PHYLOGEOGRAPHY AND TAXONOMY OF Dianthus sylvestris WULFEN s.l. ON THE BALKAN PENINSULA

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Faculty of Science
Department of Biology

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2. Prof. Sandro Bogdanović, PhD

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Prirodoslovno-matematički fakultet

Biološki odsjek

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FILOGEOGRAFIJA I TAKSONOMIJA VRSTE *Dianthus sylvestris* WULFEN s.l. NA BALKANSKOM POLUOTOKU

DOKTORSKI RAD

Mentori:

1. Dr. sc. Ivana Rešetnik
2. Prof. dr. sc. Sandro Bogdanović

Zagreb, 2022

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University of Zagreb Doctoral thesis
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PHYLOGEOGRAPHY AND TAXONOMY OF *Diantthus sylvestris* WULFEN s.l. ON THE BALKAN PENINSULA

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The Balkan Peninsula is one of the diversity centres for the morphologically highly variable and taxonomically inconsistently treated *Dianthus sylvestris* (Caryophyllaceae). In this thesis, an array of methods ranging from nomenclatural revision and morphometrics, following with phylogeographic methods (RADseq) and environmental niche analyses, to genome size estimates, were combined to explore the intraspecific relationships within Balkan populations of *D. sylvestris*, to discuss phylogeographic and historical processes that contributed to the observed genetic divergence patterns in this species, and to propose a sensible taxonomic solution. Patterns of genetic variation and species distribution models (SDM) suggested that *D. sylvestris* in the Balkan Peninsula survived the Pleistocene glaciations in two separate glacial refugia located along the eastern Adriatic coast. Two genetic groups with the split occurring around the Neretva River valley have been identified. These groups correspond to the two discerned morphological entities, the north-western and the south-eastern group of populations, distinct by the epicalyx scales shape, calyx teeth incision and petal denticulation. Migration analyses revealed relatively high rates of gene flow within each of the two groups, whereas there was almost no gene flow between them. The genetic differentiation did not support the current taxonomy, and the morphometric analyses revealed a continuous variability of quantitative morphological characters and an absence of clear-cut qualitative morphological differences among the subspecies. However, there was an obvious discontinuity in both morphological and genetic clines, therefore a taxonomic subspecies level was attributed to the two resulting groups, *D. sylvestris* subsp. *sylvestris* in the north and *D. sylvestris* subsp. *bertisceus* in the south. The thermophilous and earlier-flowering *D. sylvestris* subsp. *tergestinus* formed a separate evolutionary lineage, thus a new species-level treatment was proposed for this taxon.

(40 pages, 1 figure, 216 references, original in English)

Keywords: morphometrics, environmental niche analyses, flow cytometry, genetic variation, cline, glacial refugia

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FILOGEOGRAFIJA I TAKSONOMIJA VRSTE *Dianthus sylvestris* WULFEN s.l. NA BALKANSKOM POLUOTOKU

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Balkanski poluotok jedno je od središta raznolikosti morfološki vrlo varijabilne i taksonomski nedosljedno tretirane vrste *Dianthus sylvestris* (Caryophyllaceae). U sklopu ove disertacije korišten je niz metoda, od nomenklaturne revizije i morfometrije, preko filogeografskih metoda (RADseq) i analiza ekoloških niša, do procjene veličine genoma, kako bi se istražili unutarvrsni odnosi balkanskih populacija vrste *D. sylvestris* i utvrdili mogući filogeografski i povijesni procesi koji su pridonijeli uočenim uzorcima genetske divergencije ove vrste, te kako bi se predložilo novo taksonomsko rješenje. Obrasci genetske varijabilnosti i modeli povoljnosti staništa (SDM) upućuju da je vrsta *D. sylvestris* na Balkanskom poluotoku preživjela pleistocenske glacijacije u dva odvojena glacijalna refugija smještena duž istočne obale Jadranskog mora. Identificirane su dvije genetske skupine, gdje se granica između njih nalazi oko doline rijeke Neretve. Ove skupine odgovaraju dvjema uočenim morfološkim entitetima, sjeverozapadnoj i jugoistočnoj skupini populacija, koje se razlikuju po obliku ljuski epikaliksa, urezu zubaca čaške i nazubljenosti latice. Migracijske analize pokazale su relativno visoke stope protoka gena unutar svake od dviju skupina, dok između njih protoka gena gotovo nije bilo. Genetska diferencijacija nije u skladu s trenutačnom taksonomijom, a morfometrijske analize pokazale su kontinuiranu varijabilnost kvantitativnih morfoloških osobina i odsutnost jasnih kvalitativnih morfoloških razlika između podvrsta. Međutim, postoji jedan očiti diskontinuitet u morfološkom i genetskom gradijentu, stoga je taksonomski status podvrste pripisan dvjema skupinama populacija, *D. sylvestris* subsp. *sylvestris* na sjeveru i *D. sylvestris* subsp. *bertisceus* na jugu. Termofilni i ranije cvjetajući *D. sylvestris* subsp. *tergestinus* tvori zasebnu evolucijsku liniju te je za ovu svojtu predložen novi taksonomski tretman na razini vrste.

(40 stranica, 1 slika, 216 literaturnih navoda, jezik izvornika: engleski)

Ključne riječi: morfometrija, analize ekološke niše, protočna citometrija, genetska varijabilnost, gradijent, glacijalni refugiji

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I. **Terlević A**, Rešetnik I. 2020. Inventory of the historical *Dianthus sylvestris* herbarium materials from Herbarium Croaticum and Herbarium Ivo and Marija Horvat. *Glasnik Hrvatskog botaničkog društva* 8: 8–14. doi:10.46232/glashbod.8.1.2.

II. Domina G, Astuti G, Bacchetta G, Barone G, Rešetnik I, **Terlević A**, Thiébaut M, Peruzzi L. 2021. Typification of 14 names in the *Dianthus virgineus* group (Caryophyllaceae). *PhytoKeys* 187: 1–14. doi:10.3897/phytokeys.187.75534.

III. **Terlević A**, Bogdanović S, Frajman B, Rešetnik I. 2022. Genome Size Variation in *Dianthus sylvestris* Wulfen sensu lato (Caryophyllaceae). *Plants* 11: 1481. doi:10.3390/plants11111481.

IV. **Terlević A**, Temunović M, Bogdanović S, Grgurev M, Ljubičić I, Rešetnik I. 2022. Morphological and environmental variability of *Dianthus sylvestris* Wulfen (Caryophyllaceae) on the Balkan Peninsula. *Botanical Journal of the Linnean Society (In press)*. doi:10.1093/botlinnean/boac058.

V. Temunović M, **Terlević A**, Fior S, Luqman H, Rešetnik I, Widmer A. Genomic divergence of *Dianthus sylvestris* Wulfen s.l. on the Balkan Peninsula. *In preparation*.

LIST OF APPENDICES

I. **Terlević A**, Temunović M, Bogdanović S, Grgurev M, Ljubičić I, Rešetnik I. 2022. Environmental variability of *Dianthus sylvestris* Wulfen (Caryophyllaceae) on the Balkan Peninsula.

II. **Terlević A**, Bogdanović S, Rešetnik I. 2022. Taxonomic treatment of *Dianthus sylvestris* Wulfen (Caryophyllaceae) on the Balkan Peninsula.

THESIS SUMMARY

Phylogeographic studies across Europe have revealed Balkan, Apennine, and Iberian Peninsulas as the three main refugia for European flora and fauna of the temperate zone during Quaternary climatic fluctuations, and that these Peninsulas acted as sources for the recolonization of northern Europe after the glaciations. Moreover, due to the long-term environmental stability, these refugial areas often have high biodiversity and endemism. The hypothesis that the origin of most genera and species dates back to the Pliocene and Miocene periods is widely supported. However, the evolution within complexes of sister species and the evolution of intraspecific lineages are closely related with migration dynamics and changes in their distributions caused by cyclical climate changes during the Pleistocene. With the accumulation of evidence from phylogeographic studies, it has become clear that the Mediterranean Peninsulas harbour genetically divergent evolutionary lineages, which suggests the isolation of populations in geographically different microrefugia ('refugia-within-refugia' hypothesis). The Balkan Peninsula is a topographically and ecologically very heterogeneous area, characterised with high interspecific and intraspecific diversity. Therefore, its complex geological and climatic history, combined with heterogenous habitats, provides exceptional material for phylogeographic research. Concordant patterns of genetic variability and distribution have been observed in different species on the Peninsula, suggesting the existence of common patterns in biogeographical history. It is postulated that ecologically divergent species with different habitat requirements had different migration dynamics and distribution shifts in response to oscillations of favourable and unfavourable time periods. Specifically, the thermophilous species shifted their distribution along latitudes, while cold-adapted species probably experienced elevational range shifts. Both thermophilous and cold-adapted species experienced range expansion during favourable conditions, and range contraction during unfavourable conditions, according to their requirements. However, data on migration dynamics are lacking for species occupying habitats spanning wide latitudinal and elevational ranges. One such species is the taxonomically intricate *Dianthus sylvestris* Wulfen.

Dianthus L. is a taxonomically very complex genus and represents one of the most diverse plant groups in Europe. It is characterised by many endemic species with small and geographically very limited distributions, which indicates recent diversification. *Dianthus sylvestris* is distributed across the central Mediterranean, where it grows primarily on rocky grasslands, from lowland coastal to alpine environments. According to the available literature, numerous taxa at the species or subspecies level belonging to *D. sylvestris* s.l. have been

described across its distribution area. However, botanists have not yet agreed upon its taxonomy. Identification keys at the intraspecific level exist (e.g., Trinajstić 1979; Micevski 1993; Tutin and Walters 1993; Brullo and Guarino 2019), but the identification of subspecies is often ambiguous. *Dianthus sylvestris* on the Balkan Peninsula is currently considered as a species with seven subspecies: *D. sylvestris* subsp. *alboroseus* F. K. Mey, *D. sylvestris* subsp. *bertisceus* Rech. f., *D. sylvestris* subsp. *kozjakensis* Micevski, *D. sylvestris* subsp. *longicaulis* (Ten.) Greuter et Burdet, *D. sylvestris* subsp. *nodosus* (Tausch) Hayek, *D. sylvestris* subsp. *sylvestris* and *D. sylvestris* subsp. *tergestinus* (Rchb.) Hayek.

The main aim of this thesis was to disentangle the intraspecific relationships within *D. sylvestris* on the Balkan Peninsula, combining traditional (morphometrics) and contemporary approaches (new generation sequencing, NGS, and the application of single-nucleotide polymorphism, SNP, genome size estimations and species distribution models, SDMs). The research objectives were to: (1) Conduct morphometric and genetic analysis with the aim of solving the taxonomic problems of *D. sylvestris* s.l. and to gain insight into phylogeographic processes at the local and regional level; (2) Develop models of habitat suitability for the present and the past and to analyse the ecological niches of *D. sylvestris* subspecies with the aim of investigating the relationship between their morphological, genetic and environmental divergence; (3) Conduct a taxonomic revision of *D. sylvestris* s.l. on the Balkan Peninsula. Moreover, the following research hypotheses were tested: (1) There is genetic variability between populations of *D. sylvestris* s.l. associated with the existence of microrefugia on the Balkan Peninsula; (2) Populations belonging to different subspecies, evolutionary lineages and/or different habitat types can be identified according to their morphological diagnostic characters.

In this thesis, represented through four publications (**I – IV**), one scientific paper in preparation (**Publication V**), and two unpublished results (**Appendix I – II**), an array of complementary methods was used to disentangle relationships within *D. sylvestris* s.l.

Genomic data based on the comprehensive sampling across the Balkan Peninsula was combined with species distribution models (SDMs) generated for the present and the past (Last Glacial Maximum; LGM) to identify putative glacial refugia and assess putative migration patterns on the Balkan Peninsula during Pleistocene climatic oscillations. Several areas of pronounced relative genome size (RGS) variation at small geographic distances were revealed among the widespread diploid populations, likely indicating the intraspecific genetic divergence, whereas spatially restricted tetraploid populations of *D. sylvestris* were recorded for the first time on the Peninsula (**Publication III**). Their restricted distribution in the northern Adriatic region may be an indication of the existence of a refugium in this area. Genomic data showed a clear divergence of *D. sylvestris* subsp. *tergestinus* from the rest of the Balkan populations, despite their co-occurrence in sympatry. Furthermore, two groups of populations, the north-western and the south-eastern group, were revealed as two separately evolving evolutionary lineages, giving evidence for the existence of two separated glacial refugia on the Balkan Peninsula (**Publication V**). The two lineages might have come into contact during expansions, probably occurring throughout interglacials, in the intermediate area of Montenegro where this event resulted in the observed admixed populations. However, since *D. sylvestris* comprises both low-elevation (valleys) and high-elevation (alpine) populations, an alternative hypothesis of Pleistocene migration pathways was also suggested. It implies that the observed complex genetic structure may be a result of both range contraction of low-altitude populations and range expansion of high-altitude populations during the cold periods (**Publication V**). However, these hypotheses need to be tested in future research.

As a first step towards understanding the taxonomy of *D. sylvestris* and its tangled nomenclature synonymy, an inventory of historical herbarium material in the Croatian herbaria was conducted (**Publication I**). Furthermore, since the names *D. sylvestris* Wulfen and *D. virgineus* var. *tergestinus* Rchb. were published before 1958 (Art. 40.1; Turland *et al.* 2018), they did not have a *type*, so *types* relative to this period were chosen as *lectotypes* amongst the original material used by the authors of these names (**Publication II**). Also, a *lectotype* was chosen among the *syntypes* for the name *D. sylvestris* subsp. *bertisceus* (**Appendix II**). A taxonomic approach integrating RGS analyses, morphometrics, environmental niche analyses, phylogenetics and population genetics was applied. A continuous variability, where morphological states gradually change both with environment and geography was revealed (**Publication IV, Appendix I**). The continuity, i.e., a cline, was found in genomic data as well, except for a subset of populations identified as *D. sylvestris* subsp. *tergestinus* showing genetic divergence from all other Balkan populations (**Publication V**). Furthermore, Balkan populations showed a genetic split around the area of Neretva River valley, and this split is reflected in one morphological character, that is the shape of epicalyx scales (**Publication IV**). All above-mentioned evidence advocates for a reduction of the number of subspecies on the Balkan Peninsula (**Appendix II**).

The scientific contribution of this thesis is the advancing of general knowledge about migration routes of plants during the Pleistocene and the clarification of the role of refugia and

‘refugia-within-refugia’ in shaping high biodiversity in the Balkan Peninsula. A contemporary approach of integrating different types of data and methodologies enables testing of the hypothesis of species movement along latitudes or elevations in response to cyclical changes in Pleistocene. Moreover, this thesis contributes to the reinforcement of state-of-the-art methodology and analyses in the Croatian research community focused on evolutionary and phylogeographic issues, such as the NGS and the application of SNPs for exploring biodiversity. Finally, populations of *D. sylvestris* s.l. on the Balkan Peninsula are morphologically, ecologically, and genetically characterised, which enables defining a sensible taxonomy. Resolved taxonomic relationships contribute to the proper management and protection of natural populations.

PROŠIRENI SAŽETAK

Filogeografska istraživanja diljem Europe otkrila su Balkanski, Apeninski i Pirinejski poluotok kao tri glavna utočišta za europsku floru i faunu umjerenog pojasa tijekom klimatskih fluktuacija u Kvartaru, te da su ti poluotoci djelovali kao ishodišta za rekolonizaciju sjeverne Europe nakon glacijacija. Štoviše, zbog dugoročne stabilnosti okoliša, ova refugijalna područja često imaju visoku stopu biološke raznolikosti i endemizma. Široko je potvrđena hipoteza da podrijetlo većine rodova i vrsta seže u razdoblje Pliocena i Miocena. Međutim, evolucija unutar kompleksa sestrinskih vrsta i evolucija unutarvrsnih linija usko su povezane s migracijskom dinamikom i promjenama u njihovoj distribuciji uzrokovanom cikličkim klimatskim promjenama tijekom Pleistocena. Akumulacijom dokaza iz filogeografskih istraživanja postalo je jasno da Sredozemni poluotoci kriju genetski različite evolucijske linije, što sugerira izolaciju populacija u geografski različitim mikrorefugijima (hipoteza ‘refugija unutar refugija’). Balkanski poluotok je topografski i ekološki vrlo heterogeno područje, obilježeno visokom međuvrsnom i unutarvrsnom raznolikošću. Složena geološka i klimatska povijest, u kombinaciji s heterogenim staništima, pruža izuzetan materijal za filogeografska istraživanja ovog područja. Sukladni obrasci genetske varijabilnosti i distribucije uočeni su kod različitih vrsta na Poluotoku, što ukazuje na postojanje zajedničkih obrazaca u biogeografskoj povijesti. Pretpostavlja se da su ekološki divergentne vrste s različitim zahtjevima staništa imale različitu migracijsku dinamiku i pomake distribucije kao odgovor na oscilacije povoljnih i nepovoljnih vremenskih razdoblja. Točnije, termofilne vrste pomicale su svoju distribuciju duž geografske širine, dok su vrste prilagođene nižim temperaturama vjerojatno doživjele pomake u rasponu nadmorske visine. I termofilne i vrste prilagođene nižim temperaturama doživjele su proširenje areala tijekom povoljnih uvjeta, a smanjenje areala tijekom nepovoljnih uvjeta, u skladu sa svojim zahtjevima. Međutim, nedostaju podaci o dinamici migracije vrsta koje zauzimaju staništa širokog raspona geografskih širina i nadmorskih visina. Jedna takva je taksonomski kompleksna vrsta *Dianthus sylvestris* Wulfen.

