Bartha L., Sramko G., Volkova P.A., Surina B., Ivanov A.L., Banciu H.L. Patterns of plastid DNA differentiation in *Erythronium* (Liliaceae) are consistent with allopatric lineage divergence in Europe across longitude and latitude // Plant Systematics and Evolution. 2015. Vol. 301. P. 1747–1758.

Patterns of plastid DNA differentiation in *Erythronium* (Liliaceae) are consistent with allopatric lineage divergence in Europe across longitude and latitude

László Bartha[1,6], Gábor Sramkó[2], Polina A. Volkova[3], Boštjan Surina[4], Alexander L. Ivanov[5], Horia L. Banciu[1,6]

[1]Laboratory of Molecular Environmental Biology, Molecular Biology Center, Institute for Interdisciplinary Research in Bio-Nano Sciences, Babeş-Bolyai University, 42 August Treboniu Laurean Street, 400271 Cluj-Napoca, Romania

[2]MTA-ELTE-MTM Ecology Research Group, Pázmány Péter sétany, 1/C, H-1117, Budapest, Hungary

[3]Moscow South-West High School N 1543, 26 Bakinskikh komissarov Street, 3-5, 119526, Moscow, Russia

[4]Faculty of Mathematics, Natural Sciences and Information Technologies, University of Primorska, 8 Glagoljaška Street, SI-6000, Koper, Slovenia

[5]Department of Botany, North Caucasian Federal University, 1 Pushkin Street, 355009, Stavropol, Russia

[6]Faculty of Biology and Geology, Babeş-Bolyai University, 5-7 Clinicilor Street, 400006, Cluj-Napoca, Romania

Corresponding author: László Bartha, lbartha.ubbcluj@yahoo.com

Running title: Chloroplast phylogeography of the genus *Erythronium* in Europe

Abstract

Little attention has been paid so far to the genetic legacy of the oceanic-continental gradient across Europe. Due to this gradient steppe regions become more extensive and mesic environments become scattered towards East. The temperate mesophilic plant, *Erythronium dens-canis* (Liliaceae), is widespread in southern Europe with a distribution gap in the Pannonian Plain. Moreover, the large disjunction between *E. dens-canis* and its sister species, *E. caucasicum*, seems to partially overlap the Pontic steppe regions. By applying range-wide sampling to *E. dens-canis* and limited sampling of *E. caucasicum*, we explored their chloroplast DNA phylogeography using the plastid regions *rpl*32-*trn*L and *rps*15-*ycf*1. A striking phylogeographic structure emerged based on three major phylogroups: a Caucasian lineage, a highly structured and narrowly distributed Transylvanian lineage, and a more homogenous and widely distributed 'non-Transylvanian' lineage. Both physiographic (mountain) and climatic (steppe) barriers appear to have caused allopatric differentiation in European *Erythronium*. The Southern Carpathians constitute a latitudinal barrier and the Pannonian Plain a longitudinal barrier between the Transylvanian and 'non-Transylvanian' lineages of *E. dens-canis*, whereas the Eastern Carpathians and the Pontic steppe regions act as a longitudinal barrier between the *E. dens-canis* and *E. caucasicum lineages*. The eastern Carpathian Basin likely functioned as a combination of cryptic eastern (mesic) and cryptic northern refugia for *E. dens-canis* during glacial periods or at least during the Last Glacial Maximum. Subclades of the Transylvanian lineage conform to the refugia within refugia scenario. Two geographically deviating haplotypes of the Transylvanian lineage were recovered from a population close to the Adriatic coast and form a separate subclade likely resulting from ancient long-distance dispersal. The results reinforce the role of the Carpathian Basin in promoting differentiation in temperate species and serving as a glacial refuge for

them. Steppe-dominated gaps in the range of the genus *Erythronium* are mirrored by genetic discontinuities along longitudes thus highlighting a biogeographic role of the oceanic-continental gradient throughout Europe.

Keywords: Carpathian Basin, cryptic northern refugia, long distance dispersal, oceanic-continental gradient, phylogeography, temperate species

Introduction

The Quaternary history of temperate European biota is currently viewed in the light of two main theories. The ‘southern richness versus northern purity’ paradigm was the leading theory of the 1990s to explain the location of glacial survival and postglacial recolonisation of temperate European species. This model assumes survival in southern European (i.e. Iberian, Apennine and Balkan) peninsulas during Pleistocene glacial times which often led to genetic differentiation of species along geographical longitudes mirroring these peninsulas . The above model also postulated a northward expansion and postglacial recolonisation of deglaciated areas resulting in genetically more depauperate populations in the north relative to those in the south due to the leading edge effect . More recently, another theory has gained ground, namely that of ‘cryptic northern refugia’ of temperate species during glacial periods . This theory envisages glacial survival in ‘microclimatically favourable pockets’ at higher geographical latitudes along widespread European longitudes such as Western Europe , Central Europe and the Carpathian region . Exact locations, as well as, boundaries of such northerly refugial areas, however, are poorly understood especially for Central-East Europe partly because of the paucity of (at least) densely sampled studies and partly because of the post-glacial dispersal of species potentially blurring ancient refugial patterns.

