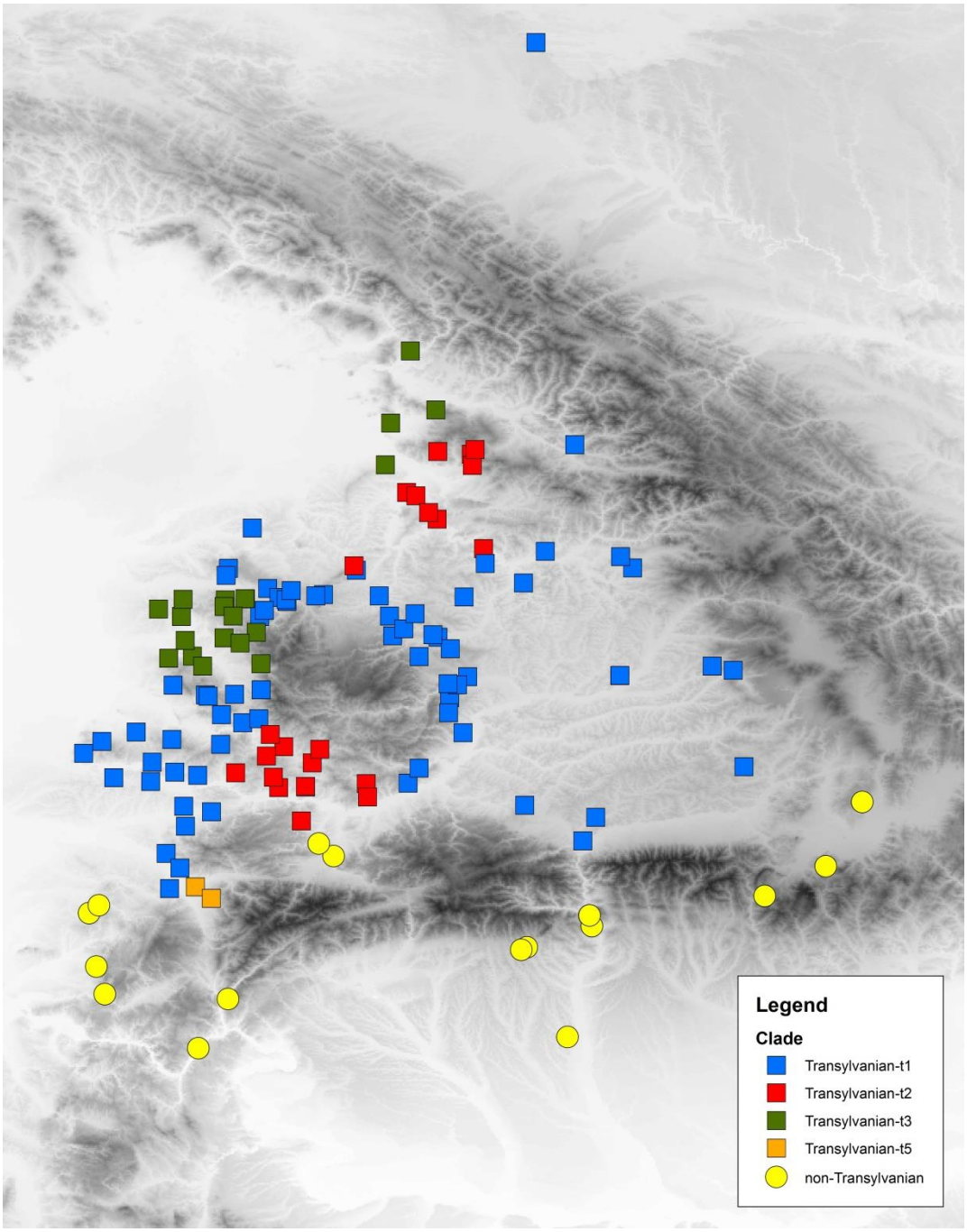

Fig. 5. Geographical distribution of the clades of *Erythronium dens-canis* in Transylvania

Close examination of clades taking into account the geographical distribution of the samples (Fig . 5) leads to the recognition that non-Transylvanian lineage, even if contains fewer samples is quite widespread. Samples belonging to this clade are spread in the Southern and Eastern Carpathians.