Dianthus L. taksonomski je veoma složen rod i predstavlja jednu od najraznovrsnijih biljnih skupina u Europi. Karakteriziraju ga mnoge endemske vrste s malom i geografski vrlo ograničenom distribucijom, što ukazuje na nedavnu diverzifikaciju. Vrsta *Dianthus sylvestris* rasprostranjena je u središnjem Sredozemlju, gdje raste prvenstveno na kamenjarskim travnjacima, od nizinskih obalnih do alpskih staništa. Prema dostupnoj literaturi, brojne svojte na razini vrste ili podvrste koje pripadaju vrsti *D. sylvestris* s.l. opisane su na području distribucije. Međutim, botaničari još nisu usuglašeni oko taksonomije tih svojti. Identifikacijski ključevi na unutarvrsnoj razini postoje (npr. Trinajstić 1979; Micevski 1993; Tutin and Walters 1993; Brullo and Guarino 2019), no identifikacija podvrsta često je dvosmislena. Trenutno se *D. sylvestris* na Balkanskom poluotoku smatra vrstom koja obuhvaća sedam podvrsta: *D. sylvestris* subsp. *alboroseus* F. K. Mey, *D. sylvestris* subsp. *bertisceus* Rech. f., *D. sylvestris* subsp. *kozjakensis* Micevski, *D. sylvestris* subsp. *longicaulis* (Ten.) Greuter et Burdet, *D. sylvestris* subsp. *nodosus* (Tausch) Hayek, *D. sylvestris* subsp. *sylvestris* i *D. sylvestris* subsp. *tergestinus* (Rchb.) Hayek.

Glavni cilj ove disertacije bio je razjasniti unutarvrsne odnose vrste *D. sylvestris* na Balkanskom poluotoku, kombinirajući tradicionalne (morfometrija) i suvremene pristupe (sekvencioniranje nove generacije, NGS, i primjena polimorfizma jednog nukleotida, SNP, procjena veličine genoma i modeliranje povoljnosti staništa, SDM). Ciljevi istraživanja bili su: (1) Provesti morfometrijsku i genetičku analizu s ciljem rješavanja taksonomskih problema vrste *D. sylvestris* s.l. te stjecanje uvida u filogeografske procese na lokalnoj i regionalnoj razini; (2) Razviti modele povoljnosti staništa za sadašnjost i prošlost i analizirati ekološke niše podvrsta vrste *D. sylvestris* s ciljem istraživanja odnosa između njihove morfološke, genetske i okolišne divergencije; (3) Provesti taksonomsku reviziju vrste *D. sylvestris* s.l. na Balkanskom poluotoku. Nadalje, ispitane su sljedeće istraživačke hipoteze: (1) Postoji genetska varijabilnost između populacija vrste *D. sylvestris* s.l. povezana s postojanjem mikrorefugija na Balkanskom poluotoku; (2) Populacije koje pripadaju različitim podvrstama, evolucijskim linijama i/ili različitim tipovima staništa mogu se identificirati prema njihovim morfološkim dijagnostičkim osobinama.

U ovoj disertaciji, predstavljenoj kroz četiri publikacije (**I – IV**), jedan znanstveni rad u pripremi (**Publikacija V**) i dva neobjavljena rezultata (**Dodatak I – II**), upotrijebljen je niz komplementarnih metoda kako bi se razjasnilo odnose unutar vrste *D. sylvestris* s.l.

Genomski podaci temeljeni na sveobuhvatnom uzorku na Balkanskom poluotoku kombinirani su s modelima povoljnosti staništa (SDM) generiranim za sadašnjost i prošlost (posljednji glacijalni maksimum; LGM) kako bi se identificirali pretpostavljeni glacijalni refugiji i procijenili pretpostavljeni migracijski obrasci na Balkanskom poluotoku tijekom klimatskih oscilacija u Pleistocenu. Među široko rasprostranjenim diploidnim populacijama otkrivena su područja izražene varijabilnosti relativne veličine genoma (RGS) na malim geografskim udaljenostima, što vjerojatno ukazuje na unutarvrsnu genetsku divergenciju, dok su prostorno ograničene tetraploidne populacije vrste *D. sylvestris* prvi put zabilježene na poluotoku (**Publikacija III**). Njihova ograničena rasprostranjenost na području sjevernog

Jadrana može biti pokazatelj postojanja refugija na tom području. Populacije podvrste *D. sylvestris* subsp. *tergestinus* u genomskim su podacima pokazale jasnu divergenciju od ostalih balkanskih populacija, unatoč njihovom pojavljivanju u simpatriji. Nadalje, dvije skupine populacija, sjeverozapadna i jugoistočna skupina, otkrivene su kao dvije zasebno razvijajuće evolucijske linije, dajući dokaze u prilog postojanju dvaju odvojenih glacijalnih refugija na Balkanskom poluotoku (**Publikacija V**). Ove su dvije linije mogle doći u kontakt tijekom ekspanzija, koje su se vjerojatno događale tijekom interglacijala, na području Crne Gore gdje je ovaj događaj rezultirao populacijama koje su pokazale miješanu genetsku strukturu. Međutim, budući da *D. sylvestris* obuhvaća i nizinske (doline) i visoko planinske (alpske) populacije, također je predložena alternativna hipoteza o Pleistocenskim migracijskim putovima, koja implicira da uočena složena genetska struktura može biti rezultat smanjivanja područja rasprostranjenosti nizinskih populacija i širenja rasprostranjenosti visoko planinskih populacija tijekom hladnih razdoblja (**Publikacija V**). Međutim, te hipoteze treba testirati u budućim istraživanjima.

Kao prvi korak prema razumijevanju taksonomije vrste *D. sylvestris* i njezine zamršene nomenklaturne sinonimije, provedena je inventarizacija povijesne herbarijske građe u hrvatskim herbarijima (**Publikacija I**). Nadalje, budući da su imena *D. sylvestris* Wulfen i *D. virgineus* var. *tergestinus* Rchb. objavljena prije 1958. (čl. 40.1; Turland *et al.* 2018) i nemaju tipski primjerak, ilustracije iz izvorne građe koju su autori ovih naziva koristili, odabrani su kao lektotipovi (**Publikacija II**). Također, među sintipovima je odabran lektotip za ime *D. sylvestris* subsp. *bertisceus* (**Dodatak II**). Primijenjen je taksonomski pristup koji uključuje RGS analizu, morfometriju, analizu ekoloških niša, filogeografiju i populacijsku genetiku. Otkrivena je kontinuirana varijabilnost, gdje se morfološka stanja postupno mijenjaju s okolišem i geografijom (**Publikacija IV, Dodatak I**). Kontinuitet, tj. gradijent, također je pronađen u genomskim podacima, osim podskupa populacija identificiranih kao *D. sylvestris* subsp. *tergestinus* koji je pokazao genetsku divergenciju od svih ostalih balkanskih populacija (**Publikacija V**). Balkanske su populacije pokazale genetsku podjelu oko područja doline rijeke Neretve, a ta podjela se ogleda u jednoj morfološkoj osobini, a to je oblik ljuski epikaliksa (**Publikacija IV**). Svi gore navedeni dokazi govore u prilog smanjenju broja podvrsta na Balkanskom poluotoku (**Dodatak II**).

Znanstveni doprinos ove disertacije je unaprjeđenje općeg znanja o migracijskim putovima biljaka tijekom Pleistocena i pojašnjenje uloge refugija i 'refugija unutar refugija' u oblikovanju visoke bioraznolikosti na Balkanskom poluotoku. Suvremeni pristup integriranja različitih tipova podataka i metodologija omogućuje testiranje hipoteze o kretanju vrsta duž geografskih širina ili nadmorskih visina kao odgovor na cikličke promjene u Pleistocenu. Nadalje, ovaj rad pridonosi razvoju analitike i najsuvremenije metodologije u hrvatskoj istraživačkoj zajednici, usmjerene na evolucijska i filogeografska pitanja, kao što su NGS i primjena SNP-ova za istraživanje bioraznolikosti. Konačno, populacije vrste *D. sylvestris* s.l. na Balkanskom poluotoku su morfološki, ekološki i genetski karakterizirane, što omogućuje definiranje smislene taksonomije. Razriješeni taksonomski odnosi doprinose pravilnom gospodarenju i zaštiti prirodnih populacija.

INTRODUCTION

INTRODUCTION

Integrative approach to systematic botany

The advancement of quantitative approaches to classification in the second half of the 20th century was a consequence of dissatisfaction with the previous intuitive approaches to classification and to science in general (Stuessy and Hörandl 2014). Phenetics appeared as a first attempt of quantitative classification (Michener and Sokal 1957), and after systematists realised that classifying species based solely on morphology has no sense, cladistics came into this challenge (Hennig 1966). Systematics is a discipline dealing with change, and the study of evolution is crucial for contemporary systematics. However, controversy regarding how taxonomists should use the results of phylogenetic analyses and incorporate the diversity of evolutionary patterns and processes into classification still exists. First, evolutionary processes characteristic for plants, such as polyploidization and hybridization, result in non-dichotomous branching patterns (Soltis and Soltis 2009), and thus generate incongruences among phylogenetic trees. However, incongruence does not have to be considered a limitation, but it may rather be regarded as a source of information for a deeper understanding of evolutionary processes (Wendel and Doyle 1998). Second, there are various concepts of species available, and different user groups use different concepts (Turelli *et al.* 2001; Fujita *et al.* 2012). Speciation is usually a process, not a singular event in time, and as such, approaches using genomic data mostly delineate lineages (fine-scale genetic structure), rather than species (Sukumaran and Knowles 2017). The confusion about species limits and the potential of taxonomic overinflation could create problems in all fields that rely on species as units of analysis, from studies of macroevolutionary dynamics to conservation biology (Sukumaran and Knowles 2017). Therefore, results based on genomic data should be considered as only one of the multiple lines of evidence which requires validation from other sources of information, such as morphology/phenotype and ecology. Overall, present-day systematic botany is a complex methodical discipline which benefits from technical development and new data sources. Only by integrating the information obtained from evidence-based multi-layered results can we discover relationships between species and/or lineages and determine underlying processes (Stuessy and Hörandl 2014). By incorporating up-to-date systematic botany, the intriguing questions on biodiversity of a certain area and on the processes involved in its creation can be uncovered.

Phylogeography

Phylogenetic analysis is the central theme of higher-level systematics, whereas population genetics permeate studies of intraspecific evolution. Throughout most of the 20th century, there was a gap between those two fields and phylogeography nowadays is helping to bridge this gap by synthesising micro- and macroevolutionary thought (Avise 2007). Phylogeography, a term coined by Avise *et al.* (1987), is a discipline concerned with the geographical distribution of genetic lineages within and among closely related species. To explore the genetic structure, phylogeography uses both information on contemporary forces of genetic exchange, such as reproductive ecology and gene flow, and historical information (Schaal *et al.* 1998). Thus, phylogeographic studies help to understand the evolutionary processes that have driven population expansion and contraction and gene movement, and that were impacted by climate fluctuations and geographic and environmental boundaries. Empirically, most intraspecific genetic lineages have proved to be non-overlapping or nearly so in geographic distribution, i.e., allopatric (e.g., Kuzmanović *et al.* 2013; Španiel, Marhold, *et al.* 2017; Šlenker *et al.* 2021). Moreover, the geographic distributions of genetic lineages frequently correspond with obvious environmental barriers to gene flow or the locations of Pleistocene refugia (Schönswetter *et al.* 2005; Slovák *et al.* 2012; Grdiša *et al.* 2014; Rešetnik *et al.* 2020; Záveská *et al.* 2021; Ninčević *et al.* 2021). Phylogeography can be also used as an insight into the relationship between genetic and morphological/taxonomic differentiation. For example, the concordance between morphological differentiation and genetic lineages was found in *Cyanus tuberosus* group (Skokanová *et al.* 2019) and in *Cardamine acris* (Perný *et al.* 2004), where genetic lineages were recognised as subspecies. On the other hand, clades inferred by chloroplast sequences in *Heliosperma pusillum* (Frajman and Oxelman 2007), and genetic groups inferred by amplified fragment length polymorphism (AFLP) in *Knautia drymeia* (Rešetnik, Frajman, *et al.* 2016), were geographically, rather than taxonomically correlated. Continuing and deepening phylogeographic research has great potential to better understand the genesis of biodiversity hotspots, and thus to help in their conservation (Radosavljević *et al.* 2012; Theodoridis *et al.* 2019; Liber *et al.* 2020).

Taxonomy

The integration of different types of data and methodologies is currently widely used for disentangling taxonomically intricate groups of organisms (Pante *et al.* 2015; Caković *et al.* 2018; Frajman *et al.* 2019; Hardion *et al.* 2020). Correct identification of a specimen is a basic prerequisite and a first step for all botanical scientific research. Thus, misidentifications of taxa can be fatal to the research results or nature protection actions (Mace 2004). Many taxa show exceptional variability associated with various evolutionary and ecological phenomena, such as polyploidy (Balao *et al.* 2009), phenotypic plasticity (Pfennig *et al.* 2010), different ecotypes (Hardion *et al.* 2020), etc., making their taxonomy very complex. Classification systems developed for such groups are temporary and prone to changes. Depending on the preferences of the authors, the taxonomic approach can result in splitting a taxon into many smaller subgroups ("splitters" approach, e.g., Lin *et al.* 2021) or combining several taxa into one large group ("lumpers" approach, e.g., Caković *et al.* 2015). Continuous splitting and lumping taxa based on subjective criteria generates an unstable taxonomy (Eriksson *et al.* 2022).

Throughout the history of plant taxonomy, the accumulation of nomenclatural synonyms is very common. The *International Code of Nomenclature for algae, fungi and plants* defines current plant nomenclature practices and describes how to name plants assigned to ranked categories (Turland *et al.* 2018). The requirement to deal with names and to solve nomenclatural issues of historical botanical research occupies a large proportion of the taxonomist's working time. The main reason is that typification works are often published in minor or general publications, making it difficult to know whether a name of a taxon has already been typified. Consequently, herbarium collections are repeatedly studied from scratch by individual researchers studying the history of a name. Historical and geographical information attached to historical herbarium collections are often vague, which makes them challenging to study. However, modern technologies, such as digitising and cataloguing, are making the collections widely accessible to scientists, and thus enable their reinterpretations (Hardy *et al.* 2020).

Intraspecific diversity

All plants display some degree of morphological variability among populations. Swedish botanist Göte W. Turesson was one of the first to show that slightly different phenotypes are the result of adaptations to local environmental conditions (Turesson 1925). To describe these mutually different populations, Turesson introduced the term *ecotype*. The biosystematists that agreed with Turesson stressed the importance of reproductive isolating barriers that limit gene flow between groups on the path to speciation (Clausen 1951). On the other hand, Huxley (1938) was concerned that the continuously distributed phenotypic variation was overlooked by classification systems of his era and thus introduced the term *cline*. In contrast to initial Turesson's concept of *ecotype* suggesting that intraspecific plant variation could be classified into discrete units, Huxley's *cline* implies the graduation of variation in traits over space. It is true that some traits can vary gradually, others discontinuously, depending on a number of factors, e.g., gene flow, number of genes involved, environmental stressors, etc. (Linhart and Grant 1996), but *ecotypes* reflect the composite response of multiple traits to the local selection pressures, whereas *clines* describe single character variation across space. The relationship of *ecotypes* to the taxonomic hierarchy and the practical need to name plants is diverse. According to some authors, it is justified for *ecotypes* to receive a status in the taxonomic hierarchy and to be nomenclaturally resolved usually at the level of subspecies, for example in *Orchis papilionacea* L. (Cozzolino *et al.* 2021) and *Dianthus superbus* L. (Hardion *et al.* 2020). On the other hand, *cline* represents a gradient in phenotypic or genetic character which is developed in the absence of barriers to gene flow (Endler 1977), which thus cannot be fitted into discrete units. The revolutionary impacts of the use of DNA sequence data and their interpretation using genomic tools, coupled with population structure and geographical patterns analyses (e.g., Dyer *et al.* 2010) has contributed to the understanding how genetic variation is partitioned within plants species (Abbott 2017; Halbritter *et al.* 2018). With new data and discoveries, we gain evidence that plant species are not just phyletic lineages that need to be taxonomically recognised but consist of dynamically evolving populations whose relationships can be fully understood by integrating different types of data and methodologies.

Data used in systematics / integrative taxonomy

Morphology

Morphological data are still the most used in plant taxonomy since morphological features can be recorded immediately and allow for a rapid identification. Morphological data can be classified in macromorphological and micromorphological characters, where the former is the most used as diagnostic in identification keys (Stuessy 2009; Beentje 2016). The latter have gained importance with the improvement of scanning electron microscopy techniques, for example characters related to trichomes (Carnicero 2017) and seeds (Bogdanović *et al.* 2016) are successfully used as diagnostic. Morphological features can also be classified into vegetative and reproductive data characters. Vegetative organs often perform several functions simultaneously (physical support, assimilation, water conduction, etc.), and therefore have greater adaptive capacity and phenotypic plasticity (Halbritter *et al.* 2018), leading to their reduced usefulness in taxonomy. However, leaf characters are often successfully used in the taxonomy of tree species (Jensen *et al.* 2002; Morales-Saldaña *et al.* 2021). Characters of reproductive organs are easy to observe and they are effective when creating identification keys and in description of taxa. These characters display less variability in relation to vegetative characters, they are more numerous, and even small differences in the structure of flower often provide reliable insight into phylogenetic relationships and classification (Stuessy 2009).

One of the principal tools in assessing morphological features is multivariate morphometrics, which in the second half of the 20th century became standard for analysing large sets of measurements from different samples, and became desirable to validate morphological descriptions of taxa (e.g., Trigas *et al.* 2018). The morphometric analyses are frequently used to explore diversity patterns at intraspecific level (Marhold 2011a). When combined with other methodological approaches such as molecular systematics and/or ploidy level estimations, multivariate morphometrics is successfully applied to disentangle complexes of closely related taxa (Đurović *et al.* 2014; Španiel, Zozomová-Lihová, *et al.* 2017; Buzurović *et al.* 2020), re-examine poorly described or dubious taxa (Rooks *et al.* 2011) and describe intraspecific variability, such as morphological differentiation between populations in heterogeneous environments (Abdusalam and Li 2018) and morphological differentiation between cytotype (Španiel *et al.* 2011). Taxonomic hypotheses can be generated using ordination methods such as principal components analysis (PCA), whereas they are tested using discriminant analyses (DAs). Principal components analysis is a technique that reduces dimensionality of the original character space. As the first two dimensions capture most of the variation of the original dataset, it is possible to visualise the relationships of individual objects on a two-dimensional plane. Although PCA was developed for quantitative characters with normal distribution, it can be used with certain reservations also for binary and semiquantitative characters and is also considerably robust with respect to departures of characters from normal distribution. The strict limitation of PCA is with regard to the number of analysed characters which should be lower than the number of analysed objects (Marhold 2011a). On the other hand, to view relationships among predefined groups in space defined by canonical axes, canonical discriminant analysis (CDA) is used. For example, groupings based on genetic markers (Kučera *et al.* 2013), genome size or ploidy levels (Koutecký *et al.* 2012) or habitat characteristics (Španiel *et al.* 2011) can be interpreted. CDA maximises separation of predefined groups and thus allows answering two questions (Marhold 2011a): 1) based on available characters, to what extent can predefined groups be morphologically distinguished, and 2) which characters contribute to this differentiation. However, it is important that the characters that define the groups should not be involved in the analysis to avoid circular argumentation.