Alpine plant phylogeography covering several European mountain ranges in the last decade usually had a strong longitudinal component (e.g. . Contrary to this, temperate plant phylogeography has rarely uncovered longitudinal differentiation patterns in a wide East-West dimension across areas north of the southern European peninsulas. Thus, it is still poorly understood to what extent the oceanic-continental gradient impacted the biogeography of temperate species in Europe during the Quaternary. Due to this gradient, steppic regions (e.g. Pannonian Plain, Pontic steppe region) become more extensive and mesic environments

85become more scattered towards East. Since the steppes were even more extensive during
86Quaternary glaciations , it can be hypothesised that they acted as barriers over longer times
87for mesophilic species, so that the main phylogeographic splits should follow them.
88According to our knowledge nobody has yet tested this hypothesis using plant species.

89 In this study we aimed to explore the genetic structure of a temperate plant species
90across a wide East-West dimension. More specifically, we wanted to test whether a species
91which can be found in mesic environments may have been distributed disjunctly in cryptic
92mesic refugia separated during glacial periods by regions characterised by more continental
93climate. To test this hypothesis we studied the Dog's tooth violet (*Erythronium dens-canis*
94L.), a typical mesic deciduous forest geophyte, for the following reasons: (i) the species is
95widespread in Europe from the Atlantic coast to western Turkey with distribution gaps in
96(ecologically unsuitable) steppe regions (Hungarian Plain and Pontic steppe regions). (ii) *E.*
97*dens-canis* is a myrmecochorous species with a consequent limited seed dispersal capacity
98and limited seed dispersal supports long-term preservation of phylogeographic structure.

99 In our study we specifically addressed the following questions: (1) Does *E. dens-canis*
100exhibit a phylogeographic structure across its wide longitudinal distribution in Europe? If so,
101is it likely that the Pannonian gap in the species range, as well as, the large geographical
102disjunction between *E. dens-canis* and its sister species, *Erythronium caucasicum* Woronow,
103contributed to this structure? (2) Does *E. dens-canis* exhibit a pattern of genetic differentiation
104reflecting survival in cryptic northern glacial refugia? If so, could the boundaries (or at least
105the southern limit) of such northern refugia be traced? In an effort to answer these questions
106we surveyed nucleotide DNA sequence variation in the plastid *rpl*32-*trn*L and *rps*15-*ycf*1
107intergenic spacer (IGS) regions among *E. dens-canis* populations sampled from most of the
108species' range and additionally among a limited number of *E. caucasicum* samples.

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Materials and methods

Plant sampling

Leaves of *E. dens-canis* and *E. caucasicum* were collected from 52 and four populations, respectively (Table 1, Fig. 1). In the case of *E. dens-canis*, samples were collected from throughout much of its distribution. One sample per population was analysed except for the Croatian population “18” for which confirmatory analysis was necessary from a second sample (see Results section). Leaf samples were dried/stored either in silica gel, alcohol or by being pressed between absorbent paper. Voucher specimens (where appropriate) are stored in herbaria BC, CL, NHMR, SANT, ZAGR (Table 1). Three ‘distant outgroups’, *Erythronium sibiricum* (Fisch. et C.A.Mey.) Krylov, *Erythronium japonicum* Decne and *Amana edulis* (Miquel) Honda, were selected (Table 1) based on the results of previous large-scale phylogenetic studies .

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from dried leaf fragments using the ZR Plant/Seed DNA kit (Zymo Research). Scarcelli et al. tested a set of 100 primer pairs in 13 species of Monocotyledons and observed that the most variable loci were located in the Small Single Copy (SSC) region of the plastid genome. We tested seven primer pairs out of those used by Scarcelli et al. , which flanked DNA regions that were located in the SSC and contained intronic or intergenic spacer portions. The seven regions tested were as follows: *rps*15-*ycf*1 IGS, *ycf*1-*rrn*5 IGS, *ndh*A Intron, *ndh*G-*ndh*I IGS, *psa*C-*ndh*G IGS, *ccs*A-*ndh*D IGS, *ndh*F-*rpl*32 IGS. Additionally, we tested the region *rpl*32-*trn*L IGS with the primers of Shaw et al. . After the screen of these regions in a preliminary sample set we selected the *rpl*32-*trn*L IGS and *rps*15-*ycf*1 IGS regions for subsequent phylogeographic analysis. Polymerase chain reaction (PCR) was performed in 25 µl reaction volumes containing 12.5 µl 2x MyTaq Red

Mix (Bioline), 8.5 µl dd water, 1 µl of each primers (10 µM) and 2 µl DNA-template solution of unknown concentration. DNA amplification was performed in a Gradient Palm-Cycler™ (Corbett Research) using the following parameters: an initial 4 min denaturation at 94 °C, followed by 40 cycles of 30 sec at 94 °C, 45 sec annealing at 61 °C, 45 sec extension at 72 °C, and a final extension for 7 min at 72 °C. Success of PCR was checked by agarose-gel electrophoresis. PCR products were column-purified using the PCR Purification Kit of Jena Biosciences. Sequencing was performed by Sanger method at Macrogen Inc. (The Netherlands) using the reverse primer for the region *rpl*32-*trn*L and the forward primer for the region *rps*15-*ycf*1. Sequence reads were clear and unambiguous in all cases except for four *rpl*32-*trn*L samples where additional sequencing efforts were needed with the forward primer to reconstruct the whole region.