Caucasian lineage samples belong to the study of Bartha et al. (2015a).

The Transylvanian lineage is highly structured:

– In clade t1 a subclade is inserted between other samples. Samples belonging to this subclade have a remote distribution from most samples distributed in Transylvania. It lies Southwest of the Apuseni Mountains, while the remaining samples come from East and North of the Apuseni Mountains.

– A similar situation exists in case of clade t2, but with different geographical positioning. Here subclade t2 has a distant position, with samples from the South of the Apuseni Mountains, while the rest of the samples come from the North of the Apuseni Mountains.

– Clade t3 includes just a few samples, but here also we can identify two subclades. Samples belonging to the basal subclade are located in the North of the Carpathian Arch, in Ukraine , while those belonging to the distant subclade are only present in a small area of the Apuseni Mountains: in the vallies of Crişul Negru and Crişul Repede Rivers.

– Clade t4 includes two samples from Croatia (samples come from the study of Bartha et al. (2015a)).

– Clade t5 is composed of only two samples from two populations from Banat region.

4.3.2. *Scilla bifolia*

Four major plastid lineages were identified after generation ML phylogenetic tree of the samples analyzed, denoted A, B , C and D (Fig . 6). A supposed ancient clade (clade A), which appears as a sister clade to a politomy, formed by the clades B, C and D.