Genomic data

Molecular/genomic data are applied in population genetic studies (geographic variability, course of gene flow, etc.; Temunović *et al.* 2012), determination of boundaries between species and higher categories (Caković *et al.* 2015), and construction of phylogenetic hypotheses on all taxonomic levels (Fassou *et al.* 2022). The recent proliferation of DNA sequencing technologies has led to a rapid increase in the volume of genomic data. Obtaining high-coverage, well-assembled and thoroughly annotated genomes is still very expensive and time-consuming, therefore subsampling methods that yield a specific set of markers chosen *a priori* provide a cost-effective solution for achieving deep sequence coverage across the sampled sites of the genome (Harvey *et al.* 2016). Restriction site associated DNA sequencing (RAD-Seq) is the most widespread method for obtaining genomic datasets from non-model organisms (Harvey *et al.* 2016). RAD-Seq is a family of methods using restriction enzymes which are often selected to cut at sites widely distributed across the genome, and thus RAD-Seq sites may come from diverse coding and non-coding regions, but not from repetitive regions (DaCosta and Sorenson 2014). Most RAD-Seq techniques focus on collecting short sequences or single nucleotide polymorphism (SNP) data from groups of short sequences.

SNP data are genomic markers used in population genetics and phylogeographic studies aimed at elucidating the history of natural populations (Trucchi *et al.* 2017; Binks *et al.* 2021; Liang *et al.* 2022; Prata *et al.* 2022). The development of efficient analytical methods has allowed variation across multiple genomes of sampled populations to be investigated and species histories to be inferred. In any project on population genetics, multiple methods have to be used and compared since this approach will provide a more comprehensive overview of the evolutionary processes acting (Bourgeois and Warren 2021). To visualize genetic distances among individuals and populations, multivariate approach such as Principal Components Analysis (PCA) can be used. This method is suitable for analysing polyploid species, since it does not have any underlying population genetic-based assumptions, such as the Hardy-Weinberg (HW) equilibrium (Dufresne *et al.* 2014). Unlike these model-free approaches, model-based approaches provide an estimate of the proportion of an individual genome originating from multiple ancestral gene pools, given a predefined number of clusters (K). Some of these tools, based on a Bayesian framework, are STRUCTURE (Pritchard *et al.* 2000) and

FASTSTRUCTURE (Raj *et al.* 2014), which group individuals in clusters maximizing HW equilibrium and linkage disequilibrium between loci. An alternative to STRUCTURE may be the Discriminant analysis of principal components (DAPC, ADEGENET; Jombart *et al.* 2010), which maximizes divergence between groups identified by PCA and it does not require that populations are in HW equilibrium. However, methods for assessing population structure that make use of genotype information are in principle more powerful than multivariate approaches (Dufresne *et al.* 2014). Tools for demographic inference that are based on allele frequencies are, for example, TREEMIX (Pickrell and Pritchard 2012), which infers most likely admixture events in a tree, and divMigrate (Sundqvist *et al.* 2016), which estimates directional relative migration (=gene flow) and tests asymmetry between migrating rates. Asymmetric migration is common in natural populations and information about the symmetry of gene flow becomes important for understanding genetic structuring of populations and make inference on past demographic changes (Sundqvist *et al.* 2016).

Phylogenetic trees are commonly used to reconstruct the relationships between populations, and can be constructed based on restriction-digest methods that primarily focus on SNPs (Herrera and Shank 2016; Harvey *et al.* 2016; Bombonato *et al.* 2020; Bateman 2021). SNP data has been once considered reserved for population genetic studies, however their potential to both resolve phylogenetic problems and investigate population demographics, makes them an important source of genomic information (Leaché and Oaks 2017). One tool for phylogenetic inference based on the maximum likelihood (ML) search criterion is IQ-TREE (Minh *et al.* 2020), which estimates divergence time and phylogenetic relationships. The task of phylogenetics is to find the best way to translate evolutionary changes in DNA sequences and other characters into models that effectively represent the underlying mechanisms for genome-scale variation (Bravo *et al.* 2019).

Cytology

Out of the multitude of cytological characteristics, data related to chromosomes are mainly used in plant taxonomy – their number and morphology, aspects of polyploidy and number and position of satellite chromosomes (Behroozian *et al.* 2012). The use of flow cytometry (FCM) for estimating genome size of plants has rapidly expanded in taxonomic studies since the late 1980s (Suda, Kron, *et al.* 2007). It is generally used for ploidy estimation of individuals (Greilhuber *et al.* 2005; Suda and Trávníček 2006), in which DNA content of a sample is compared to a reference of known ploidy and expressed as multiples of a single chromosome complement. Understanding the course of evolution, adaptive radiation, the emergence of new species etc., is often directly related to cytological changes. FCM is now an essential tool for quantifying these changes and identifying cryptic taxonomic structure by providing distinction of morphologically indiscernible taxa (Suda, Krahulcová, *et al.* 2007; Šmarda *et al.* 2008; Slovák *et al.* 2009; Kolár *et al.* 2009; Frajman *et al.* 2015; Hodálová *et al.* 2020; Martínez-Sagarra *et al.* 2021). Combining DNA content measurements with chromosome counts allows direct verification of the estimated ploidy level. Information on ploidy is often important in guiding taxonomic delineation of plants since allopolyploids are often morphologically distinct and reproductively isolated from their diploid progenitors. Consequently, allopolyploids are frequently circumscribed at the species level (Soltis *et al.* 1993). On the other hand, autopolyploids often morphologically resemble their diploid ancestors and hence they are not recognised taxonomically (Soltis *et al.* 2007) or in some cases are identified at subspecific level (Mosquin 1967). Additional benefit of FCM is that it can be used to differentiate between taxa with the same chromosome number (i.e., homoploid taxa) but different DNA amount (i.e., genome size; Murray 2005). However, the methods of FCM for distinguishing related homoploid taxa must be more stringent than when estimating ploidy, as the differences in DNA content are often small (Nunvářová Kabátová *et al.* 2019).

Environmental data

To avoid mistaken description of taxa, the integrative taxonomy approach considers also using evidence from ecology. Ecological data are different from other data used in systematic botany because they do not come from the plants themselves, but from the interactions of plants with the environment and other plants and animals. Knowledge from the field of ecology is often used to interpret the distribution of taxa (Guisan and Thuiller 2005; Soberón 2007; Barve *et al.* 2011; Sikora *et al.* 2013; Đurović *et al.* 2021), variability in plants (Knight and Ackerly 2002; Souza *et al.* 2019; Alcántara-Ayala *et al.* 2020; Li *et al.* 2020; Stephens *et al.* 2022), development of adaptive traits (Guzmán *et al.* 2009; Halbritter *et al.* 2018; Ninčević *et al.* 2021), etc. Furthermore, ecological data helps to describe the emergence of *ecotypes* (Bertel *et al.* 2018; Cozzolino *et al.* 2021), specialisation on certain soil types (edaphic; Piedallu *et al.* 2016; Konečná *et al.* 2020), the effect of pollination mechanisms (Shrestha *et al.* 2018), certain types of reproductive isolation (Owens and Samuk 2020), etc. Ecological indicators have major influence on classification systems and phylogenetic interpretations at lower taxonomic levels, primarily below the genus (Aguirre-Gutiérrez *et al.* 2015; Jaime *et al.* 2015; Janišová *et al.* 2018).

The recent availability of environmental data from databases containing climate, soil and topographic GIS layers (Geographic Information System; e.g., Chelsa, SoilGrids and EarthEnv; Hengl *et al.* 2017; Karger *et al.* 2017, 2020; Amatulli *et al.* 2018) allow the quantification of species ecological niches, as well as niche overlap between different species (Rödder and Engler 2011; Broennimann *et al.* 2012). Niche quantification and niche comparison between species are usually performed with an ordination method in environmental space (Broennimann *et al.* 2012). Ecological niches of taxa can be characterised and the output used to answer fundamental questions about niche evolution and speciation and for validating taxon boundaries (Dagnino *et al.* 2017; Collart *et al.* 2020). Consequently, the generated knowledge provides additional line of evidence for disentangling taxonomically challenging groups (Mota-Vargas and Rojas-Soto 2016; Otero *et al.* 2019; Lin *et al.* 2021). Furthermore, niche equivalency and niche similarity tests assess the statistical significance of a measured niche difference against null model niches taken randomly within a given background area (Warren *et al.* 2008). These tests are used for interpreting the significance of ecological niche differentiation among species or subspecies, and for testing specific hypotheses about niche conservatism/divergence (Warren *et al.* 2008). The above-mentioned analyses and tests are also known as pre-modelling analyses. On the other hand, core-modelling analyses are based on the quantification of species-environment relationship, calibrating the models and using them to make spatial predictions (Guisan and Zimmermann 2000; Di Cola *et al.* 2017). This later approach of bringing together the quantified ecological niche of a species and then projecting it onto geography to identify regions where the species' requirements are manifested is known as 'species distribution modelling' (SDM; Barve *et al.* 2011).

SDM is also known as 'ecological (or environmental) niche modelling' (ENM) or habitat suitability modelling. Two primary types of SDM techniques are regression methods, such as Generalized Linear and Generalized Additive Models (GLM/GAM, Guisan *et al.* 2002), and machine learning (ML) methods, such as Maximum Entropy (MaxEnt, Merow *et al.* 2013), Random Forest (Evans *et al.* 2011) and Boosted Regression Trees (BRT/GBM, Elith *et al.* 2008). Its correlative/statistical models are used for understanding and explaining species distributions (Glasnović *et al.* 2018), predicting rare species occurrences (McCune 2016), predicting the impacts of climate change on species distribution (Đurović *et al.* 2021), assessing invasion risk (Jaźwa *et al.* 2018), understanding niche dynamics and evolution (Yesson and

Culham 2006; Luqman *et al.* 2022) and in landscape genetics for evaluating the effects of landscape features on gene flow patterns (Cruzan and Hendrickson 2020). In the context of climate change, a large number of general circulation models (GCMs) have been developed, representing physical processes in the atmosphere, ocean and land surface, allowing to simulate the climate responses in the past and in the future. In phylogeographic studies, GCMs/climate data usually used for modelling potential habitat suitability are those developed for the Last Glacial Maximum period (LGM, ~21 kya, Karger *et al.* 2017). Hence, SDM is increasingly used in identifying potential refugia along the Quaternary climatic oscillations for past populations (Pinto-Carrasco *et al.* 2022). The integration of both molecular and past and present ecological data are required for getting a better understanding of the possible mechanisms that generated species' diversity patterns and their current distribution patterns (Kutnjak *et al.* 2014; Nieto Feliner 2014; Đurović *et al.* 2021; Reich *et al.* 2021).

Phylogeography of the Balkan Peninsula

A myriad of genetic and species diversity is found on the three Mediterranean peninsulas, the Iberian, Apennine and Balkan Peninsulas (Hewitt 2011). Among them, the Balkan Peninsula is one of the biodiversity hotspots containing more endemics than other peninsulas (Hewitt 2011; Nieto Feliner 2014), accumulated in a comparative small space over extended time (Médail and Diadema 2009). Its geological history included several cycles of land connections and disconnections, from Miocene to Pleistocene (Tzedakis 2004), including events such as the Messinian Salinity Crisis (MSC = 5.96–5.33 Mya; Krijgsman *et al.* 1999) and the inland formation of lakes of various sizes following the desiccation of the Pannonian Sea occurring on the same time (Krstić *et al.* 2012). The majority of genera diversified during these events (Vargas *et al.* 2018), however, the within species diversification most likely occurred in response to Pleistocene glacial oscillations.

During recent years, an accumulation of phylogeographic studies on species complexes from the Balkan Peninsula have revealed various intricate phylogeographic patterns, where different lineages occurring in separate microrefugia correspond to phylogeographic breaks within the Peninsula (Španiel and Rešetnik 2022). Barriers such as mountains, river canyons and valleys fostered the formation of multiple microrefugia within the Peninsula during Pleistocene glacial cycles, in which multiple genetic lineages have diverged (e.g., Surina *et al.* 2011; Kutnjak *et al.* 2014; Caković *et al.* 2015; Đurović *et al.* 2017, 2021). This pattern has become known as 'refugia within refugia' (Gómez and Lunt 2007; Médail and Diadema 2009).

The mode and tempo of postglacial recolonisation of genetic lineages is difficult to generalise, since different species inhabited different geographic locations of microrefugia and were isolated by different barriers. Moreover, the genetic differentiation during population expansions and/or reductions underwent different selection processes. However, two main patterns of genetic differentiation during postglacial recolonisation events have been considered. One is the 'leading edge' hypothesis (Hewitt 2000), and the second one is the hypothesis of glacial persistence in two separate, i.e., the north-western and the south-eastern Balkan refugia. The first pattern implies higher genetic diversity in southern populations and a corresponding reduction towards north due to repeated founder events from long-distance colonisers, and it was observed in e.g., *Edraianthus tenuifolius* (Surina *et al.* 2011) and *Euphorbia myrsinites* (Falch *et al.* 2019). The second pattern implies the persistence in two different refugia hosting populations with similar genetic diversity, and it was documented in e.g., *Viola suavis* s.l. (Mereďa *et al.* 2011).

In the western Balkan Peninsula, specifically along the Dinaric Mountains, most studied taxa exhibit the differentiation into two or three genetic lineages, as seen in e.g., *Tanacetum cinerariifolium* (Grdiša *et al.* 2014), *Salvia officinalis* (Rešetnik, Baričevič, *et al.* 2016; Jug-Dujaković *et al.* 2020), *Silene saxifraga* (Đurović *et al.* 2017), *Cerastium grandiflorum* and *C. decalvans* (Đurović *et al.* 2021). The Dinaric Mountains have several deep and narrow canyons formed by karstic rivers which likely acted as barriers to gene flow. Evidence for such allopatric diversification has been found between populations of e.g., *T. cinerariifolium* (Grdiša *et al.* 2014) separated by the Zrmanja canyon in the Northern Dinaric Mountains; *Alyssum austrodalmaticum* (Španiel, Zozomová-Lihová, *et al.* 2017; Zozomová-Lihová *et al.* 2020), *Euphorbia myrsinites* (Falch *et al.* 2019) and *Campanula pyramidalis* and *C. austroadriatica* (Lakušić *et al.* 2013) separated by Neretva river valley in the Central Dinaric Mountains; and *Heliosperma pusillum* group (Frajman and Oxelman 2007) separated by the karst rivers Drina and Sutjeska in the Southern Dinaric Mountains. Secondary postglacial contacts between populations that have experienced some differentiation during glacial or interglacial periods, without developing a complete reproductive barrier, lead to admixture of lineages and thus reflect complex patterns (Rešetnik *et al.* 2020; Zozomová-Lihová *et al.* 2020; Reich *et al.* 2021).

Dianthus sylvestris Wulfen (Caryophyllaceae)

Dianthus is a taxonomically difficult genus (Tutin and Walters 1993) with over 100 species occurring in Europe, and with more than 70 endemics in geographically restricted areas (Valente *et al.* 2010). Tutin and Walters (1993) largely follow the systematic treatment of *Dianthus* by Pax and Hoffmann (1934), who estimated the number of species to be close to 300. Moreover, recent taxonomic research has been largely focused on the description of new taxa (Mazzola *et al.* 2004; Meyer 2011; Brullo *et al.* 2015).

According to the scenario proposed by Valente *et al.* (2010), *Dianthus* diversified with a constant rate until 2.0−1.3 Myr ago (Early−Mid-Pleistocene), when an acceleration in diversification took place, coinciding with a period of profound climatic transformations in the Pleistocene (Willis 1994). Furthermore, the diversification of the Eurasian lineage of *Dianthus* is assumed to be still an ongoing process (Valente *et al.* 2010), which is reflected in the taxonomic complexity due to uncertainties in species boundaries. Its rapid radiation did not yet allow for the accumulation of mutations in the sequenced genomic regions, while phenotypic characters evolved more quickly (Fassou *et al.* 2022). Since most of the available species' treatments are based on classical morphology, most of the subgenera and sections do not represent natural groups (Fassou *et al.* 2022). The most widely used intrageneric classification has been that of Pax and Hoffmann (1934), until the work of Fassou *et al.* (2022), which showed little correlation with previous systematic treatments.

One of the taxonomically intricate species of the genus is *Dianthus sylvestris* (Figure 1). This species has divergent habitat preferences, and it grows from the sea level to the alpine zone and it is associated with both carbonate and serpentinite flora (Gammella 2016). Due to its high morphological and ecological variability, it has been getting more attention recently from taxonomists (Bacchetta *et al.* 2010; Domina *et al.* 2021) and researches in the field of plant ecological genetics (Gammella 2016; Luqman *et al.* 2022). *Dianthus sylvestris* was described and named as a species in 1786 by Wulfen. One or more forms of this plant were previously known to Séguier (1745), who recorded its occurrence between Trent and Verona, and formed a phrase: "*Caryophyllus silvestris flore rubro inodoro*". It is probably the plant mentioned by Linné (1753) as an unscented variety of *D. Caryophyllus;* Linne cited the Séguier's depiction for *D. Caryophillus* var. *inodorus,* and extracted the name "*inodorus*" from the Séguier's phrase. The majority of Wulfen's collection is deposited at W (herbarium acronyms follow Thiers 2021). However, twenty percent of collections at W have been lost in World War II, including parts of Caryophyllaceae, and it is assumed that the Wulfen's original material of *D. sylvestris* was destroyed. Consequently, the original material used for the description of several taxa was unknown. The first push forward to elucidating the taxonomic uncertainties of this taxon came from Bacchetta *et al.* (2010), who treat *D. sylvestris* occurring on the central and southern Apennine Peninsula, Sardinia and Sicily as a species complex.