Haplotype calling and their parsimony network reconstruction

Sequences were aligned manually in MEGA5.2 . One inverted motif (TATTCTAT) randomly occurred in outgroup and ingroup *rpl*32-*trn*L sequences whereas one inversion (ATGTTTGAAATA) occurred in the *Amana edulis rps*15-*ycf*1 sequence. These were reverse-complemented prior to any analyses. Because exploratory phylogenetic analyses suggested an unresolved relationship between *E. caucasicum* and certain intraspecific groups of *E. dens-canis*, definition of haplotypes was based on these two species in concert. Haplotype calling and the simultaneous parsimony network reconstruction was performed in TCS at 95% connection limit without taking into consideration gaps and structural mutations due to their putative homoplasious nature.

Phylogenetic tree and molecular clock analyses of haplotypes

After haplotype definition, alignments were made with sequences of *E. sibiricum*, *E. japonicum* and *A. edulis* for phylogeny reconstruction. Phylogenetic tree analyses were based on maximum likelihood, parsimony and Bayesian criteria. Because preliminary phylogenetic

analyses based on individual *rpl*32-*trn*L and *rps*15-*ycf*1 datasets yielded congruent topologies, incongruence between these was not tested statistically. Maximum likelihood (ML) analysis was based on RAxML using the RAxML GUI version 1.2 under the GTR + Γ model of sequence evolution (as recommended 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 . Maximum parsimony analysis was run in *PAUP** and relied on heuristic search using 1000 random addition of sequence replicates and TBR branch swapping with MULTREES option in effect, MAXTREES set to 15,000 (without possibility of increasing the tree buffer) and a limit of ten trees retained for each iteration step. The statistical robustness of tree branches was estimated via bootstrapping; 1000 pseudo-replicates were performed in *PAUP** with MAXTREES re-set to 1000 and with the retention of one tree per replicate. Prior to Bayesian analysis, MrModeltest v2. was used to select the nucleotide substitution models (under the Akaike Information Criterion) which best fitted each plastid dataset (Suppl. Table S1). Bayesian analysis relied on a partitioned dataset and involved two simultaneous runs of 6,000,000 generations of Monte Carlo Markov chains by saving every one thousandth tree and with chain heating parameter set to 0,2. Each run employed four simultaneous chains. After checking convergence in Tracer 1.5 , i.e. effective sample sizes (ESS) were >5000, a 50% majority-rule consensus phylogram was generated in MrBayes 3.2 with a ‘burn-in’ of 1500 trees (25%).

To obtain a proxy on the timing of coalescence of the main *E. dens-canis/E. caucasicum* lineages, we performed synthetic dating across a range of substitution rates (0,001–0,005 substitutions/site/my) that had been tentatively selected in similar studies . Our slowest rate (0,001 subst/site/my) is relatively close to the rate (0,0008 subst/site/my) calculated by Yamane et al. (2006) for indel evolution in certain monocot species and arbitrarily applied by de Lima et al. (2014) in the monocot genus *Mauritia*. We opted for this rate-exploratory

approach because there was a lack of information on the age of the split between *Amana* and *Erythronium*, or indeed pollen or macrofossil evidence for *Erythronium*, which could have been used for calibrating substitution rates more directly. Dating relied on Beast v.1.8.1 , using a strict clock method and the substitution models already applied in case of the MrBayes analysis. We assumed a strict clock model because a pilot analysis using an uncorrelated lognormal relaxed clock model resulted in a value of the ucld.stdev parameter close to zero (0.2), a strategy that is commonly encountered in the literature. Input data files were created in BEAUti v.1.8.1 and included only the *E. dens-canis* and *E. caucasicum* haplotype sequences. A coalescent tree prior was used and rates were set as priors. Markov chain Monte Carlo (MCMC) chains were run for 10 million generations, sampling every 1000th generation. Log files were analysed using TRACER v1.6.0 to assess convergence and confirm that effective sample size for all parameters was above 200. A maximum-credibility tree was produced using treeAnnotator v.1.8.1 with a burn-in of 2500 trees.

Molecular diversity and population demographic analyses of phylogroups

DnaSp 5 was used to estimate indices of genetic diversity (number of haplotypes, h; haplotypic diversity, Hd; number of polymorphic sites, S; and nucleotide diversity, π) for the main intraspecific groups of *E. dens-canis*. To detect possible signs of a past demographic expansion, we calculated Tajima's D and Fu's F_S statistics in Arlequin 3.5 . Evidence for population expansion may come from significantly negative values of D and F_S . Significance was determined by a permutation test of 1000 permutations in Arlequin.