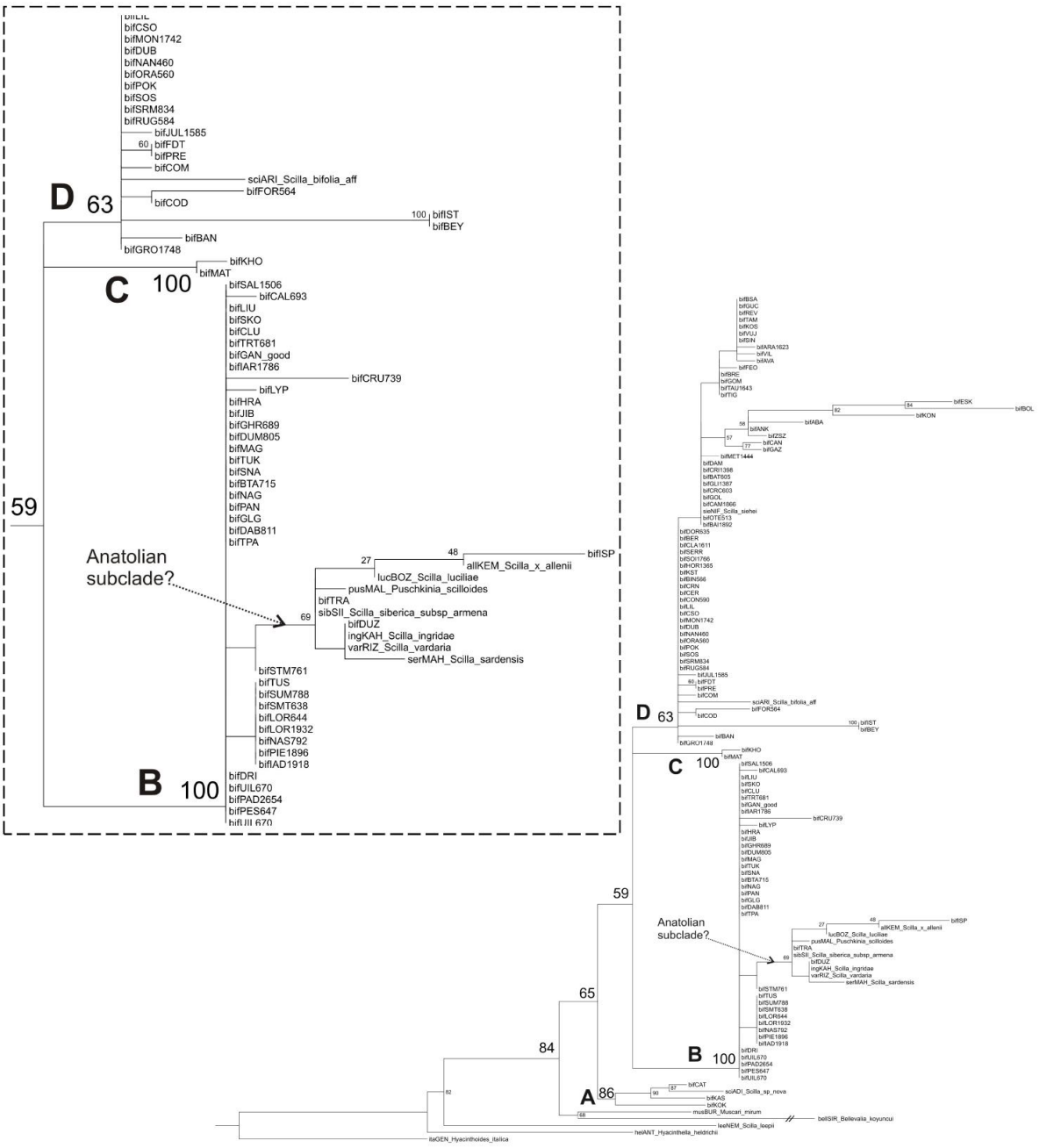

Fig. 6. ML Phylogram based on *rpl32-trnL* plastid sequences of *Scilla bifolia* s.l.

Analysing the geographical distribution of clades (Fig . 7), we can conclude that in this case, as in *E. dens- canis* clade B has Transylvanian origin. Clade C is the Caucasian one and clade D the non-Transylvanian one.