Figure 1. *Dianthus sylvestris* from Jacquin (1781-1787, Tav. 82).

Regarding the area of the Balkan Peninsula, numerous intraspecific entities on different taxonomic levels (subspecies, varieties, forms) were reported by many authors over the last 200 years, and the authors' opinions on their taxonomic treatment is often conflicting (Schlosser and Vukotinović 1869; Borbás 1876; Beck-Mannagetta 1909; Hayek 1924; Gjurašin 1933; Mayer and Trpin 1965; Trinajstić 1979). According to Marhold (2011b), seven subspecies have been recorded for the Balkans: *D. sylvestris* subsp. *bertisceus* Rech. f., *D. sylvestris* subsp. *kozjakensis* Micevski, *D. sylvestris* subsp. *longicaulis* (Ten.) Greuter et Burdet, *D. sylvestris* subsp. *nodosus* (Tausch) Hayek, *D. sylvestris* subsp. *siculus* (C. Presl) Tutin, *D. sylvestris* subsp. *sylvestris* and *D. sylvestris* subsp. *tergestinus* (Rchb.) Hayek. Four subspecies have been recorded on the Croatian territory (Flora Croatica Database – FCD, Nikolić 2020); *D. sylvestris* subsp. *longicaulis*, *D. sylvestris* subsp. *nodosus*, *D. sylvestris* subsp. *sylvestris*, *D. sylvestris* subsp. *tergestinus*). However, there is still a dispute concerning the number of taxa and in-depth analyses are required for acquiring a sensible taxonomic circumscription of Balkan populations. Type specimens are known for five taxa. The iconography from Jacquin (1781-1787, Tav. 82) was designated by Bacchetta *et al.* (2010) as a neotype for the typical subspecies; syntypes of *D. sylvestris* subsp. *bertisceus* are kept in G (Geneva, Switzerland) and K (Kew, London UK); the holotype of *D. sylvestris* subsp. *kozjakensis* is in the University Herbarium in Skopje; the lectotype of *D. sylvestris* subsp. *longicaulis* is in NAP (Naples, Italy); and the holotype of the last described subspecies *D. sylvestris* subsp. *alboroseus* is in JE (Jena, Germany). For *D. sylvestris* subsp. *nodosus* the type specimen has not been designated yet.

THESIS OUTLINE

This thesis summarizes four published scientific publications (**I – IV**), one scientific paper in preparation (**Publication V**), and two unpublished results (**Appendix I – II**) in which all the research hypotheses and objectives are addressed.

Research hypotheses: (1) There is genetic variability between populations of *D. sylvestris* s.l. associated with the existence of microrefugia on the Balkan Peninsula; (2) Populations belonging to different subspecies, evolutionary lineages and/or different habitat types can be identified according to their morphological diagnostic characters.

Research objectives: (1) Conduct morphometric and genetic analysis with the aim of solving the taxonomic problems of *D. sylvestris* s.l. and gaining insight into phylogeographic processes at the local and regional level; (2) Develop models of habitat suitability for the present and the past and analyse the ecological niches of *D. sylvestris* subspecies with the aim of investigating the relationship between their morphological, genetic and environmental divergence; (3) Conduct a taxonomic revision of *D. sylvestris* s.l. on the Balkan Peninsula.

Publications contributing to the first and second objectives are **III-V**, and all the included publications contribute to some extent to the third objective.

First objective: A detailed exploration of morphological characters (**Publication IV**) coupled with genetic analyses (**Publication V**) revealed a gradual change that follows geography, that is a southeast – northwest cline of both morphological diversity and genetic structure. The morphometric analyses based on 15 morphological characters did not show any clear groupings of populations that would correspond to the subspecies, but rather revealed two groups, namely one group consisting of north-western, and the other group including the south-eastern Balkan populations. Although showing morphological overlap with two sympatric subspecies, populations identified as *D. sylvestris* subsp. *tergestinus* were genetically divergent from all other Balkan populations. **Publication V** explores the potential phylogeographic process that could have driven the diversification of *D. sylvestris* on the Balkan Peninsula, that is allopatric diversification and speciation promoted mainly by geographic barriers.

Second objective: By quantifying niche overlap and performing niche equivalency and similarity tests (**Appendix I**), niche conservatism was revealed to be more common, since there was not a single significant case of niche divergence. In **Publication V**, two separated glacial refugia are identified and potential migration patterns are discussed, contributing to understanding the phylogeography of *D. sylvestris* s.l. on the Balkan Peninsula. The finding of tetraploid populations only within the north-western Balkan group (**Publication III**) implies that the northern Balkan Peninsula was under more pronounced climatic oscillations compared to the more stable conditions in the south, and that these climatic transitions could have been the driving force for polyploidization.

Third objective: Firstly, nomenclatural issues were addressed within the first two publications. Results of **Publication I** represent a detailed inventory of the historical herbarium material, the current composition of *D. sylvestris* taxa in Croatian herbarium collections (ZA and ZAHO) and their distributions across the Balkans. In **Publication II** lectotypes were chosen for the names *D. sylvestris* Wulfen and *D. virgineus* var. *tergestinus* Rchb., strengthening the applications of names lacking type material. Also, in **Appendix II** a lectotype was chosen among the syntypes for the name *D. sylvestris* subsp. *bertisceus*. **Publication III** revealed several areas of pronounced genome size variation at small geographic distances on the Peninsula, likely indicating intraspecific divergence. However, these divergences are not indicative of the subspecies, thus genome size did not show taxonomic value. **Publication IV** indicates that some morphological characters are variable even within the population level, and thus their taxonomic importance becomes questionable. **Publication V** gives the final evidence which advocates for a comprehensive reduction of the number of subspecies on the Balkan Peninsula. Based on all obtained results, a new taxonomic treatment and a key for the identification of *Dianthus sylvestris* taxa on the Balkan Peninsula were proposed in **Appendix II**.

INDIVIDUAL PUBLICATIONS

Publication I

Inventory of the historical *Dianthus sylvestris* herbarium materials from Herbarium Croaticum and Herbarium Ivo and Marija Horvat

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Povijest članka / article history: primljeno / received: 4.12.2019., prihvaćeno / accepted: 26.4.2020.
URL: https://doi.org/10.46232/glashbod.8.1.2

Terlević, A., Rešetnik, I. (2020): Inventory of the historical *Dianthus sylvestris* herbarium materials from Herbarium Croaticum and Herbarium Ivo and Marija Horvat. Glas. Hrvat. bot. druš. 8(1): 8-14.

Abstract

In order to initiate a taxonomical revision of the *Dianthus sylvestris* group, an inventory of historical herbarium materials from ZA and ZAHO collections was conducted. Herbarium specimens of *D. sylvestris* group from these two herbaria were digitized and the data from the original herbarium labels were inserted in the Flora Croatica Database. A total of 344 herbarium sheets were digitized and six taxa (*D. sylvestris* Wulfen in Jacq. ssp. *sylvestris*, *D. sylvestris* ssp. *tergestinus* (Rchb.) Hayek, *D. sylvestris* ssp. *longicaulis* (Ten.) Greuter et Burdet, *D. sylvestris* ssp. *nodosus* (Tausch) Hayek, *D. siculus* C. Presl and *D. arrosti* C. Presl) were registered within studied collections. Inventory of herbarium sheets from ZA and ZAHO historical collections provided a significant insight into historical distributional data of *D. sylvestris* taxa related to the area of the Balkan Peninsula, which is a prerequisite for accurate taxonomic/geographic sampling for further morphological and molecular analyses.

Keywords: Balkan Peninsula, digitization, distribution, Flora Croatica Database

Terlević, A., Rešetnik, I. (2020): Pregled povijesnog herbarijskog materijala vrste *Dianthus sylvestris* u zbirkama Herbarium Croaticum i Herbarij Ive i Marije Horvat. Glas. Hrvat. bot. druš. 8(1): 8-14.

Sažetak

Kako bi se započelo s taksonomskom revizijom grupe svojti koje pripadaju *Dianthus sylvestris*, provedeno je popisivanje povijesnog herbarijskog materijala iz zbirki ZA i ZAHO. Herbarijski primjerci vrsta koje pripadaju grupi *D. sylvestris* su digitalizirani, a podaci s originalnih herbarijskih etiketa su uneseni u bazu podataka Flora Croatica. Digitalizirano je ukupno 344 herbarijskih listova i zabilježeno je šest svojti (*D. sylvestris* Wulfen in Jacq. ssp. *sylvestris*, *D. sylvestris* ssp. *tergestinus* (Rchb.) Hayek, *D. sylvestris* ssp. *longicaulis* (Ten.) Greuter et Burdet, *D. sylvestris* ssp. *nodosus* (Tausch) Hayek, *D. siculus* C. Presl i *D. arrosti* C. Presl). Popisivanje herbarijskih listova ovih svojti iz ZA i ZAHO pruža uvid u povijesne podatke o rasprostranjenosti svojti iz grupe *D. sylvestris* na području balkanskog poluotoka, što je preduvjet za ispravno taksonomsko/geografsko uzorkovanje za daljnje morfološke i molekularne analize.

Ključne riječi: Balkan, baza podataka Flora Croatica, digitalizacija, rasprostranjenost

Introduction

Dianthus sylvestris group is considered to be one of the most complex groups within the genus *Dianthus* L. (Caryophyllaceae). An important diversity centre is the central Mediterranean area (Balkan and Apennine Peninsula) where the number of taxa belonging to this group is high. They mainly occur in rupestrian habitats, as well as on rocky and sunny grasslands, garigues, steppes and mesic meadows (Bacchetta et al. 2010). Real taxonomical value of taxa belonging to this group is often doubtful, and synonymy confusion is common. The group is morphologically characterized by woody stocks, shortly branched, usually with dense terminal leaf rosettes, linear and acute leaves, flowers not or slightly fragrant, arranged in loose cymes, epicalyx scales 2-6(-8), glabrous, abruptly contracted into a short mucro, 3-5 times shorter than calyx, glabrous petals with limb denticulate at apex or entire (Pignatti 1982, Tutin & Walters 1993). Subspecies listed by Marhold (2011), regarding the Euro-Mediterranean area, are *Dianthus sylvestris* ssp. *bertisceus* Rech. f., *D. sylvestris* ssp. *kozjakensis* Micevski, *D. sylvestris* ssp. *longicaulis* (Ten.) Greuter et Burdet, *D. sylvestris* ssp. *nodosus* (Tausch) Hayek, D. sylvestris ssp. *siculus* (C. Presl) Tutin, *D. sylvestris* ssp. *tergestinus* (Rchb.) Hayek and *D. sylvestris* ssp. *sylvestris*. In addition, *D. sylvestris* ssp. *alboroseus* F.K. Meyer, a pale pink to white flowering form, was described from southern Albania (Meyer 2011). According to Bacchetta et al. (2010), in central and southern Italy, Sardinia and Sicily this group is represented by 17 morphologically and ecologically well differentiated species.

The *D. sylvestris* group is very polymorphic and no reasonable key on subspecific level can be presented. Since an in-depth taxonomical review of this group needs to be done, the first step is to analyse the existing herbarium data from ZA (Herbarium Croaticum) and ZAHO (Herbarium of Ivo and Marija Horvat) collections. Therefore, the aim of this article is to present: (i) an update of the distribution data based on herbarium data, (ii) taxa analysis in terms of spatial distribution, collectors and collecting dates.

Materials and methods

The herbarium specimens of *D. sylvestris* group were searched for and used for the purpose of digitization within two Croatian herbaria, ZA and ZAHO (acronyms are according to Thiers 2019). A regular procedure within these herbaria include the following steps: mounting, systematisation and digitization. According to Šegota et al. (2017), first the plant material was placed on a new paper, mounted with pH neutral adhesive tape on herbarium sheets while the herbarium labels were glued with Gaylord pH neutral white adhesive. The original metadata for *D. sylvestris* group herbarium sheets were inserted within the Flora Croatica Database (Nikolić 2019). The metadata include: herbarium ID, taxon name, locality, habitat, geographical coordinates, collector(s) and identifier(s) name, and collection dates. Most of these data were provided from the original label's text, while data such as geographical coordinates were derived from an up-to-date interpretation of collection locality. Finally, the prepared sheets were scanned using available scanning equipment (inversed Epson Expression 11000XL Pro A3 scanner) with the image resolution of 300 dpi (tiff). A stamp with the ZA

Figure 1. Herbarium sheets of *Dianthus sylvestris* group taxa after digital imaging, (a) ZA 47545, (b) ZAHO 46581.

and ZAHO herbarium ID, colour plate and ruler were added to each sheet prior to scanning (Fig. 1). Finally, the images were uploaded in the Flora Croatica Database and are accessible on virtual portal of the Herbarium Croaticum.

Results and discussion

In total, 344 herbarium sheets were found and digitized within the two studied collections. In the ZA *Herbarium Generale* (collection formed via exchange with other world herbaria) 25 specimens of *D. sylvestris* group were discovered. The ZA collection holds 207 herbarium sheets (60%) and ZAHO collection holds 137 sheets (40%). Altogether, six taxa belonging to *D. sylvestris* group were registered, four of them being native to Croatia. Specimens determined as *D. sylvestris* s.l. occurred on the largest number of herbarium sheets (235), followed by *D. sylvestris* ssp. *tergestinus* (69), *D. sylvestris* ssp. *nodosus* (33), *D. sylvestris* ssp. *longicaulis* (3), *D. arrosti* C. Presel (2), *D. siculus* C. Presel (1) and *D. sylvestris* ssp. *sylvestris* (1) (Tab. 1).

Dianthus arrosti and *D. siculus* are not native to Croatia. The species *D. arrosti* occurs in Sicily, Sardinia and southern Calabria (Bacchetta et al. 2010), and according to Marhold (2011) it is also native to Algeria and Morocco. One herbarium specimen found in ZA *Herbarium Generale* originates from Dr. L. Nicotra – Herbarium Siculum (PAL, Palermo, Italy), and the other from Dr. C. Baenitz, Herbarium Europeum (MNHM, Paris, France). However, locality descriptions on herbarium labels are not clear. *D. siculus* is common in Italy, Corse, Spain, Morocco, Algeria and Tunisia (Bacchetta et al. 2010, Marhold 2011). In Euro+Med PlantBase (Marhold 2011) it is registered as *D. sylvestris* ssp.

Table 1. The number of digitized herbarium sheets for each taxon across the studied collections.

Taxon name	ZA	ZAHO	total
Dianthus sylvestris Wulfen in Jacq.	103	132	235
Dianthus sylvestris Wulfen in Jacq. ssp. *tergestinus* (Rchb.) Hayek	66	3	69
Dianthus sylvestris Wulfen in Jacq. ssp. *nodosus* (Tausch) Hayek	32	1	33
Dianthus sylvestris Wulfen in Jacq. ssp. *longicaulis* (Ten.) Greuter et Burdet	3	0	3
Dianthus arrosti C. Presl	2	0	2
Dianthus sylvestris Wulfen in Jacq. ssp. *sylvestris*	0	1	1
Dianthus siculus C. Presl	1	0	1
Grand total	**207**	**137**	**344**

siculus (C. Presl) Tutin. The single herbarium specimen found in ZA Herbarium Generale originate from the collection of O. Debeaux – Plantes d`Algerie.

Herbarium specimens originate from 12 European countries and from Algeria (Fig. 2); however, the majority were collected in Croatia (57%). As much as 23 herbarium sheets in ZA, and 7 herbarium sheets in ZAHO collection could not be georeferenced

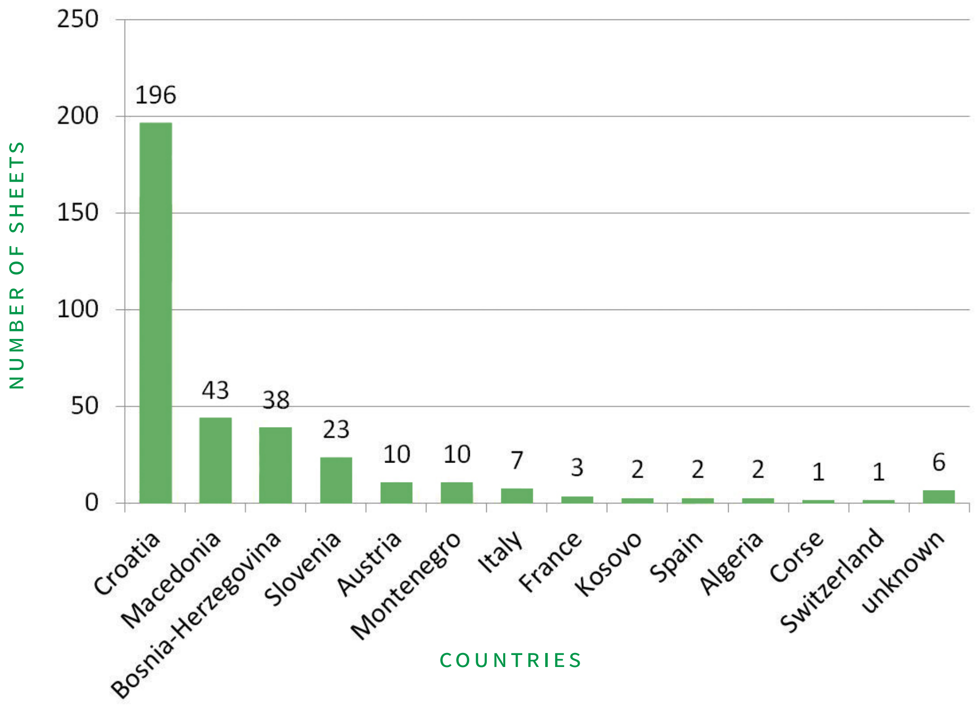

Figure 2. Geographical origin of *D. sylvestris* group herbarium specimens stored across studied collections.