Results

A total of 122 *rpl*32-*trn*L and *rps*15-*ycf*1 sequences were newly generated for the study. Summary statistics of sequences are presented in Electronic Supplementary Material 1. Sequence alignment file used for the phylogenetic analysis is available in TreeBASE under the study number 16526. GenBank accession numbers of sequences are listed in Table 1.

Number, structuring and distribution of haplotypes

TCS analysis retrieved 29 *E. dens-canis* and three *E. caucasicum* haplotypes (Table 1, Fig. 2). The haplotype network showed pronounced structuring (Fig. 2). Combining information on the geographic origin of haplotypes and the network topology led to the recognition of three main haplogroups: (i) a Caucasian group (*E. caucasicum*), (ii) a group containing haplotypes broadly from most of the distribution range of *E. dens-canis*, i.e. from the Atlantic coast to the southern slopes of Southern Carpathians, but excluding most samples from Transylvania, (iii) a Transylvanian group which also contained two haplotypes (H23, H24) from 'non-Transylvanian' samples (i.e. from a single Croatian population, pop. "18") (Figs. 1 and 2, Table 1). The 'non-Transylvanian' haplogroup is typically star-shaped with the most frequent central haplotype, H1 found throughout the entire range, connected through few mutational steps to more rare haplotypes (Fig. 2). The Transylvanian haplogroup exhibits a different pattern and is markedly more structured than the 'non-Transylvanian' one. The relationship of the Caucasian haplogroup to the Transylvanian and 'non-Transylvanian' haplogroups was not resolved by the network analysis.

Haplotype phylogeny and molecular clocks

All of the methods used in phylogenetic reconstruction yielded highly congruent topologies. A Bayesian majority-rule consensus phylogeny is presented in Fig. 3. *Erythronium sibiricum* branches before a well supported clade that includes an unresolved trichotomy formed by *E.*

230*caucasicum*, and the Transylvanian and ‘non-Transylvanian’ lineages of *E. dens-canis*.
231Whereas relationships between haplotypes within the ‘non-Transylvanian’ lineage of
232haplotypes were mostly unresolved, in the Transylvanian lineage there was a polytomy of
233four moderately to well-supported clades (t1-t4, Fig. 3) three of which were confined to
234Transylvania (Fig. 1c), whereas the fourth (t4) comprised the two haplotypes found in the
235Croatian population “18” (Fig. 1a). Repeat analyses of samples from this Croatian population
236(from DNA extraction to sequence generation) were conducted at a different laboratory
237(University of Debrecen) and yielded the same results.
238Exploratory dating of the phylogeny indicated that the Caucasian, Transylvanian and ‘non-
239Transylvanian’ lineages split from their common ancestor between 0.93 Mya (95% HPD
240interval 0.63 to 1.33 – assuming the fastest rate) and 4.66 Mya (95% HPD interval 3.12 to
2416.66 – assuming the slowest rate) (Electronic Supplementary Material 2).
242***Lineage diversity and population demographics***
243Indices of genetic diversity (Hd, S, π) and population demographic statistics for the
244recognised lineages within *E. dens-canis* are summarized in Table 2. The Caucasian clade was
245omitted from these tests because of its small sample size. Genetic diversity was considerably
246higher in the Transylvanian than in the ‘non-Transylvanian’ lineage despite the lower sample
247size of the former (Table 2), while Tajima’s D and Fu’s F_S statistics were negative and
248significant only for the ‘non-Transylvanian’ lineage (Table 2).

26

Discussion

Allopatric lineage divergence in European Erythronium

Lineage differentiation in European *Erythronium* appears to have been heavily influenced by allopatric divergence (vicariance). Thus, on the basis of the geographical distribution of haplogroups (Fig. 1), the Eastern and Southern Carpathians, as well as the Pannonian Plain, would seem to have greatly impacted the formation of the Transylvanian lineage, whereas the Ukrainian and Russian (Pontic) steppes were likely to have acted as geographical barriers in the origin of *E. caucasicum*. During Quaternary glaciations a more pronounced oceanic-continental gradient existed across Europe than today which manifested in an extended ‘steppe-tundra’ biome . Steppe regions most likely acted as dispersal barriers for mesic species as evident here from major phylogeographic splits even if the (current) extent of a barrier not necessarily correlates with the degree of genetic divergence (see also Surina et al. (2014)). Similarly, the partially glaciated Southern Carpathians undoubtly also acted as dispersal barriers for temperate species. Such climatic (steppe) and physiographic (mountain) barriers likely acted synergistically in the Eastern Carpathian Basin leading to a combination of cryptic northern and cryptic eastern mesic glacial refugia for the temperate and moisture-needing *E. dens-canis*.

There is currently a lack of other good examples showing genetic breaks of mesic species along ecologically unsuitable steppe regions in Europe such as we have found in *Erythronium.* As far as a north-south genetic division along a physiographic barrier is considered (like in case of the Southern Carpathians), a study by Huck et al. (2009) using AFLPs revealed a genetic division along the Alps in the temperate species *Meum athamanticum* Jacq.