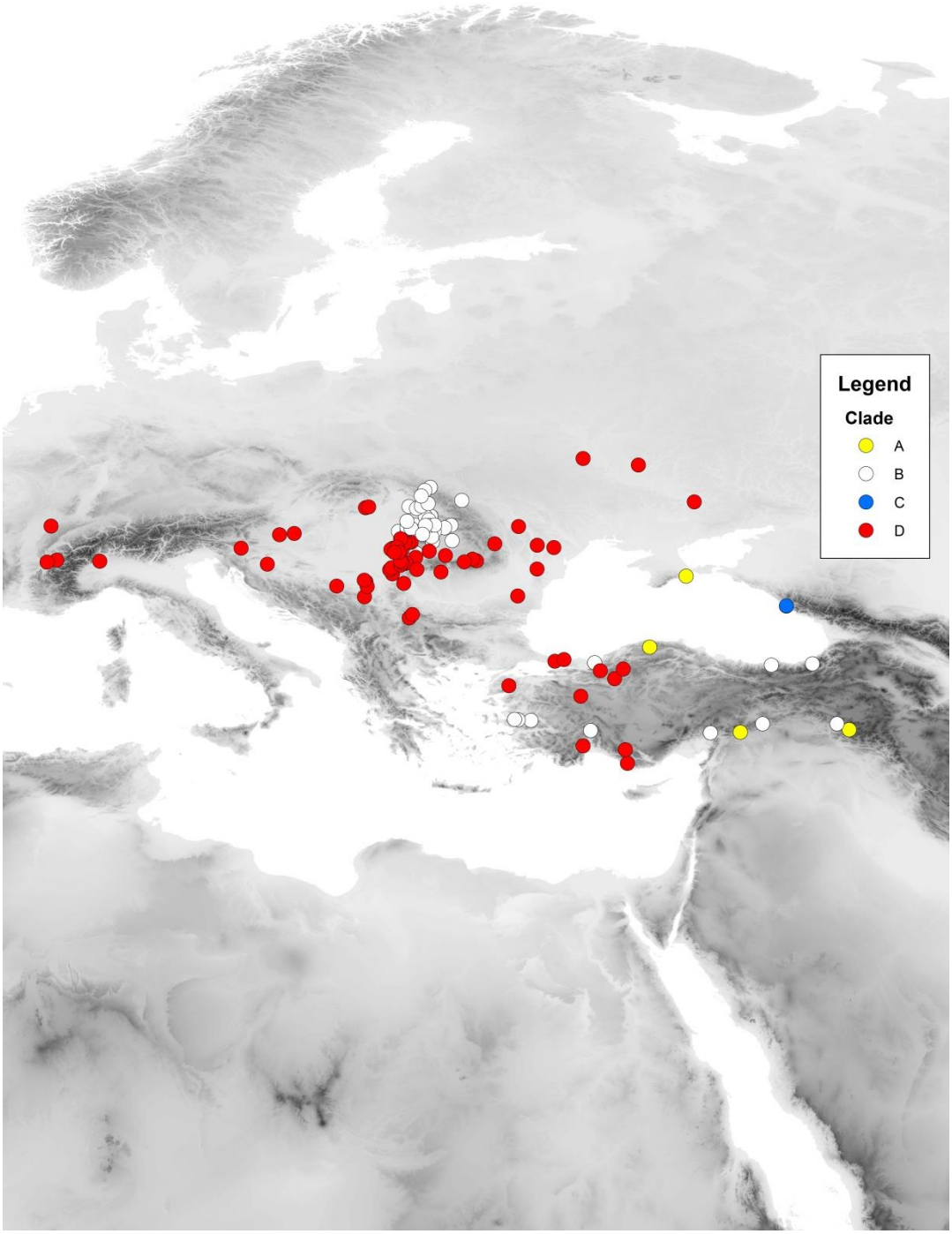

Fig. 7. Geographic position of different clades of *Scilla bifolia* s.l.

Clade A is composed of only 4 samples, 3 of which appear at South of the Black Sea and the fourth in the Crimean Peninsula.

Clade B includes all of the 37 samples from the interior of the Carpathian Arch, so this clade has a narrow distributional range. Clade B presents an Anatolian subclade, nested in the

Transylvanian samples. This subclade is composed mainly of the additional putative closely related taxa, but also of 3 samples of *S. bifolia*.

Clade C, the Caucazian one is composed of only two samples from Russia.

Clade D is the biggest clade, with large distributional range. It includes 64 samples of *S. bifolia* and two samples of putative closely related species.

4.4. Genetic diversity indices of the clades in case of E*rythronium dens-canis*

Table 1 presents the indices of genetic diversity calculated for the four specific clades of *Erythronium dens-canis* phylogeography. No calculations have been made for clades t4, t5 and Caucasian clade, because they comprise a very small number of samples.

Tab. 1. Genetic diversity indices of the clades of *E.dens-canis*

	Clades			
Diversity indices	**nT**	**t1**	**t2**	**t3**
Number of sequencies	42	66	23	21
Number of variable sites (S)	17	26	13	7
Number of haplotypes (h)	16	24	13	6
Haplotype diversity (Hd)	0.7	0.856	0.897	0.757
Nucleotide diversity (Pi)	0.00105	0.00136	0.00168	0.00166

5. DISCUSSION

5.1. *Erythronium dens-canis*

The structure of the identified lineages and clades can be explained by vicariance or by long distance dispersal.

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Lineage differentiation in European *Erythronium* appears to have been heavily influenced by allopatric divergence (Bartha et al., 2015a). Geographical barriers that have contributed to the formation of this lineage are the Pannonian Plain in the West, the Southern Carpathians in the South and the Eastern Carpathians in the East.

In case of clades t1-t4, in the absence of geographical barriers, long distance dispersal can give the explanation of their structure.