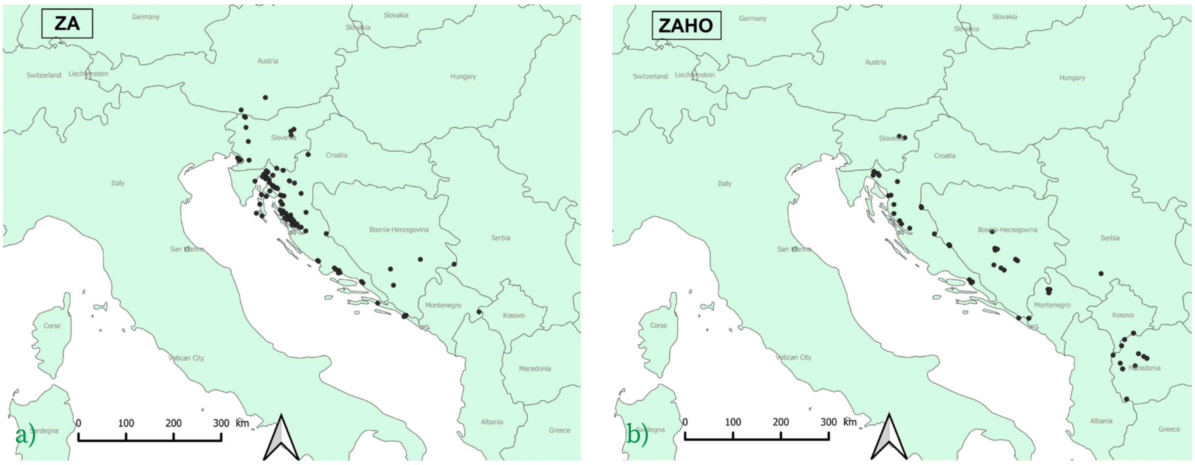

Figure 3. Distribution maps of *D. sylvestris* group herbarium specimens from (a) ZA and (b) ZAHO collection.

due to ambiguous locality description or due to the lack of any locality description, except the country. Likewise, all of the 25 herbarium sheets from *Herbarium Generale* have not been georeferenced for the same reasons. Regarding the Croatian territory, the collection sites of taxa are localized mostly on Mt Velebit, which reflects field activities of collectors of those two collections (Fig. 3). The distribution of taxa in Croatia based on historical herbarium data (Fig. 4) showed overlaps with the field record data made by the authors during 2018 and 2019 fieldworks (location data not shown).

The most productive collector of the studied herbarium materials was Ivo Horvat, the lone collector of ZAHO, with 135 herbarium sheets. Ljudevit Rossi stored 40 herbarium sheets in ZA, Dragutin Hirc 30, Ivo Pevalek 17, Josip Klasancije Schlosser 13, Ljudevit Vukotinović 12, while other 44 collectors contributed with less than 10 herbarium sheets each. The majority of specimens were collected between 1910s and 1940s (Fig. 5) which is in line with the previous reports (Strgulc Krajšek et al. 2009, Šegota et al. 2017). As much as 49 herbarium specimens lack data on collecting period, i.e. the year of collecting was either missing from the label or it was illegible. The oldest specimen kept at ZAHO was collected in 1920, whereas the oldest specimens from ZA date back to 1853 for *Herbarium Generale* (unknown collector), and to 1852 for the main ZA collection (specimen collected by Lj. Vukotinović and K. Schlosser).

Taxa of *D. sylvestris* group which have not been found in ZA and ZAHO collections, but are known from the Balkan Peninsula, are *D. sylvestris* ssp.

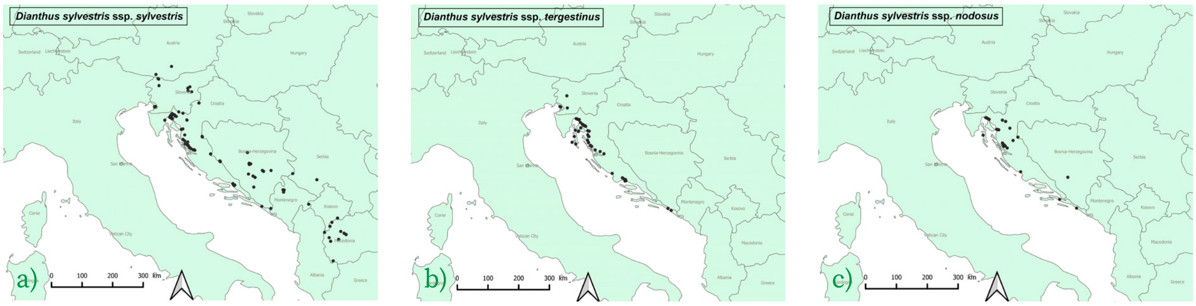

Figure 4. Individual distribution maps for (a) *D. sylvestris* (including the single herbarium specimen stored as *D. sylvestris* Wulfen in Jacq. ssp. *sylvestris*), (b) *D. sylvestris* ssp. *tergestinus*, and (c) *D. sylvestris* ssp. *nodosus* stored across studied collections.

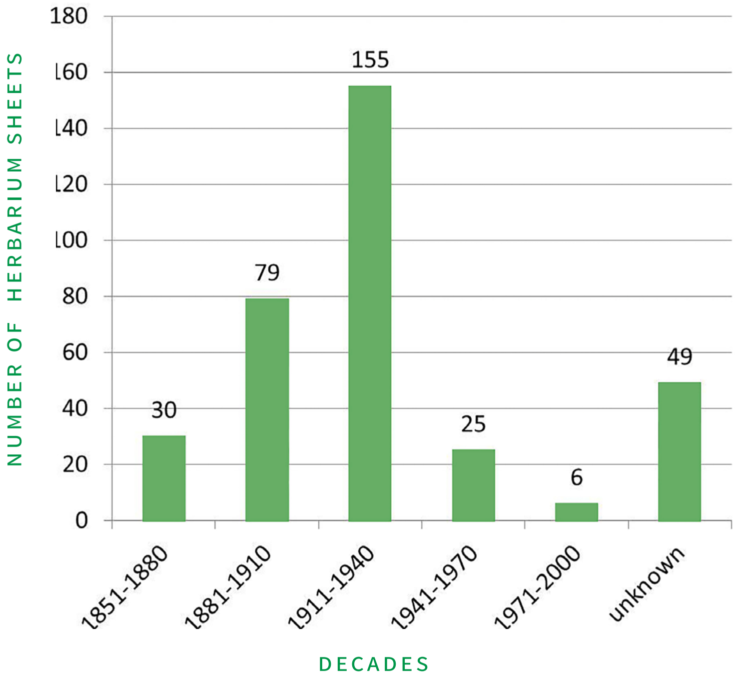

Figure 5. Temporal distribution of the herbarium specimens based on collection period shown in decadal scale.

alboroseus, D. sylvestris ssp. *bertisceus* and *D. sylvestris* ssp. *kozjakensis.* However, these taxa have rather restricted distribution areas that were rarely covered by field activities of main ZA collectors. Moreover, taxa *D. sylvestris* ssp. *kozjakensis* and *D. sylvestris* ssp. *alboroseus* were described only recently by Micevski (1990) and Meyer (2011), respectively.

These results represent the current state of *D. sylvestris* group taxa composition within ZA and ZAHO collections. Apart from historical herbarium specimens, *D. sylvestris* collections from ZA have recently been growing due to extensive sampling of *D. sylvestris* taxa throughout the Balkans (Croatia and neighbouring countries). Sampling of *D. sylvestris* material for morphological and molecular analysis is under way, in order to resolve taxonomic, phylogenetic as well as phylogeographic relationships among taxa. After the morphological characterisation of taxa, it will be possible to carry out a thorough taxonomic revision, from which a different view on taxa composition in these herbaria could be obtained.

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Typification of 14 names in the *Dianthus virgineus* group (Caryophyllaceae)

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Abstract

The nomenclature of 14 taxa from Central and Southern Europe within the *Dianthus virgineus* group is discussed. *Dianthus aggericola* Jord., *D. collivagus* Jord., *D. consimilis* Jord., *D. orophilus* Jord., *D. saxicola* Jord., *D. juratensis* Jord. are here lectotypified by specimens from the Jordan herbarium in LY, while *D. godronianus* Jord. by a specimen in P. *Dianthus subacaulis* Vill. is neotypified by a specimen collected on Mont Ventoux (S. France) and housed in MPU. For *D. sylvestris* Wulfen, a lectotype is here designated and its previous neotypification is discussed. *Dianthus caryophyllus* var. *tenuifolius* Moris, *D. caryophyllus* f. *minor* Moris and *D. sylvestris* var. *garganicus* Ten. are lectotypified by specimens housed in herbarium Moris (TO) and herbarium Tenore (K). *Dianthus virgineus* var. *tergestinus* Rchb. is lectotypified by a drawing from the *Icones florae Germanicae & Helveticae,* while *D. contractus* var. *evolutus* Lojac. is neotypified by a specimen in P. For each taxon the currently accepted name is provided including new synonymies. The type indication is followed by nomenclatural and taxonomic notes, in which the original material found is commented and the reasons for the identification of the types are discussed.

Keywords

Dianthus, France, Italy, Slovenia, nomenclature

Introduction

Dianthus L. (Caryophyllaceae) includes about 300 species from the temperate regions of the Old World, many of which are narrow endemics (Hardion et al. 2020). This genus still shows challenging systematics. A large part of recent taxonomic research, in fact, has been focused on the description of new taxa. Since 2000, 64 new species and subspecies have been described for the Euro-Mediterranean area, and a total of 98 new names have been published (IPNI 2021). Conversely, the taxonomic contributions on *Dianthus* that have taken into consideration groups of species with statistical analyses of morphological data or molecular investigations are very few (Domina et al. 2017; Hardion et al. 2020). Furthermore, the genus-level taxonomic treatments date back to more than 50 years ago (Williams 1890, 1893; Pax and Hoffmann 1934; Reeve 1967), and the recently published treatments of *Dianthus* in national flora (Bernal et al. 1990; Tison and de Foucault 2014; Vangjeli 2015; Brullo and Guarino 2017; Nikolić 2020) have not brought any significant change to the taxonomy of this genus. In several cases, the nomenclatural types for these taxa are not yet designated (Domina & al. 2021). This created a situation of taxonomic uncertainty. *Dianthus* is an interesting genus, both from a biological and economical point of view (Hardion et al. 2020). Hence, an integrated approach to the taxonomy of this genus is needed.

The *D. virgineus* L. group can be considered as one of the richest and most complex in the genus. Many taxa have been described from Central and Southern Europe, albeit their actual taxonomical value is often doubtful. The genus has undergone extensive taxonomic investigations since the 18th century (Smith 1794), but in many cases the original material used for the description of the taxa is not known and the nomenclatural types have not been designated yet. The lectotype of *Dianthus virgineus* L., the oldest available name that applies to wild plants in this group, has been designated only recently (Domina et al. 2021). The nomenclature and morphology of the large part of taxa described from Southern Italy, Sardinia, and Sicily have been investigated by Bacchetta et al. (2010). Other typifications were published by Camarda and Corrias (1987), Brullo et al. (2000), Arrigoni (2006), and Clementi et al. (2015). However, most of the taxa described in this group are still to be investigated.

In this study, the nomenclature of 14 taxa is discussed: *Dianthus aggericola* Jord., *D. collivagus* Jord., *D. consimilis* Jord, *D. godronianus* Jord., *D. orophilus* Jord., *D. saxicola* Jord., *D. juratensis* Jord., and *D. subacaulis* Vill. from S. France; *D. sylvestris* Wulfen from NE Italy/SW Slovenia; *D. virgineus* var. *tergestinus* Rchb. from NE Italy; *D. caryophyllus* var. *tenuifolius* Moris and *D. caryophyllus* f. *minor* Moris from Sardinia; *D. sylvestris* var. *garganicus* Ten. from S Italy; and *D. contractus* var. *evolutus* Lojac. from Sicily.

As part of an ongoing project aimed to push forward the taxonomic knowledge on selected genera of the Italian vascular flora, this study aims to lay the foundations for further taxonomic investigations by an integrated approach based on morphometric and molecular data (Domina et al. 2021; Giacò et al. 2021).

Material and methods

We examined the scientific literature for the effective place of publication of surveyed *Dianthus* names described from Central and South Europe. The bibliographic data was searched in the available digital sources and in the libraries of the European institutions, while the original material of the investigated species was searched in the main European herbaria: B, C, FI, G, K, MA, MPU, NAP, P, PAL, RAB, RO, TO, W, WU, and ZA; herbarium acronyms follow Thiers (2021). A start-up online screening was possible thanks to digital herbarium specimens' images provided by GBIF (https://www.gbif.org), Jstor (http://plants.jstor.org), and ReColNat (https://www.recolnat.org/fr/). More thorough investigations were conducted in the Jordan herbarium at LY. The articles of the International Code of Nomenclature for Algae, Fungi, and Plants (hereafter ICN) follow Turland et al. (2019). Until more in-depth, integrated morphometric, genetic, karyological, and ecological information comes to light, our judgments should be considered provisionally accepted, according to current knowledge. In this group, the characters that have been proven to best discriminate species (Bacchetta et al. 2010) are: leaf length and width, number of flowers per scape, shape and length of outer and inner bracts. These characters have been used to check the morphological features of the selected types. The anther and petal length have been proposed as additional discriminant characters, but these can be easily appreciated on fresh plants and not on herbarium samples.

Typification of the names

Dianthus aggericola Jord. in Billot, Annot.: 48. 1856. [December 1856]

= *D. virgineus* L., Sp. Pl. 1: 412. 1753. Ind. Loc.: "du Reculet (Ain)".

Type. (lectotype here designated): *Dianthus aggericola* Jord., du Reculet, 8 July 1854 [A. Jordan], LY0079734!

Note. No other original material was found in the surveyed herbaria. According to the label, this plant was originally collected in Reculet and then grown in Jordan's garden, where it was collected in July. As a matter of fact, besides his huge herbarium and library, Alexis Jordan owned a one-hectare experimental garden. We know that he used it to sow most species every year, while maintaining alive perennial ones, and that he regularly made herbarium sheets from these cultivated plants. In this context, the notion of original material requires particular attention (Thiébaut and Tison 2016).

The lectotype designated here matches the protologue and corresponds to the current application of the name, which is considered a heterotypic synonym of *D. godronianus* Jord. in Kerguélen (1993), in turn, currently considered a heterotypic synonym of *D. virgineus* (Domina et al. 2021). The lectotype of *D. aggericola* and that of *D. virgineus*, have the same leaf length and width, uniflowered scapes, the same length and shape of outer and inner bracts, the same calyx length and shape. We, therefore, confirm this synonymy.

Dianthus caryophyllus var. *tenuifolius* Moris, Fl. Sardoa 1: 231. 1837. [April 1837]

≡ *D. siculus* subsp. *tenuifolius* (Moris) Arrigoni, Parlatorea 7: 20. 2005.
= *D. genargenteus* Bacch., Brullo, Casti & Giusso, Nordic J. Bot. 28(2): 145. 2010.
Ind. Loc.: "In sterilibus frequens [Sardinia]".

Type. (lectotype here designated): *Dianthus caryophyllus tenuifolia*, prope Belvì, July, inter rupe / Mus. Bot. Horti Taurinensis, Herb. Moris Barbey Cat Sard. N.156, TO!

Note. Three herbarium sheets are kept in TO, with several individuals each. All three specimens bear labels handwritten by Moris but lack the year of collection. Two of them come from generic localities ("in arenis maritimis" and "in collibus"), while one is from Belvì in the centre of Sardinia (Nuoro). All the specimens are complete and in good condition but refer to different collections: two specimens have been collected in inner localities, whereas another one comes from the coast. Moris reports that the scape bears a single flower and that another taxon (*D. caryophyllus* var. *tenuifolius* f. *minor*) grows in arenosis maritimis [sandy coast]. Thus, here we propose the specimen from Belvì as lectotype, despite not being dated, assuming that the herbarium in TO hosts the original material by Moris as already done by Arrigoni (1979), Rizzotto (1989), Escobar García et al. (2010) in other similar cases,

Based on the specimen in TO coming from the coast (referring actually to f. *minor*), Valsecchi (1985), and then Bacchetta et al. (2010), refer *D. caryophyllus* var. *tenuifolius* to *D. morisianus* Vals. Based on the diagnosis and the lectotype designated here, *D. caryophyllus* var. *tenuifolius* does not belong to *D. morisianus*. The former taxon shows short scapes bearing one or few flowers and epicalyx scales with mucro 0.5–1.5 mm long, while the latter shows longer multiflowered scapes with epicalyx scales with mucro 2.0–3.5 mm long. This interpretation agrees with Arrigoni (2010). According to the lectotype of *D. caryophyllus* var. *tenuifolius*, which shows woody stocks contracted with branches, epicalyx scales with an evident mucro, and small calyx, this taxon is a heterotypic synonym of *D. genargenteus* Bacch., Brullo, Casti & Giusso.

Dianthus caryophyllus f. *minor* Moris, Fl. Sardoa 1: 231. 1837. [April 1837]

= *D. morisianus* Vals., Boll. Soc. Sarda Sci. Nat. 24: 333. 1985. Ind. Loc.: "In arenosis maritimis [Sardinia]".

Type. (lectotype here designated): *Dianthus caryophyllus* var *tenuifolia*, in arenosis maritimis, S. Nicolai flumini major Majo junio / Mus. Bot. Horti Taurinensis, Herb. Moris Barbey Cat Sard. N.156, TO!

Note. A single sheet was found in TO. Albeit it may represent the holotype, it is cautiously designated here as a lectotype.

The selected specimen, uniflorous, has fixed seven portions of plants whose leaves and flower scapes are smaller than those of the typical form. All other characters of the flowers correctly match the protologue. This taxon is a heterotypic synonym of

D. morisianus, a species described by Valsecchi (1985) for the same area and habitat (Peruzzi et al. 2015), that shows the same leaf length and width and, albeit with multiflowered scapes, the same length and shape of outer and inner bracts, and the same calyx length and shape.