In our study Beast analysis provided a broad time frame (from the early Pliocene to the mid-late Pleistocene) in which a most common ancestor of *E. dens-canis* and *E. caucasicum* lived

(Electronic Supplementary Material 2). This prevented us from making precise estimates of the age of lineages of European *Erythronium*. In any case, establishment of these lineages might be linked with the disruption of the temperate, broad-leaved (mesophytic) forests which were once widely distributed across the Northern Hemisphere and became fragmented during aridification in the late Tertiary and during Quaternary glaciations . The genus *Erythronium* has already been mentioned in this context by Allen et al. (2003).

Quaternary glacial survival in the Eastern Carpathian Basin

Since Stewart and Lister (2001) introduced the 'cryptic northern refugia' concept, the number of studies dealing with extra-Mediterranean refugia of temperate animal and plant species in Europe has continually increased . Some of the plant species for which cryptic northern refugia has been previously suggested include *Fagus sylvatica* L. , *Meum athamanticum* Huck et al. (2009) or *Cyclamen purpurascens* Mill . The present study adds to the growing body of evidence by reporting a deep genetic lineage almost exclusively confined to a well delimited region (the Eastern Carpathian Basin) that can be best interpreted as an *in situ* glacial extra-Mediterranean refugium for a temperate forest herb occurring at a relatively high latitude. The Transylvanian lineage of haplotypes contained three well-supported subclades, t1-t3, suggesting a refugia-within-refugia scenario in the Carpathian Basin. Indices of genetic diversity that in the Transylvanian lineage are higher than in the 'non-Transylvanian' lineage (both in term of haplotype and nucleotide diversities (Hd, π) (Table 2)) might also be linked with a multiple microrefugia concept. The higher diversity indices in the Transylvanian lineage, however, may also be due to long-term large ancestral population size. The locations of any potential microrefugia, as well as the putative partitioning role of the Apuseni Mountains among lineages t1-t3 (Fig. 1c) remain to be established using denser sampling of populations with multiple samples collected per population.

Previous studies addressing cryptic northern glacial survival of temperate plants in Europe have neglected comprehensive sampling in the Eastern Carpathian Basin despite its particular physiographic feature. However, a study by Schmitt et al. (2007) reported evidence of a differentiation centre for the animal species, *Erebia medusa* (D. & S.), in the eastern part of the Carpathian (i.e. Transylvanian) Basin, which likely resulted from the glacial survival of this species there during the LGM. In future, more studies, particularly on plants, should focus on this part of Europe and especially in the surroundings of the Apuseni Mountains where cryptic lineages with high conservation value might prevail in greater abundance than previously thought.

The southern counterpart of the Transylvanian lineage

In an attempt to explain the genetic homogeneity of the 'non-Transylvanian' lineage of *E. dens-canis* we pose two hypotheses that are not mutually exclusive from each other. On the one hand, this lineage may have experienced past demographic expansion and may have gone through bottlenecks in the course of such (possibly westward) expansion as suggested by the estimated population demographic indices (Table 2). On the other hand, a large population size of the lineage may have tempered its genetic differentiation . The current dataset does not allow us to distinguish between these phenomena as possible causes of the lack of genetic structure in the lineage. Additionally, we are aware of the fact that the low dispersal capability cannot be fully reconciled with the range expansion hypothesis. A future study should investigate whether intra-populational genetic diversity shows any gradient along geographical longitudes in this widespread species. A westward expansion hypothesis would be supported by a westward decline of genetic diversity as commonly found in Mediterranean species . Despite not showing any particular structuring associated with possible refugia, populations of the 'non-Transylvanian' lineage of *E. dens-canis* most probably survived the Last Glacial Maximum (LGM) in multiple 'southern' refugia (i.e. South of the Alps, the

Pannonian Lowlands and the Carpathians) as suggested for the ecologically similar *Cyclamen purpurascens* for which the foothills of the Southern Limestone Alps and the karst area of North-Western Dinarides were inferred as primary refugial areas.

Origin of the deviant Adriatic Dog's tooth violet population and implications to conservation

Contrary to the possibility of the Transylvanian and 'non-Transylvanian' lineages of *E. dens-canis*, as well as subclades t1-t3 of the Transylvanian lineage, originating by allopatric divergence, we here suggest that the occurrence of the deviant Adriatic *E. dens-canis* population is best viewed as a consequence of long distance dispersal. In support, the geographic coherence of the Transylvanian lineage is clear and this lineage has been reasonably well sampled in the Eastern Carpathian Basin without recovering any t4 clade-like haplotype from there.

Despite the proximity of this population to the ancient settlement Rijeka, we consider that humans did not contribute to its founding. In our opinion, if a human-mediated origin had occurred, haplotypes 23 and 24 should constitute the terminals of the t1, t2 or t3 clades, but this was not the case. By taking into consideration the generally conservative nature of chloroplast DNA evolution, the establishment of the t4 lineage should have preceded the LGM. Additionally, plants of this population thrive in perfectly natural vegetation on slopes of a karst doline (N 45.38537° E 14.535749° (WGS)) adding further support to the hypothesis of non-human introduction.