The nested position of a genetic line within clade t1, with a Southwest distribution of the Apuseni Mountains show that this subclade has formed later. Therefore, taking into account the geographical location of subclades we can conclude that clade t1 has formed East of the Apuseni Mountains, in a putative cryptic glacial refugia from the Eastern Transylvanian Basin, then due to long distance dispersal events it established and spread to the Southwest of the Apuseni Mountains (Fig. 8.).

In case of clade t2, as in the former one, we deal with crytpic refugia, but this time in North of the Apuseni Mountains, followed by a long distance dispersal South of the Apuseni Mountains (Fig. 9).

In clade t3 populations from the North of the Eastern Carpathians are the ancestral ones, those from the Crişul Repede and Crişul Negru Rivers` Vallies settled later, due to long distance dispersal (Fig. 10).

We can conclude therefore, that in case of *Erythronium dens-canis* the existence of the Transylvanian lineage gives evidence of the existence of extra-Mediterranean glacial cryptic refugia in the Eastern Carpathian Basin. According to the refugia-within-refugia theory (Gómez and Lunt, 2007) the highly structuring of this lineage with 4 clades, indicate the existence of microrefugia with unknown exact geographic position. These were located in East (t1 clade) and North (t2 clade) of Apuseni Mountains and in the Easterm Carpathians in Ukraine (t3 clade). From these microrefugia the species expanded its range in South-west and South of the Apuseni Mountains and in Crişul Negru and Crişul Repede Rivers` Vallies respectively, by long-distance dispersal.

The higher haplotype diversity in clade t1 and t2 (Table 1) shows that these clades were present with larger populations in LGM. The narrow actual distribution of clade t3 and the smaller probable dimension of population in LGM is underlined by the lower diversity of this clade than those of clades t1 and t2 (Table 1).