Dianthus collivagus **Jord. in Billot, Annot.: 46. 1856. [December 1856]**

≡ *D. caryophyllus* var. *collivagus* (Jord.) Cariot & St-Lager Étude Fl., éd. 8, 2: 104. 1889.
=? *D. sylvestris* Wulfen in Jacq., Coll. 1: 237. 1786. [January-September 1786]. Ind. Loc.: “abonde sur les côteaux du Rhône près de Lyon”.

Type. (lectotype here designated): *Dianthus scheuchzeri* Rchb., *Dianthus sylvestris* auct. Gall. ex parte non Wulf, Lyon a Néron, Jordan, odor levis, folia ramis trigemina semper angustissima; *Dianthus scheuchzeri* Jord. non Rchb., *Dianthus collivagus* Jord., Lyon à Neron, ex herbis Jordan, July 1854, CLF056818!

Note. Other six specimens collected by Jordan are preserved at LY, but they are not original material, since they are lacking a date or reporting dates later than the protologue.

The lectotype designated here matches the protologue and corresponds to the current application of the name, which is considered as a heterotypic synonym of *D. sylvestris* subsp. *sylvestris* in Kerguélen (1993). The lectotype of *D. collivagus*, concerning the shape of calyx teeth, is very similar to the lectotype of *D. inodorus* (L.) Gaertn., which in turn is currently included within the variability of *D. sylvestris* (Domina et al. 2021).

Dianthus consimilis **Jord. in Billot, Annot. 47. 1856. [December 1856]**

≡ *D. caryophyllus* var. *consimilis* (Jord.) Rouy & Foucaud in Rouy, Fl. Fr. 3: 195. 1896.
=? *D. sylvestris* Wulfen in Jacq., Coll. 1: 237. 1786. [January-September 1786]. Ind. Loc.: “Alpes de l’Oisans”.

Type. (lectotype here designated): *Dianthus consimilis* Jord., June-July 1855, [A. Jordan] Roux, Herbier Jordan, LY0079676!

Note. At LY we found another specimen citing “Lautaret (H. Alpes, May, June-July 1855, ex Horto Alexis Jordan, LY0079674!” but lacking basal leaves.

The lectotype designated here matches the protologue and corresponds to the current application of the name, which is considered as a heterotypic synonym of *D. sylvestris* subsp. *sylvestris* in Kerguélen (1993). The lectotype of *D. consimilis*, concerning the shape of calyx teeth, is very similar to the lectotype of *D. inodorus* (L.) Gaertn., which, in turn, is currently included within the variability of *D. sylvestris* (Domina et al. 2021).

***Diantbus contractus* var. *evolutus* Lojac., Fl. Sicul. 1(1): 165. 1888. [September 1888]**

= *D. arrostoi* C.Presl, Delic. Prag. 60. 1822. Ind. Loc.: "Sulle più alte vette delle Nebrodi sui terreni ghiaiosi o sulle rupi calcaree di Serre di Quacedda. Juntera Minà Pal!".

Type. (neotype here designated): *Dianthus contractus* Jan., *Dianthus constrictus* Janka, In asperis calcareis elatioribus montis Nebrodes, Julio, M. Lojacono Pojero, P05052873 (photo!).

Note. Neither the original material nor traces of this taxon were found in the herbaria consulted and among the documents accompanying the centuries distributed by Lojacono (Aghababyan et al. 2012; Domina et al. 2014). We chose to designate as a neotype the single specimen found, which is at least collected by Lojacono.

The neotype designated here matches the protologue and allows to consider this name as an heterotypic synonym of *D. arrostoi* C.Presl. Compared to the lectotype of *D. contractus* designated by Bacchetta et al. (2010: 151: s.l., s.d., Jan, NAP-GUSS!), and to the lectotype of *D. arrostoi* designated by Camarda and Corrias (1987: 417), this variety differs only by the more elongated scapes.

***Dianthus godronianus* Jordan in Mém. Acad. Roy. Sci. Lyon, Sect. Sci., ser. 2, 1: 241. 1851. [January 19851]**

≡ *D. caryophyllus* subsp. *godronianus* (Jord.) P.Martin, Soc. Ech. Pl. Vasc. Eur. Bassin Médit. 19: 93. 1984.

≡ *D. sylvestris* var. *godronianus* (Jord.) Kerguélen, Lejeunia, Nouv. Sér., 120: 81. 1987.

= *D. virgineus* L., Sp. Pl. 1: 412. 1753. Ind. Loc.: Coteaux stériles de la région des oliviers. Provence, Hyères, Marseille, Toulon, Apt, mont Ventoux, Vaucluse, Villeneuve; Dauphinée, Rabou près de Gap, Valence, Avignon, Languedoc, Viviers, pont du Gard, Uzés, Montpellier, Mende, Perpignan Corse, Calvi, Bastia, Cervione, Evisa, Otta, Campitello.

Type. (lectotype here designated): Soleirol, Herb. Cors., 959 *Dianthus virgineus* L. (Gren. et Godr.), *Dianthus sylvestris* Duby, Bastia - mai 1823, P05000349 (photo!).

Note. – Jordan (1851, 1856) believed that the plants referred by Godron (1847, 1848) to *D. virgineus* L. actually represent a different species, which he renamed *D. godronianus*. According to Godron (1848), this species grows in the surroundings of Montpellier, South France, and Corsica. A duplicate of the collection no. 959 by Soleirol, explicitly cited as seen by Godron (1848), was chosen as lectotype.

This specimen corresponds with the protologue and with the current application of the name. In Kerguélen (1993), this taxon is considered accepted at varietal rank (*D. sylvestris* subsp. *longicaulis* var. *godronianus*). In Jauzein (2014), this taxon is instead included in *D. caryophyllus* subsp. *longicaulis* (Ten.) Arcang., but the author argues that it could constitute a distinct subspecies (*D. caryophyllus* subsp. *godronianus* (Jord.) P.Martin). *Dianthus godronianus* is instead considered a distinct species by Tison and de Foucault (2014), although these authors note that some coastal populations in Provence differ for a few morphological features. According to the lectotype features and the recent lectotypification of the latter name (Domina et al. 2021), this species can be regarded as a heterotypic synonym of *D. virgineus*.

***Dianthus orophilus* Jord. in Billot, Annot.: 43. 1856 [December 1856]**

≡ *D. caryophyllus* var. *orophilus* (Jord.) Rouy & Foucaud, Fl. Fr. 3: 195. 1896 [July-August 1896]
=? *D. sylvestris* Wulfen in Jacq., Coll. 1: 237. 1786. [January-September 1786]. Ind. Loc.: "schistes au Lautaret et dans le province de Maurienne (Savoie)".

Type. (lectotype here designated): *Dianthus orophilus*, *Dianthus sylvestris* an var. *gracilior*, du Lautaret May [18]53-June [18]55 […], LY0825955!

Note. Two syntypes from Col de Lautaret are housed at LY: LY0825955 and LY0087623, both in good condition. We have designated here the most complete one as lectotype. The selected type comes from Jordan's garden, where it was cultivated since its first collection in 1853.

This specimen conforms to the description of the protologue and corresponds to the current application of the name, which is considered as a heterotypic synonym of *D. sylvestris* subsp. *sylvestris* in Kerguélen (1993). The lectotype of *D. orophilus* concerning the shape of calyx teeth, is very similar to the lectotype of *D. inodorus* (L.) Gaertn., which in turn is currently included within the variability of *D. sylvestris* (Domina et al. 2021).

***Dianthus saxicola* Jord., Pugill. Pl. Nov.: 29. 1852 [October 1852]**

≡ *D. caryophyllus* var. *saxicola* (Jord.) Cariot & St-Lager, Étude Fl., éd. 8, 2: 103. 1889.
=? *D. sylvestris* Wulfen in Jacq., Coll. 1: 237. 1786. [January-September 1786]. Ind. Loc.: "in lapidosis et rupestribus calcareis Beugesi et Delphinatus prope Lyon ubi eum legi".

Type. (lectotype here designated): *Dianthus saxicola* Jord., Serrières (Ain) près de Lyon, 7 June 1852, A. Jordan, LY0682162!

Note. Two specimens belonging to the original material are housed at LY: LY0682162 and LY0088790. Both are in good condition. We have designated here the most complete one as the lectotype.

This specimen conforms to the description of the protologue and corresponds to the current application of the name, which is considered a distinct species by Tison and de Foucault (2014). The lectotype of *D. saxicola* has 10–15 cm long basal leaves and multiflorous scapes; concerning the shape of calyx teeth, it is very similar to the lectotype of *D. inodorus* (L.) Gaertn., which in turn is currently included within the variability of *D. sylvestris* (Domina et al. 2021). Further research is needed to clarify the relationships between these two taxa.

Dianthus juratensis Jord. in Billot, Annot.: 47. 1856. [December 1856]

≡ *D. caryophyllus* var. *juratensis* (Jord.) Gren., Fl. Chaîne Jurass.: 105. 1865.
=? *D. sylvestris* Wulfen in Jacq., Coll. 1: 237. 1786. [January-September 1786]. Ind. Loc.: "du Mont Reculet (Ain)".

Type. (lectotype here designated): *Dianthus juratensis* Jord., mont Reculet (Ain), 24 August 1854, [A. Jordan], LY0083755!

Note. Another herbarium sheet (LY08259243) is preserved at LY; it contains plants collected in 1855 in Villeurbanne, where they were cultivated after being originally collected in the wild at Reculet (Ain).

The lectotype designated here matches the protologue and corresponds to the current application of the name, which is considered as a heterotypic synonym of *D. sylvestris* subsp. *sylvestris* in Kerguélen (1993). The lectotype of *Dianthus juratensis*, concerning the shape of calyx teeth, is very similar to the lectotype of *D. inodorus* (L.) Gaertn., which in turn is currently included within the variability of *D. sylvestris* (Domina et al. 2021).

Dianthus subacaulis Vill., Hist. Pl. Dauphiné 3(2): 597. 1789. [September-October 1789]

≡ *D. sylvestris* var. *subacaulis* (Vill.) W.D.J.Koch, Syn. Fl. Germ. Helv. 1: 97. 1835.
≡ *D. virgineus* var. *subacaulis* (Vill.) Ser., Prodr. [A. P. de Candolle] 1: 361. 1824.
≡ *D. pungens* subsp. *subacaulis* (Vill.) Bernal, Laínz, Muñoz Garmendia & Pedrol, Anales Jard. Bot. Madrid 44(2): 571. 1987.
=? *D. sylvestris* Wulfen in Jacq., Coll. 1: 237. 1786. [January-September 1786]. Ind. Loc.: "aux environs du Buis, sur le Mont Ventoux".

Type. (neotype here designated): Herbier A. Dubuis, *Dianthus subacaulis* Vill. subsp. *subacaulis*, Pentes rocailleuses dénudées près du somment du Mont Ventoux (1912 m). (Vaucluse), 7 July 1955, MPU329773 (photo!).

Note. No original material was found in GRM and in the other surveyed herbaria. Also A. P. V. Mutel's Herbarium was checked because he used to include Villars specimens in his own herbarium (M. Lefebvre, pers. comm.).

The neotype designated here matches the protologue and corresponds to the current application of the name, which is accepted by both Kerguélen (1993) and Tison and de Foucault (2014). This species is characterized by having 1 cm long basal leaves, very short, 1–5 cm long single-flowered scapes and epicalyx scales lanceolate with a linear mucro. Concerning the shape of calyx teeth, it is very similar to the lectotype of *D. inodorus* (L.) Gaertn., which in turn is currently included within the variability of *D. sylvestris* (Domina et al. 2021). Further research is needed to clarify the relationships between these two taxa.

Dianthus sylvestris **Wulfen in Jacq., Coll. 1: 237. 1786. [January-September 1786]**

≡ *D. caryophyllus* subsp. *sylvestris* (Wulfen) Rouy & Foucaud, Fl. France 3: 193. 1896. Ind. Loc.: – "in montibus illis prope Ponewitsch Baronis Wolkensberg in Carniolia, tum in M. Utocsek prope Pillichgraz; in iis. Vallis Rablensis; denique & in iis Vallis Canalensis &c.".

Types. (lectotype here designated): The water-coloured iconography published by Jacquin (1781–1786, t. 82, the small individual on the right).

Note. The iconography designated by Bacchetta et al. (2010) as neotype is actually part of the original material as uncited illustration (Art. 9.12 of the ICN), since Jacquin's Icones and Collectanea work are interrelated. Therefore this neotypification must be corrected in lectotypification. This illustration depicts two individuals: one small with a 2 branched single-flowered stem and one large, unbranched but with multiflowered stems and basal leaves three times longer, exemplifying morphological variation in this species. In the protologue, it is clearly stated that the larger plant was seen only once in Monte Re, near Lake of Predil, NE Italy ("Uno duntaxat, quod miratus sum, loco Montis regii Rablensis, giganteum inveni, caulibus cubitalibus bi- & trifloris"), while smaller plants are common elsewhere in Carniola. Accordingly, we can conclude that the two drawings depict plants originating from two different areas, thus belonging to two different gatherings. Consequently, the type designated by Bacchetta et al. (2010: 143), neotype or lectotype, belongs to more than one gathering and cannot be accepted as a type (Art. 8.1, 8.2, 9.3 of the ICN). Thus, the name remains to be typified. No other original material for this name exists (de Langen et al. 1984), so that we select here as lectotype only the small specimen of the water-coloured iconography published by Jacquin at table 86 that better fits the description "folia ... pollicari aut circiter longitudine… Caulis subquinquepollicaris... Flos plerumque unicus [Leaves … one inch or about one inch long, stem less than 5 inches ... flower generally single]".

The lectotype here selected agrees with the current application of the name by numerous authors, e.g., Kerguélen (1993), Bacchetta et al. (2010), Tison and de Foucault (2014), Brullo and Guarino (2017), who consider *D. sylvestris* as an accepted species. The overall size of the plant, and the length of the leaves are not stable characters for taxonomic discrimination. The shape and relative size of calyx and epicalyx scales are better discriminating taxonomic characters and are evident in the lectotype. These features allow to distinguish *D. sylvestris* subsp. *sylvestris* from *D. sylvestris* subsp. *tergestinus* (Bacchetta et al. 2010).

Dianthus sylvestris **var.** ***garganicus*** **Ten., Fl. Napol. Syll.: 208. 1831. [July-August 1831]**

≡ *D. caryophyllus* subsp. *garganicus* (Ten.) Grande, Boll. Soc. Bot. Ital. 1912: 178. 1912.

≡ *D. caryophyllus* var. *garganicus* (Ten.) Fiori, Nuova Fl. Italia 1: 512. 1924.
≡ *D. sylvestris* subsp. *garganicus* (Ten.) Pignatti, Giorn. Bot. Ital. 107: 211. 1973.
≡ *D. garganicus* (Ten.) Brullo, Braun-Blanquetia 2: 31. 1988.
= *D. tarentinus* Lacaita, Nuovo Giorn. Bot. Ital. n.s., 18(4): 511. 1911. Ind. Loc.: "Gargano".

Type. (lectotype here designated): *Dianthus sylvaticus*, *D. sylvestris* Ten. Fl. Neap. Prodr. (1811) p. xxv. - Eiusd. Fl. Nap. I (1811–1815) p. 231, Gargano, Tenore misit Nov 1827 / Herb. J. Gay., Presented by Dr. Hooker, February 1868, K000725365 (photo!).

Note. In the same herbarium sheet three herbarium specimens, sent by Michele Tenore to Jaques Étienne Gay, are mounted. K000725363 was collected by Tenore from Calmaldoli (Campania, Italy) in November 1825; K000725364 by Nicolas Bové from La Calle (Algeria) in June 1839, and K000725365 by Tenore from Gargano (Apulia, Italy) in November 1827. In NAP there is a specimen from Gargano with the handwriting by Michele Tenore, lacking a date.

The lectotype designated here matches the protologue and corresponds to the current application of the name, which is considered as a heterotypic synonym of *D. tarentinus* Lacaita (Bacchetta et al. 2010; Brullo and Guarino 2017; Bartolucci et al. 2018). This synonymy is here confirmed based on the shape and size of the leaves, of the scales of the epicalyx and of the calyx which are observable on the types of the two taxa.

***Dianthus virgineus* var. *tergestinus* Rchb., Icon. Fl. Germ. Helv. 6: 47, pl. 266 fig. 5049β?. 1842–1844. [1844 publ. 1842–1844]**

≡ *D. tergestinus* (Rchb.) A.Kern., Sched. Fl. Exs. Austro-Hung. 2: 71. 1883.
≡ *D. caryophyllus* var. *tergestinus* (Rchb.) Tanfani in Caruel, Fl. Ital. 9(2): 283. 1892.
≡ *D. sylvestris* subsp. *tergestinus* (Rchb.) Hayek, Repert. Spec. Nov. Regni Veg. Beih. 30(1, 2): 247. 1924. Ind. Loc.: none [but Trieste, Italy, can be easily inferred from the epithet "tergestinus" that means "from Trieste"].

Type. (lectotype here designated): Rchb., Icon. Fl. Germ. Helv. 6: pl. 266 fig. 5049β. 1842–1844.

Note. The main text (Icon. Fl. Germ. Helv. 6: 47. 1842–1844. [1844 publ. 1842–1844]) lacks a written diagnosis or description, and, in any case, it is not clear if the plate was published simultaneously with the main text. Stafleu and Cowan (1983) reports that the volume 6 was published between 1842 and 1844, even though the title page shows 1844. However, this name was validly published on plate CCLXVI (= 266) by an illustration with analysis (Arts. 38.7 and 38.8 of the ICN), which is obviously part of the original material.

This taxon is considered as a subspecies of *D. sylvestris* by Vangjeli (2015), Brullo and Guarino (2017), Bartolucci et al. (2018), Peruzzi et al. (2019), and Nikolić (2020). It differs from *D. sylvestris* subsp. *sylvestris* by having a poorly developed mucro of the epicalyx scales and entire petals. Its distribution (Trieste area and along the north-eastern Adriatic coast), separated from the main range of *D. sylvestris* subsp. *sylvestris*, is compatible with the rank of subspecies.