We propose that clade t4 is a distinct evolutionary unit with high conservation value. *Erythronium dens-canis* is a protected species in Croatia which may facilitate implementation of certain conservation strategies. Médail and Diadema (2009) have emphasized the importance of identifying areas for plant conservation at the population level in northern parts of the Mediterranean (e.g. in Adriatic coast) and our study has done this for *E. dens-canis*.

34

348***Concluding remarks***

349The present study documents two biogeographical oddities among the three equally ranked
350lineages of the temperate European *Erythronium*. The first concerns the genetic splits between
351*E. caucasicum* and lineages of *E. dens-canis*. The split between *E. dens-canis* and *E.*
352*caucasicum* is of similar magnitude (in terms of number of substitutions) to that between the
353lineages of *E. dens-canis* from inside and outside the Carpathians. This phenomenon (i.e. the
354depth of genetic breaks does not correlate with the strength of geographic barriers) has been
355documented previously in arctic-alpine species in this part of Europe . The second oddity
356concerns the fact that the Transylvanian lineage is much more structured than the 'non-
357Transylvanian' lineage despite the former having a much smaller range than the latter. This
358highlights the fact that interconnectivity of populations could be more limited at the margins
359of the species' area than at the species 'optimal' range especially during unfavorable (e.g.
360glacial) conditions.

361Our results exemplify the recognition of Stewart et al. (2010) according to which longitudinal
362and traditional latitudinal gradients have acted in concert to create refugia for species. The
363Eastern Carpathian Basin likely provides an example for this phenomenon by combining
364signs of cryptic eastern mesic ('oceanic') and cryptic northern glacial refugia.

365Although this study has broadened our knowledge of the Quaternary history of temperate
366European biota, future studies should test whether the patterns revealed here are generally
367found across ecologically similar taxa. We further suggest that future studies should focus on
368similar forest geophytes with high ecological amplitude, limited dispersal capacity and wide
369distribution range.

36

370**Acknowledgements**

371

372The authors are grateful to Richard Abbott (University of St. Andrews) for his scientific
373comments and linguistic improvements on the manuscript. The constructive comments of the
374two anonymous reviewers on the manuscript also are appreciated. Special thanks go to the
375following persons for their help in providing plant material (herbarium curators have
376herbarium codes in parentheses): Ettore Balocchi, Sergey Banketov, Csaba Bartha, Attila
377Bartók, Sorina Bartok, Sandro Bogdanović, Paolo Caciagli, Ioana Ciortan, Mihály
378Debreczeni, Simon Dubreul, Darko Ermenc, Xermán García Romai, Schivo Gianluca, Nicole
379Grau, Zsolt Hegyeli, Bogdan-Iuliu Hurdu, Neus Ibáñez Cortina (BC), Satoko Kawarasaki,
380Nevena Kuzmanović, Dominique Lanari-Menager, Ferran Llorrens, Dejan Mandjukovski,
381Patrizio Maniero, Attila Molnár V., Tetsuo Ohi-Toma (TI), David García San León (SANT),
382Nikolaj Stepanov, Andrada Paşcu, Milutin Praščević, Mihai Puşcaş (CL), Mirko Ruščić,
383Zvone Sadar, Toni Safner, Ivan A. Schanzer (MHA), Pierre-Olivier Templier, Lorenzo Testa,
384Stefano Zerauschek. Sampling on the Russian Caucasus was partly made during Spring
385educational practices of Moscow South-West High School (No. 1543) and partly funded by
386“Sovremennoe estestvoznanie” foundation. This work was supported by a grant of the
387Romanian Ministry of National Education, CNCS – UEFISCDI, project number PN-II-ID-
388PCE-2012-4-0595.

References

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391**Figures**

392

393**Fig. 1** 1a-1b. Geographic origin of samples analysed in the study. Numbers follow those from

394Table 1. Color codes indicate to which lineage they belong to (red: ‘non-Transylvanian’

395lineage of *E. dens-canis*, white: Transylvanian lineage of *E. dens-canis* and blue: *E.*

396*caucasicum*). 1c. Distribution of samples among subclades of the Transylvanian lineage of *E.*

397*dens-canis*

398**Fig. 2** TCS parsimony network of haplotypes. Color codes follow those from Fig. 1. Size of

399circles is proportional with the number of samples they contain. Small hollow circles