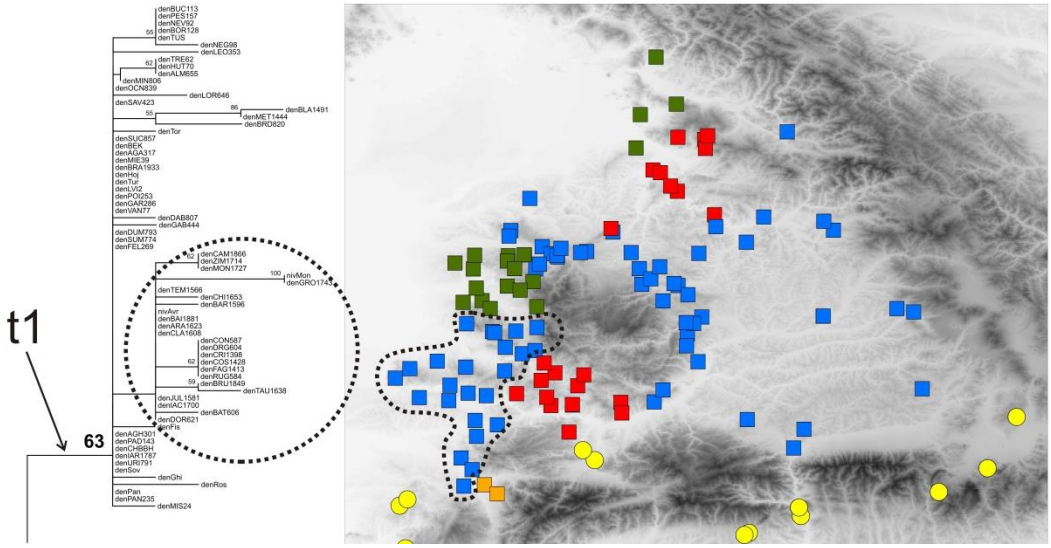

Fig. 8. Position of subclades t1 on the phylogram and their geographic position

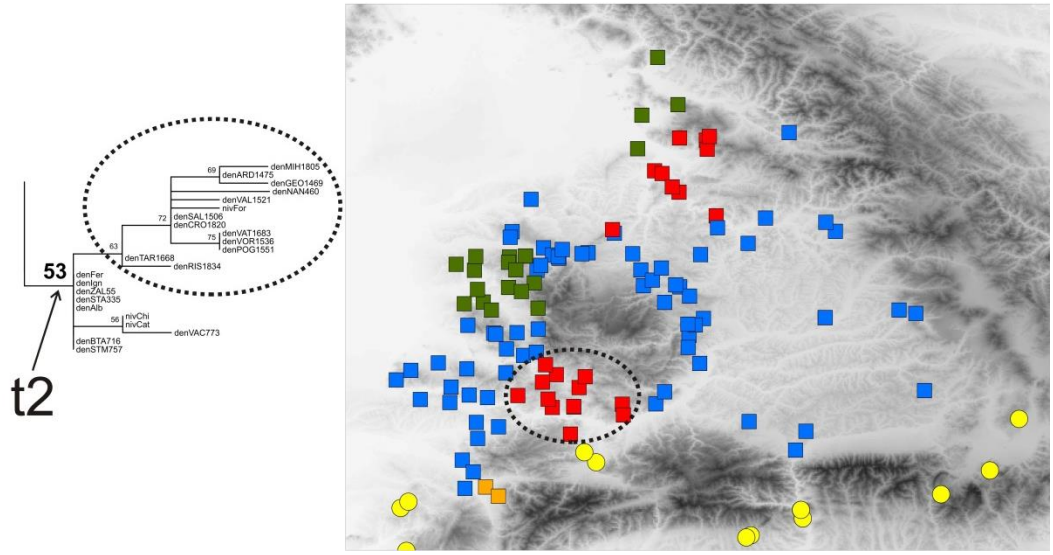

Fig. 9. Position of subclades t2 on the phylogram and their geographic position

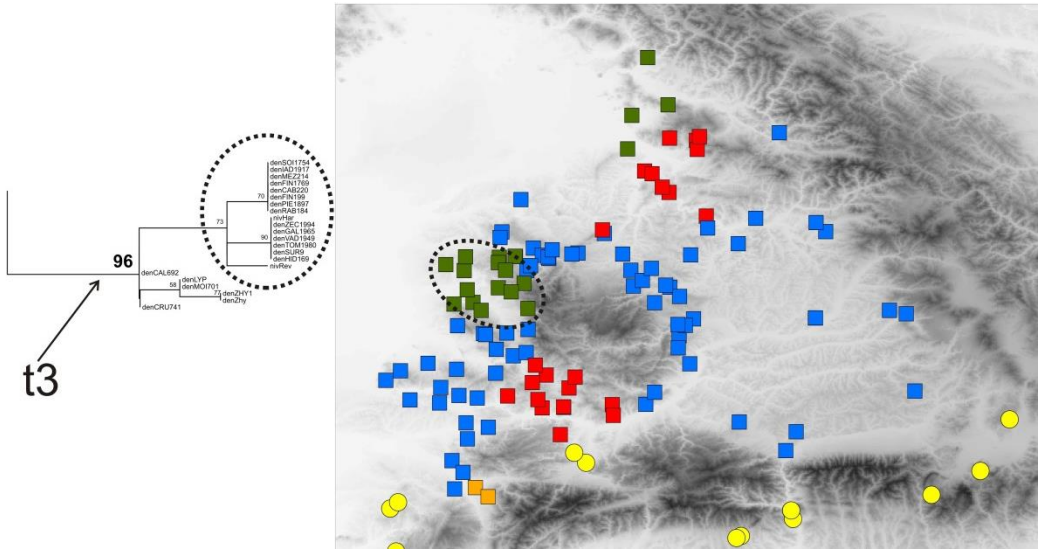

Fig. 10. Position of subclades t3 on the phylogram and their geographic position

Samples from clade t4 come from a population from Croatia – interesting is their presence in the Transylvanian lineage. According to Bartha et al. (2015a) this clade is a distinct evolutionary unit, and as a consequence has high conservation value.