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Publication III

plants

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Article

Genome Size Variation in *Dianthus sylvestris* Wulfen sensu lato (Caryophyllaceae)

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Abstract: Genome size (GS) is an important characteristic that may be helpful in delimitation of taxa, and multiple studies have shown correlations between intraspecific GS variation and morphological or environmental factors, as well as its geographical segregation. We estimated a relative GS (RGS) of 707 individuals from 162 populations of *Dianthus sylvestris* with a geographic focus on the Balkan Peninsula, but also including several populations from the European Alps. *Dianthus sylvestris* is morphologically variable species thriving in various habitats and six subspecies have been recognized from the Balkan Peninsula. Our RGS data backed-up with chromosome counts revealed that the majority of populations were diploid ($2n = 30$), but ten tetraploid populations have been recorded in *D. sylvestris* subsp. *sylvestris* from Istria (Croatia, Italy). Their monoploid RGS is significantly lower than that of the diploids, indicating genome downsizing. In addition, the tetraploids significantly differ from their diploid counterparts in an array of morphological and environmental characteristics. Within the diploid populations, the RGS is geographically and only partly taxonomically correlated, with the highest RGS inferred in the southern Balkan Peninsula and the Alps. We demonstrate greater RGS variation among the Balkan populations compared to the Alps, which is likely a result of more pronounced evolutionary differentiation within the Balkan Peninsula. In addition, a deep RGS divergence within the Alps likely points to persistence of the alpine populations in different Pleistocene refugia.

Keywords: genome size; Balkan Peninsula; European Alps; tetraploids; glacial refugia

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1. Introduction

Genome size (GS; size of the monoploid chromosome set, [1]) is an important cytogenetic characteristic that may be helpful in delimitation of taxa [2–10]. Numerous studies using GS data, often in combination with chromosome counts, have explored diversification of polyploid species complexes [11–14] or genera with high incidence of polyploidy [15–18]. Flow cytometric GS estimation has become an established method, as it allows rapid estimation of nuclear DNA content of large numbers of individuals in either absolute or relative units [2,19]. It is a faster and more convenient method for ploidy level estimations compared to conventional chromosome counting, and it can be used for detecting rare cytotypes [20] or to provide evidence of GS intraspecific variability [21].

The existence of intraspecific variation in GS has been acknowledged [7,22–24] and reported for many species, e.g., *Festuca pallens* Host [23], *Senecio carniolicus* Willd. [25], *Tephroseris longifolia* (Jacq.) Griseb. & Schenk [26], and *Minuartia verna* (L.) Hiern [8], albeit sometimes argued to be of minor evolutionary relevance [24] or suggested to be a result of an experimental artefact [27]. Nevertheless, this variation can be a result of microevolutionary differentiation and can reflect taxonomic heterogeneity [28]. The reasons for GS variation in the absence of polyploidy may be sought in the increased activity of retrotransposons [29] and the accumulation of retrotransposons and other repetitive elements are considered the main factors of GS increase in angiosperms [7].

Multiple studies have shown correlations between intraspecific variation of GS and morphological or environmental factors, as well as geographical distribution [5,26,30–35], but exact causes of this variation, and thus the interpretation of GS heterogeneity, remains a challenging task [35–37]. For instance, GS is a characteristic that may be related to the variation in plant phenology [38] and water availability [36], and may affect morphological characteristics such as seed size, nuclear and cell volumes, and duration of mitotic and meiotic cycles [39]. Evidence concerning GS variation across environmental gradients may point to the involvement of GS in adaptive evolution [18,34] or speciation and diversification [40]. Thus, GS data can facilitate taxon delimitation at sectional, specific, and intraspecific levels [3,4,6,9,12,15].

Dianthus is one of the largest genera of Caryophyllaceae comprising over 300 species distributed throughout Eurasia and northern Africa [41]. This high diversity is a result of rapid radiation and diversification, which was pronounced especially in the Mediterranean Basin [42], where several polymorphic *Dianthus* groups with high intraspecific diversity, and thus unresolved taxonomy exist [43–48]. One of them is *Dianthus sylvestris* Wulfen s.l., which is one of the most taxonomically challenging groups of the European flora [49,50]. Its main diversity centers are the Balkan and the Apennine Peninsulas, where large morphological variation has led to description of several taxa growing in various habitats from the Mediterranean coast to the alpine belt [44,45,47,51]. Due to high morphological variability and subtle morphological transitions, there is a dispute concerning the number of taxa. Several authors have tried to develop a sensible intraspecific classification for *D. sylvestris* on the Balkan Peninsula [52–56], leading to recognition of six subspecies [57–59]: *D. sylvestris* subsp. *alboroseus* F.K. Mey., *D. sylvestris* subsp. *bertisceus* Rech. f., *D. sylvestris* subsp. *kozjakensis* Micevski, *D. sylvestris* subsp. *nodosus* (Tausch) Hayek, *D. sylvestris* subsp. *sylvestris*, and *D. sylvestris* subsp. *tergestinus* (Rchb.) Hayek. The morphometric study of Terlević et al. (submitted) [60] performed on 97 populations of *D. sylvestris* s.l. across its range on the Balkan Peninsula showed that the states of several morphological traits deemed diagnostic for subspecies, i.e., number and shape of epicalyx scales, calyx length, petal characteristics, and indumentum density, frequently overlap, making the reliable identification of subspecies often difficult and ambiguous. However, the combination of morphological characteristics (i.e., entire or slightly eroded petals and usually one pair of epicalyx scales) and different flowering time clearly distinguishes the thermophilus *D. sylvestris* subsp. *tergestinus* from all other subspecies including the sympatric *D. sylvestris* subsp. *sylvestris* and *D. sylvestris* subsp. *nodosus*.

Within *Dianthus*, the most frequent chromosome number is diploid ($2n = 2x = 30$) [61–64], although polyploid taxa including series with up to seven ploidy levels have been documented ($2n = 2x$, $3x$, $4x$, $5x$, $6x$, $8x$, and $12x$) [11,65]. In the Chromosome Counts Database (CCDB) [63], chromosome numbers for 162 *Dianthus* taxa have been registered, of which 89 (55%) are diploid ($2n = 30$), 17 (10%) tetraploid ($2n = 60$), and 11 (7%) hexaploid ($2n = 90$), whereas for 45 taxa (28%) multiple ploidy levels have been recorded. The karyological features of *Dianthus* chromosomes have been rarely reported due to their small size (0.6–2.7 μm long) and large number in polyploids, but most of them are metacentric and of similar size [61,66–68].

For *D. sylvestris* two ploidy levels have been reported: diploids ($2n = 30$) throughout the distribution area [61,62,69] and tetraploids ($2n = 60$) from Gorges de Daluis in the Maritime Alps in France [70]. For diploids from Mt. Jahorina in Bosnia and Herzegovina, the GS of 1C = 0.61 pg has been reported by Siljak-Yakovlev et al. [69] and consequently by Pellicer & Leitch [64]. Due to the low number of chromosomally investigated populations, precise information about the incidence of polyploidy within *D. sylvestris* remains unclear and it is unknown how both ploidies, as well as GS variation, correlate to taxonomic entities within the species and if there is a geographic pattern of GS variation that could be of evolutionary significance.

The main aim of this study was thus to investigate GS and ploidy-level variation within *D. sylvestris* s.l., with a geographic focus on the Balkan Peninsula and to a lesser extent the Alps. To this end, we intersect the GS data of 162 populations, calibrated with chromosome counts, with taxonomic entities and explore its geographic variation. More specifically, we (i) ask if there are polyploid populations present in the area and how they are distributed, (ii) explore whether the pattern of relative genome size (RGS) variation correlates to current taxonomic treatment and geography, and (iii) investigate if there is a relationship between RGS and environmental variation. The obtained data, together with other evidence, will help to disentangle the complex relationships within *D. sylvestris* s.l.

2. Results

2.1. Chromosome Numbers

We estimated the diploid chromosome numbers $2n = 2x = 30$ for two populations from Karlobag (D32) and Krk island (D185) in Croatia and the tetraploid numbers $2n = 4x = 60$ for three populations (D12, D20, and D21) from Istria (Italy and Croatia; Figure 1, Table S1). Chromosomes were small, 1–2 µm long.

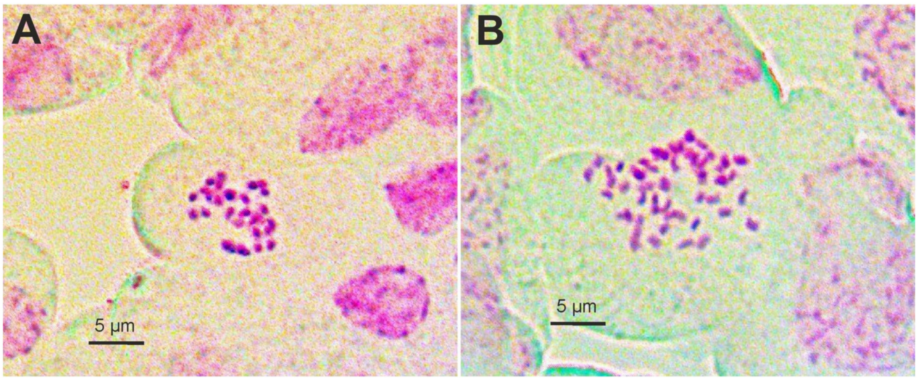

Figure 1. Mitotic chromosomes from *Dianthus sylvestris* root tips. (**A**) A diploid ($2n = 2x = 30$) from the population D185 (Punat, Krk island, Croatia) and (**B**) a tetraploid ($2n = 4x = 60$) from the population D12 (Plomin, Mt Učka, Croatia).

2.2. Relative Genome Size Estimation and DNA Ploidy Level

RGS was analyzed for 707 individuals from 162 populations of *D. sylvestris* s.l. from the Balkan Peninsula and the Alps (Figure 2, Figure S1). High-resolution histograms of DNA content comprised two large G1 peaks representing nuclei of the sample and the reference (Figure 3), and the ratio of their positions determined the sample's RGS. In addition to the main peaks, the minor peaks of the sample corresponded to endopolyploid nuclei [71] that are common in Caryophyllaceae [72]. The coefficient of variation (CV) of the sample's G0/G1 peak of the majority of 707 measurements was between 1.36 to 6 (4.7 on average). In further analyses, we also included 17 populations that exceeded this threshold and had a CV of up to 10, as their peaks were clearly visible and their RGS values fitted well to the remaining data.

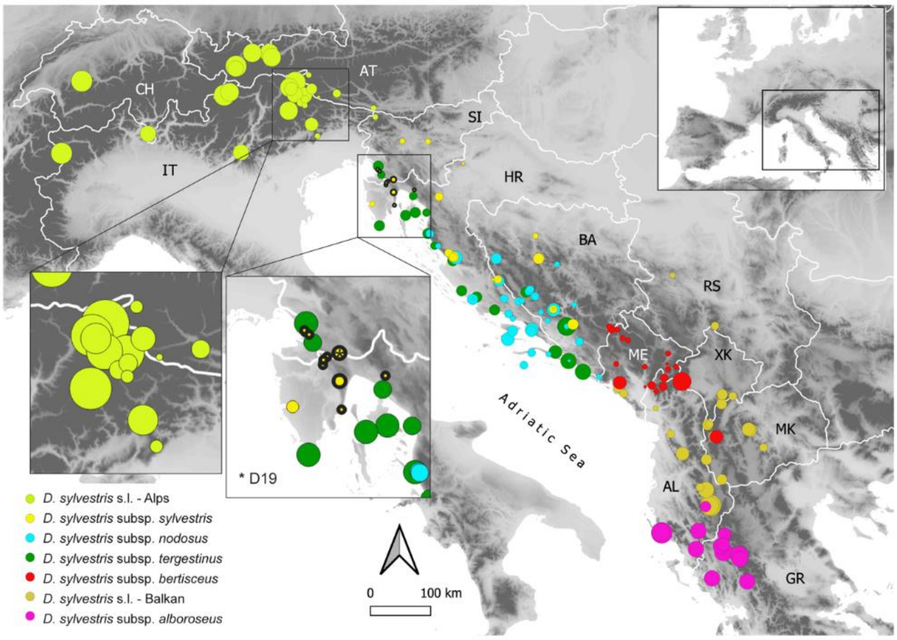

Figure 2. Geographical distribution of the monoploid relative genome size (RGS) variation of 162 diploid and tetraploid (circles with thick black outline) populations of *Dianthus sylvestris* s.l. in the Balkan Peninsula and the Alps. Only populations with at least three measured individuals and a standard deviation of RGS < 0.01 are shown. The size of the dots is proportional to the mean monoploid RGS of the corresponding populations. Asterisk indicates the ploidy-mixed population D19. Color coding indicates different taxa.

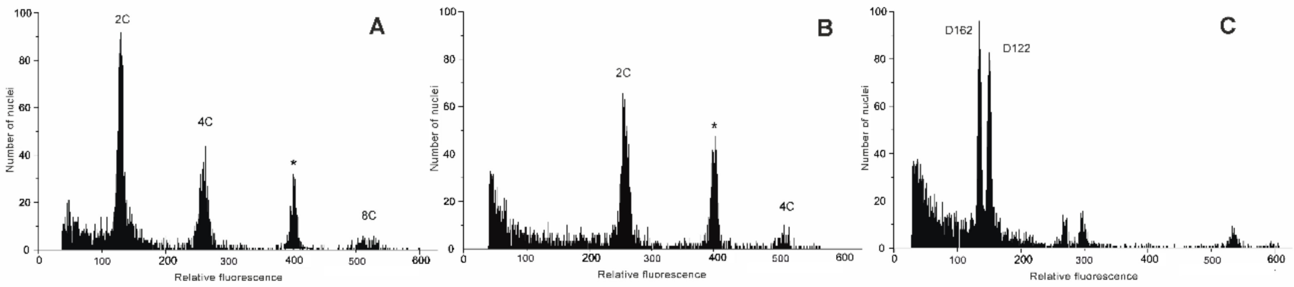

Figure 3. Histograms of fluorescence intensities of diploid (**A**) (population D19) and tetraploid (**B**) (population D17) accessions of *D. sylvestris*, together with the internal reference standard *Bellis perennis* (asterisk), from which relative genome size (RGS) was calculated. The peaks are labelled following Greilhuber et al. [1]. Multiple peaks of the sample correspond to nuclei after one (4C) and two (8C) rounds of endoreplication. (**C**) Histogram showing genuine difference between diploid populations D162 (0.325 ± 0.001) and D122 (0.360 ± 0.004).

Flow-cytometry screening resulted in two discrete groups of RGS values that corresponded to the estimated diploid and tetraploid chromosome numbers. Diploid populations occurred throughout the sampled area and all subspecies, at elevations from 8 to 2274 m. On the other hand, all tetraploid populations belonged to *D. sylvestris* subsp. *sylvestris*, and were limited to Istria and Kvarner (Croatia and Italy), from 240 to 941 m (Figure 2). A total of 152 populations (94%) were DNA-diploid, with RGS ranging from 0.324 to 0.376 (mean: 0.341 ± 0.011), whereas ten populations (6%) were DNA-tetraploid with RGS ranging from 0.640 to 0.657 (mean: 0.649 ± 0.006; Table 1). A 1.16-fold variation in RGS was thus revealed among diploids with a 1.03-fold variation among tetraploids (Figure 3C, Table 1). Only one population from Istria (Vodice-D19; Figure 2) was ploidy-mixed, with two individuals being DNA-diploid and eight DNA-tetraploid. The monoploid RGS of 15 DNA-diploid populations of *D. sylvestris* subsp. *sylvestris* ranged from 0.162 to

0.188 and was significantly higher compared to ten DNA-tetraploid populations with values between 0.160 and 0.164 (Figure 4, Kruskal–Wallis test = 10.58, $p < 0.01$).

Table 1. Descriptive statistics of relative genome size (RGS) variation in diploid (2*x*) and tetraploid (4*x*) populations of *Dianthus sylvestris* s.l., for which at least three individuals were analyzed. SD, standard deviation; mRGS, monoploid RGS.

Ploidy	No. Measurements	Populations	Individuals	Min. No. Individuals	Max. No. Individuals	Mean No. Individuals	Mean RGS	SD RGS	Min RGS	Max RGS	Mean mRGS	SD mRGS
2*x*	536	152	657	3	12	4.3	0.341	0.011	0.324	0.376	0.17	0.005
4*x*	45	10	50	3	8	5	0.649	0.006	0.64	0.657	0.162	0.001

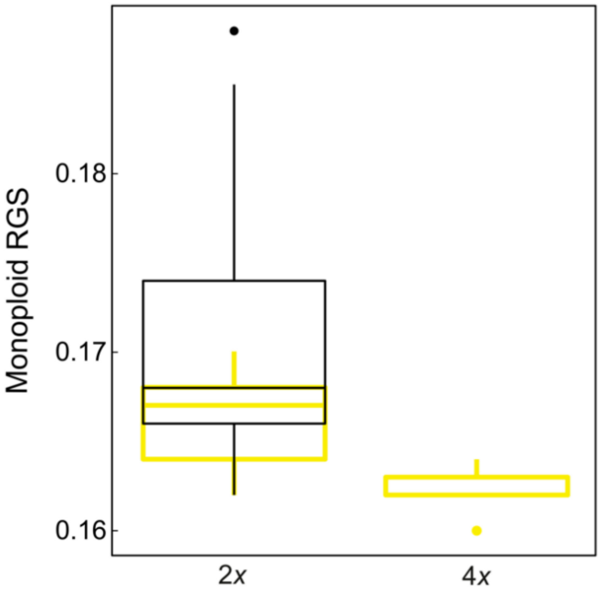

Figure 4. Monoploid relative genome size (RGS) variation in *Dianthus sylvestris* s.l. in the Balkan Peninsula and the Alps. Black colored box indicates the variation of the complete diploid dataset (152 DNA-diploids, 2*x*), whereas yellow color corresponds to 15 DNA-diploids (2*x*), and 10 DNA-tetraploid (4*x*) populations of *D. sylvestris* subsp. sylvestris. Boxes correspond to 25 and 75 percentiles, horizontal lines to medians, whiskers 5 to 95 percentiles, and circles to outliers.