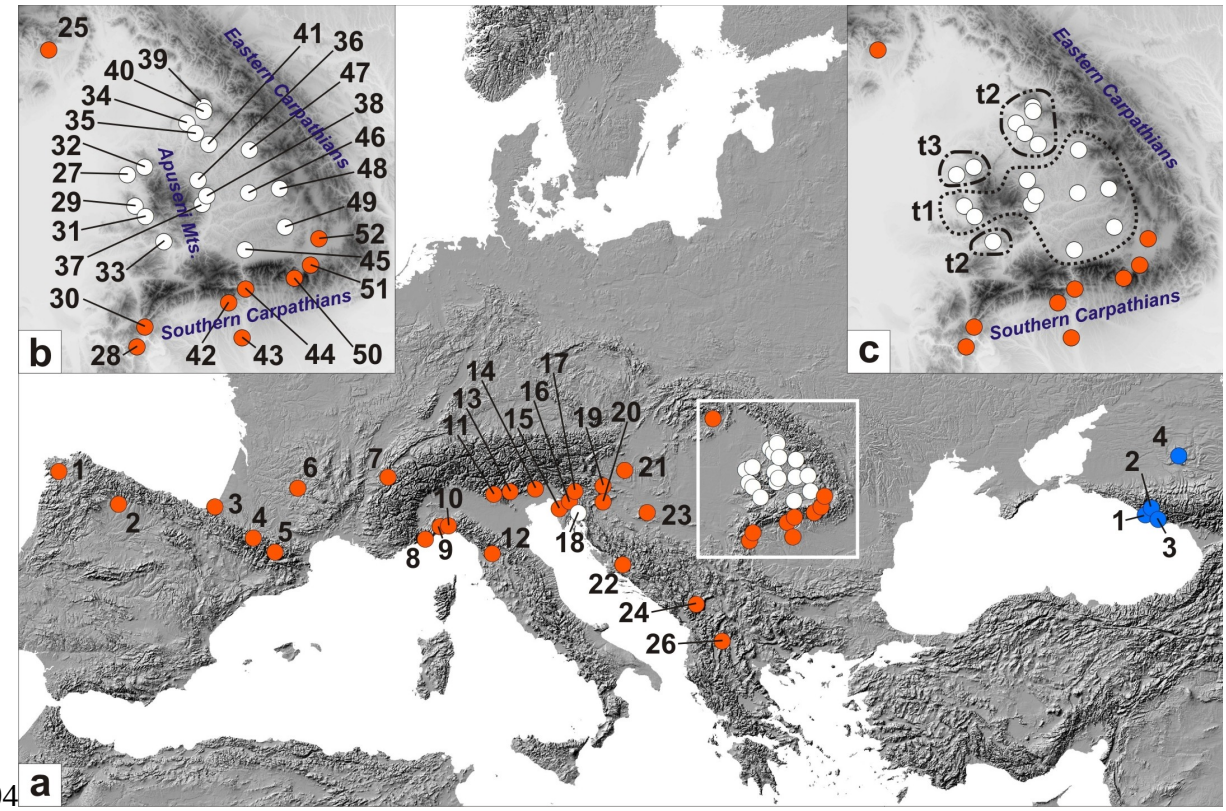
400represent unsampled haplotypes

401**Fig. 3** Fifty percent majority-rule consensus tree of haplotypes resulting from the Bayesian

402analysis. Numbers adjacent to nodes represent ML bootstrap, MP bootstrap and BI posterior

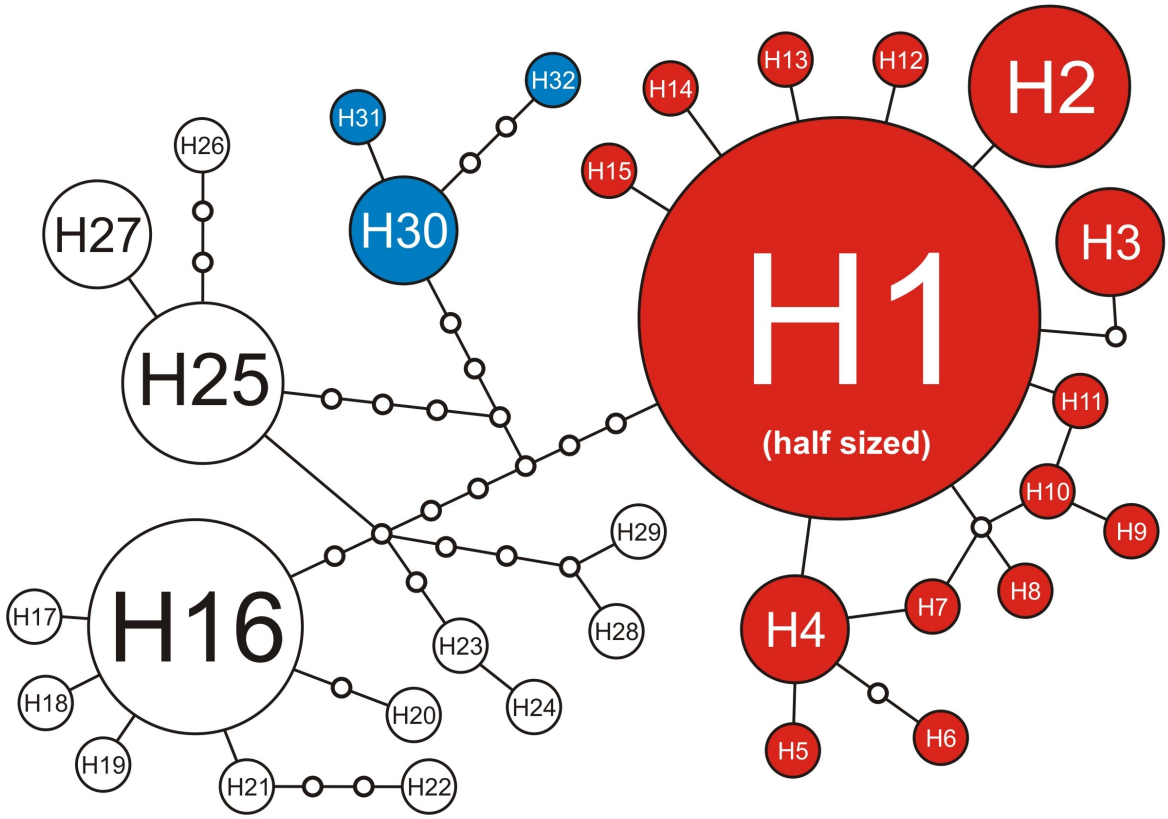
403probability values (expressed as percentages)