All identified clades represent unique genetic lineages, and serve as phylogenetic conservation units. Clades t1 and t2 have the highest genetic diversity. Areas with high genetic diversity are important in conservation of a significant proportion of a species diversity and its evolutionary potential (Taberlet and Cheddadi, 2002). As a result, these two clades have high conservation value.

Since clade t5 has the most restricted distribution area, at the southern limit of the Transylvanian lineage, specimens of these populations have high conservation values, as well as populations from the Crişul Negru and Crişul Repede Rivers` Vallies.

5.2. *Scilla bifolia*

The factors explaining the structuring of the clades of *Scilla bifolia* are those presented at the former species: biogeographic factors (barriers, cryptic refugia) and stochastic events. The Transylvanian clade is precisely delimitated – the species survived the LGM in extra-Mediterranean cryptic refugia from the Eastern Carpathian Basin.

Clade B have an Anatolian subclade, nested in the Transylvanian samples, suggestive of its secondary establishment in Asia Minor. There may be two different explanations for the

formation of these taxa in Anatolia: 1. processes of radiative evolution, after the establishment of specimen in Anatolia; 2.these new taxa suffered introgressive chloroplast capture. In the current state of research on the *Scilla bifolia* s.l. we can not decide which of these two explanations is correct.

6. CONCLUSIONS

- Phylogeography structure of the two species is clear.
- The two studied species present highly similar structure. For both species topology shows clearly the existence of a narrowly distributed Transylvanian lineage limited in the Eastern Carpathian Basin. The existence of these lineages gives evidence of the existence of cryptic glacial extra-Mediterranean refugia in the studied region.
- In case of *E. dens-canis* the Transylvanian lineage presents four distinct clades, which, according to the refugia within refugia theory indicate the existence of microrefugia with unknown exact geographic position. These were located in East (t1 clade) and North (t2 clade) of Apuseni Mountains and in the Ukrainian Carpathians (t3 clade). From these microrefugia the species expanded its range in South-west and South of the Apuseni Mountains and in Crişul Negru and Crişul Repede Rivers` Vallies respectively, by long-distance dispersal.
- The Quaternary history of *E. dens-canis* in the Carpathian Basin was affected both by biogeographic factors (microrefugia, barriers – formation of the Transylvanian lineage) and by stochastic events (long-distance dispersal – formation of the clades).
- In case of *Scilla bifolia* four genetic lineages were identified: an ancient lineage, one of Transylvanian origin (with restricted distribution), a Caucasian one and a non – Transylvanian one (with wide distribution).
- The Transylvanian lineage has a nested Anatolian subclade (of secondary origin in Asia Minor) within the samples from Transylvania. There may be two different explanations for the formation of these taxa in Anatolia :
    1. processes of radiative evolution, after the establishment of specimen in Anatolia

2. These new taxa suffered introgressive chloroplast capture. In the current state of research on the *Scilla bifolia* s.l. we can not decide which of these two explanations is correct.

- All identified clades are unique and therefore serve as phylogenetic conservation units.
- Clades t1 and t2 have the greatest genetic diversity. As a consequence, these clades have the greatest chance to have evolutionary potential and ability to adapt to future climate change conditions. Therefore populations belonging to these clades have high conservation values.
- t5 clade from southern Banat is a very small one and it is situated at the Southern limit of the Transylvanian lineage. Therefore, these are probably the most endangered populations under the ongoing climate change.
- The populations from Crişul Negru and Crişul Repede Rivers` Vallies from t3 clade have a narrow distribution, and therefore have high conservation values.
- Currently, the lowland populations have become rare due to vast woodland habitat replacement with farmland. Mass deforestation of forests from lowland to mountain areas can lead to fragmentation of populations, decreasing their size (with all the negative consequences on population genetics) and finally to their extinction.

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8. TABLE OF CONTENTS OF THE PHD THESIS