42


b
Eastern Carpathians
Apuseni Mts.
Southern Carpathians
c
Eastern Carpathians
Southern Carpathians
t1
t2
t3
a

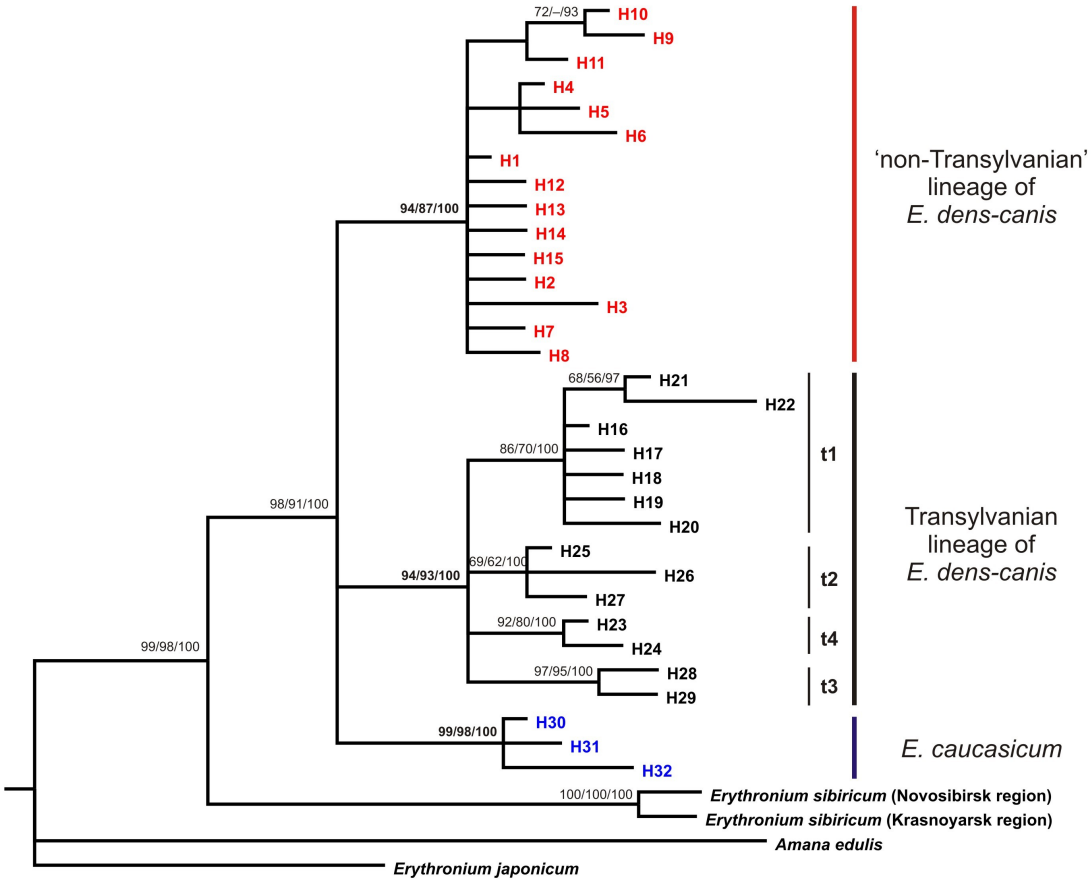
404

44


H1
(half sized)
H2
H3
H4
H5
H6
H7
H8
H9
H10
H11
H12
H13
H14
H15
H16
H17
H18
H19
H20
H21
H22
H23
H24
H25
H26
H27
H28
H29
H30
H31
H32

405

46


72/–/93
H10
H9
H11
H4
H5
H6
H1
H12
H13
H14
H15
H2
H3
H7
H8
94/87/100
'non-Transylvanian' lineage of E. dens-canis
68/56/97
H21
H22
H16
86/70/100
H17
H18
H19
H20
t1
98/91/100
H25
69/62/100
H26
94/93/100
H27
t2
92/80/100
H23
H24
t4
99/98/100
97/95/100
H28
H29
t3
Transylvanian lineage of E. dens-canis
99/98/100
H30
H31
H32
E. caucasicum
100/100/100
Erythronium sibiricum (Novosibirsk region)
Erythronium sibiricum (Krasnoyarsk region)
Amana edulis
Erythronium japonicum

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