2.3. Morphological and Environmental Differences between Diploids and Tetraploids

Comparison of morphological characteristics from the study of Terlević et al. (submitted) [60] between the nine diploid and seven tetraploid populations of *D. sylvestris* subsp. *sylvestris* showed a statistically significant difference in five vegetative characteristics (Table S2). The first two PCA axes explained 75.64% and 18.06% of the total morphological variation (Figure 5A) and the characteristics contributing most to the separation along the first axis were plant height (PH) and height of the first branching (FBH; component scores 0.49 and 0.50). The same variables had the highest scores in the DA (0.80 and 0.93), even though there was overlap between the scores of the discriminant functions (Figure 5B). The tetraploid plants were thus higher and had their lowermost lateral shoots higher. In addition, they had longer cauline and basal leaves (CLL, BLL), as well as longer internodes (UIL).

Although the correlation test failed to show any association between RGS of 152 diploid populations and environmental variables, the Kruskal–Wallis test showed significant difference in 14 environmental variables between 15 diploid and ten tetraploid populations of *D. sylvestris* subsp. *sylvestris* (Table S3 and Figure S2). The environmental variables contributing most to the separation of diploid and tetraploid populations were those describing temperature and precipitation preferences, as well as the terrain geomorphology. The first two PCA axes explained 40.32% and 18.24% of the total environmental variation

(Figure 5C) and the variables contributing most to the separation along the first axis were soil clay content and number of snow days in a year (component scores 0.50 and −0.53). Number of snow days in a year, slope and eastness had the highest scores in the DA (−0.59 and −0.64), without overlap between diploids and tetraploids (Figure 5D).

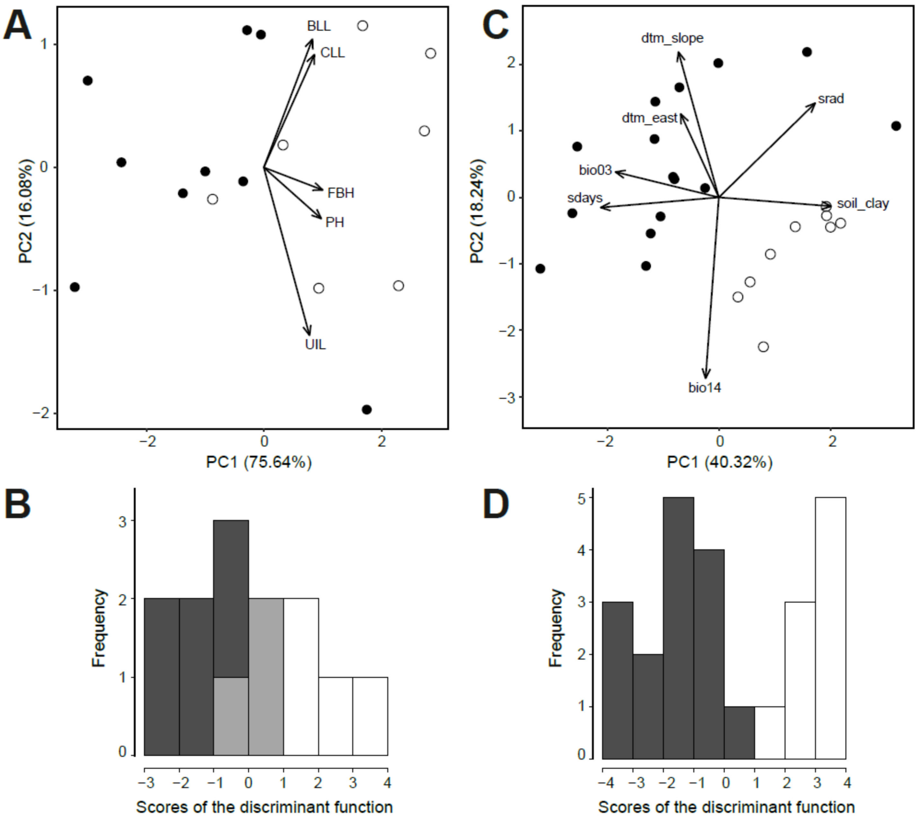

Figure 5. Morphological and environmental differentiation between diploid (black) and tetraploid (white) populations of *Dianthus sylvestris* subsp. *sylvestris*, with their overlap in B shown in grey. (**A**) Principal component analysis (PCA) and (**B**) histogram of discriminant analysis (DA) based on five morphological characteristics. (**C**) PCA and (**D**) histogram of DA based on seven environmental variables. Characteristic abbreviations in (**A**,**C**) are explained in Tables S2 and S3.

2.4. *RGS Variation across Intraspecific Entities*

Differences in RGS among the five subspecies and two geographic groups of populations of *D. sylvestris* s.l. were significant (Kruskal–Wallis = 54.1, $p < 0.01$, Figure 6A). The Tukey post-hoc test showed that populations of *D. sylvestris* subsp. *alboroseus* and those of *D. sylvestris* s.l. from the Alps had significantly larger monoploid RGS values than other groups ($p < 0.01$, Figure 6A). Additionally, the RGS of *D. sylvestris* subsp. *tergestinus* was significantly higher than the RGS of *D. sylvestris* subsp. *sylvestris*, whereas there was no significant difference between *D. sylvestris* s.l. from the Balkans, *D. sylvestris* subsp. *bertisceus*, *D. sylvestris* subsp. *nodosus*, and *D. sylvestris* subsp. *sylvestris*.

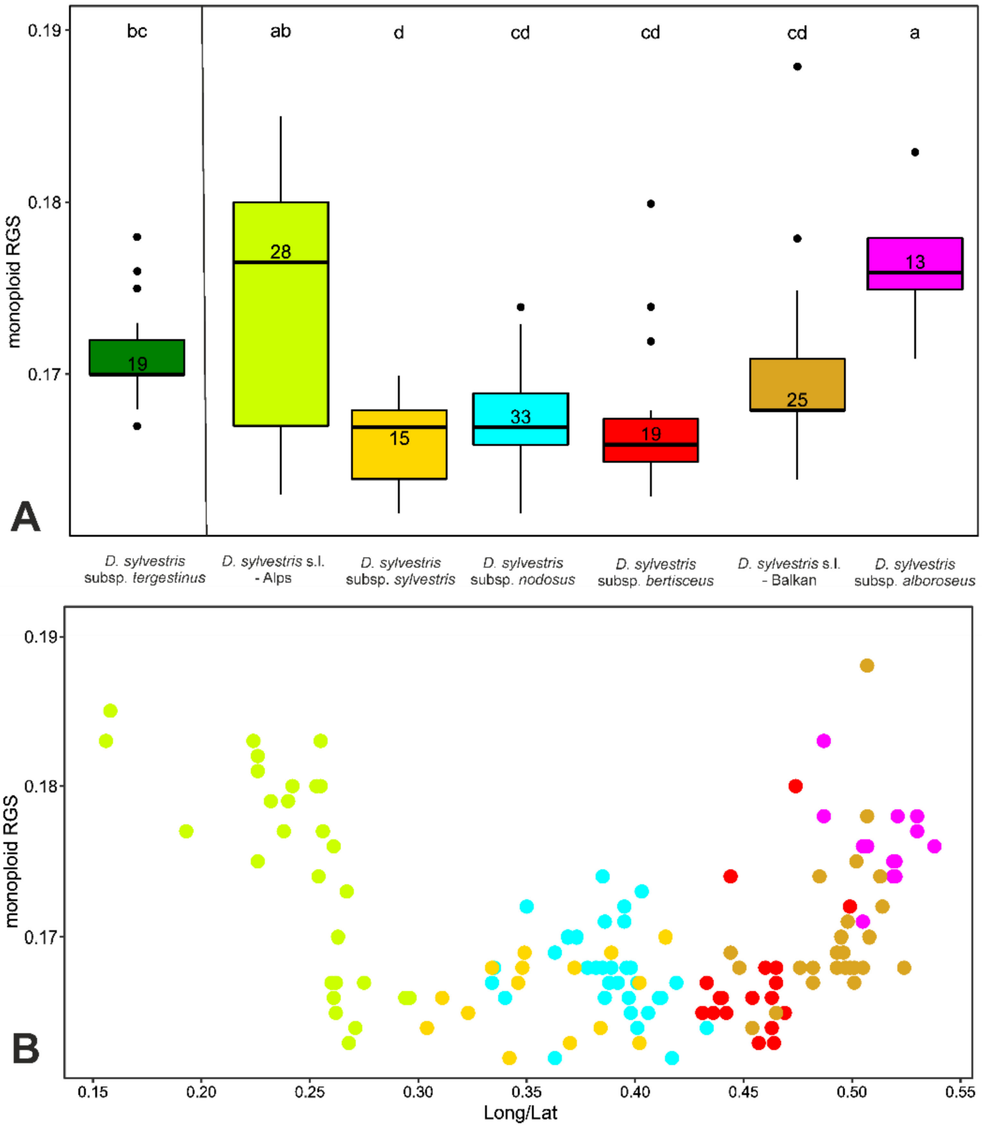

Figure 6. Monoploid RGS variation based on population means of 152 DNA-diploid populations of *Dianthus sylvestris* s.l. Color coding for taxa corresponds to Figure 2. (**A**) Boxes in the boxplot and (**B**) points in the scatterplot are arranged in geographical order from the north-west to the south-east. Being the most clearly distinguished subspecies, D. sylvestris subsp. tergestinus is singled out on the left of plot (**A**) and excluded from the plot (**B**). Boxes correspond to 25 and 75 percentiles, horizontal lines to medians, whiskers 5 to 95 percentiles, and circles to outliers. Means not significantly different at $p < 0.01$, according to the Tukey post-hoc test, are indicated by the same letter. Numbers adjacent to the median lines represent the sample size.

3. Discussion

3.1. Tetraploidization within D. sylvestris Populations in the Northern Balkan Peninsula

The extensive RGS measurements combined with confirmatory chromosome number estimations revealed the prevalence of diploid populations of *D. sylvestris* throughout the investigated area, as well as the occurrence of tetraploid populations in the northwesternmost Balkan Peninsula (Istria and Kvarner in Croatia and Italy; Figure 2, Table S1). Therefore, this is the first report of tetraploid populations within *D. sylvestris* s.l. in the Balkan Peninsula, which likely originated separately from the tetraploid populations reported from France [70], given the geographic distance between them. The confirmation of independent origin of these two groups of tetraploid populations requires additional evidence based on genetic data or detailed examination of RGS values of French populations as their different values might indicate a separate origin. However, multiple polyploidization events within single species are common and have been reported, for example in *Astragalus onobrychis* [73], *Cerastium decalvans* [74], and *Euphorbia montenegrina* [10]. In addition, within *Dianthus*, multiple and independent origin of polyploids within the *Dianthus broteri* complex [75,76] and several heteroploid species of *Dianthus* section *Plumaria* [11], that even occur sympatrically, have been reported. Diploidization, a process following polyploidization, is commonly accompanied by elimination of parts of the genome [77,78], termed genome downsizing [79]. Reduction of monoploid GS has been observed in many different plant groups (e.g., [8,15,79,80]) and our data suggest that it is occurring also in tetraploid *D. sylvestris*, as its monoploid RGS was significantly smaller compared to the diploids (Kruskal–Wallis test = 10.58, $p < 0.01$).

Given the morphological similarity of tetraploid populations to their diploid counterparts occurring in the same area, both being identified as *D. sylvestris* subsp. *sylvestris*, we suggest an autopolyploid origin of tetraploids, as it was also suggested for polyploids within *Dianthus* sect. *Plumaria* [11]. A tetraploid origin from the diploid *D. sylvestris* subsp. *sylvestris*, rather from *D. sylvestris* subsp. *tergestinus* that also occurs in the same area (Figure 2), is further supported by more similar monoploid RGS of the tetraploids with the former taxon. Alternatively, an allopolyploid origin involving putative diploid parents of northern i.e., alpine and eastern i.e., Balkan provenance is possible. Despite a clear identification of tetraploid populations as *D. sylvestris* subsp. *sylvestris*, we demonstrated that tetraploid individuals differ from diploids in stem and leaf size characteristics. This is in line with other studies, where it has been shown that polyploidization can substantially affect morphological variation and, although the relationship between ploidy and body/organ size is complex, polyploid plants are often larger than their diploid parents [81,82].

In addition to morphological divergence, ecological differentiation among different ploidies within the same species has also been evidenced [76,83,84]. Given that polyploids may be more competitive compared to diploids [85], they are expected to have higher dispersal potential and may thrive in different ecological niches. It has been suggested that diploids tend to be restricted to refugia, whereas polyploids show better ability to re-colonize deglaciated regions [86]. However, contradicting cytogeographic patterns were also observed [8,87], and in *D. sylvestris* the tetraploids were also found to be geographically and environmentally restricted compared to the diploids that thrive in a broad range of environments. Diploid and tetraploid populations of *D. sylvestris* subsp. *sylvestris* were environmentally segregated by different temperature and precipitation preferences, as well as different geomorphology of the terrain. Tetraploids were collected in warmer habitats with less fluctuation in temperature, and more precipitation during the driest month. Furthermore, they thrive on significantly less steep, westerly exposed slopes with higher clay content in the soil and in habitats with a lower number of frost and snow days per year and with higher surface solar radiation. Nevertheless, one mixed-ploidy population from north-west Croatia (Vodice-D19) provides evidence that tetraploids and their diploid progenitors can occupy similar environments [88] and further studies are needed to reveal whether the observed ecological divergence is adaptive or simply a result of much wider distribution of diploids inhabiting a broader array of environments. Interestingly, the area of Istria, where tetraploids occur, is known as an important Pleistocene refugium for plants [89,90]. It is likely that Pleistocene climatic oscillation also triggered polyploidization in *D. sylvestris*, given the Pleistocene radiation in Eurasian *Dianthus* [42].

3.2. Geographic and Intraspecific Variation of RGS within Diploid *D. sylvestris* s.l.

Despite the fact that RGS estimations were performed with DAPI, which is an AT-content dependent, and cannot be, with 100% reliability, translated to absolute values and thus used in comparative studies [91], such a conversion based on the GS of our standard *Bellis perennis* (2C = 3.38 pg, [92]) revealed a variation of 1C in *D. sylvestris* s.l. ranging between 0.55 and 0.64 pg (mean: 0.58 pg, N = 152 populations). These values therefore correspond well to the estimated absolute GS of 1C = 0.61 pg [69]. *Dianthus sylvestris* is thus a small-genome species and it has been suggested that plants with small genomes display more pronounced morphological variation and thrive in wide environmental gradients, compared to large-genome taxa [37]. *Dianthus sylvestris* exhibits a high morphological variability and grows from the coastal Mediterranean environment to the alpine belt both in the Alps as well as the Balkan Peninsula [60] and hence supports the available evidence, but further studies across flowering plants are needed to bring more evidence for this hypothesis.

Our comprehensive sampling revealed an indicative spatial pattern of RGS variation within diploid populations of *D. sylvestris* (Figures 2 and 6B). Although the general pattern is complex and often populations with clearly different RGS occur in vicinity, larger RGS values predominate in the southern Balkan Peninsula and the central part of the Alps, i.e., at the south-eastern and northern margin of the species distribution. A clear geographic pattern in RGS variation with smaller monoploid RGS in the distribution center and its increase towards the distribution margins were also observed at the genus level in *Knautia* [15] and *Sesleria* [16]. Causes for such patterns remain unclear, but it has been suggested that larger GS can limit adaptive and competitive abilities of populations at the distribution margins and might thus represent a factor limiting further range expansion [37], however further studies are needed to rigorously test this hypothesis.

The southernmost populations with large RGS correspond to *D. sylvestris* subsp. *alboroseus*. Therefore, this subspecies also exhibited highest RGS among the Balkan taxa (Figure 6B). Towards the north-west, the RGS decreases in populations morphologically intermediate between *D. sylvestris* subsp. *alboroseus* and *D. sylvestris* subsp. *bertisceus* and thus not clearly classified to any subspecies and reaches the smallest values in *D. sylvestris* subsp. *bertisceus*. Further to the north-west, a slight, although statistically non-significant, increase in geographically adjacent *D. sylvestris* subsp. *nodosus* and *D. sylvestris* subsp. *sylvestris* can be observed. The latter two subspecies can hardly be distinguished morphologically, they have similar environmental niches [60] and their highly similar RGS renders their recognition as two subspecies questionable. Interestingly, these two subspecies with similar morphology and RGS appear to form a unique phylogenomic cluster based on preliminary analyses of the RADseq data (Temunović et al., unpublished), separated from more southern populations, which are genetically more diverse. More pronounced genetic differentiation of southern compared to northern populations has been observed in several plant groups (e.g., [10,74,93–95]) and is, in *D. sylvestris*, also reflected in more pronounced RGS variation in this geographic region. Along the same line, ecologically and morphologically distinct *D. sylvestris* subsp. *tergestinus* [60] growing along the Adriatic coast, had higher RGS compared to geographically partly sympatric *D. sylvestris* subsp. *nodosus* and *D. sylvestris* subsp. *sylvestris*. Preliminary analyses of the genomic RADseq data (Temunović et al., unpublished) suggests that *D. sylvestris* subsp. *tergestinus* forms an evolutionary lineage distinct from all other Balkan populations of *D. sylvestris*. Its divergent RGS, which is significantly different from *D. sylvestris* subsp. *sylvestris*, is thus likely a result of divergent evolutionary histories.

Also, in the Alps, there is a pronounced variation in RGS within *D. sylvestris*, with a clear trend in its geographical distribution (Figure 2). Whereas the majority of the analyzed samples scattered across the western parts of the Eastern Alps, and a few populations from the Western Alps, exhibit larger RGS ranging between 0.346 and 0.369, the easternmost alpine populations (most of them from the eastern part of the Southern Alps) have smaller RGS ranging between 0.326 and 0.339 that correspond to the RGS of the populations from the north-western Balkan Peninsula. Therefore, the most prominent RGS variation among all groups analyzed was within the alpine group (Figure 6A). This relatively abrupt change in RGS within the Alps could be a result of divergence due to persistence of the species in two (or more) separate glacial refugia. It is likely that the eastern populations from the Southern Alps shared their refugium with the northern Balkan populations of *D. sylvestris* subsp. *sylvestris* in the north-western Balkan Peninsula, whereas the other alpine populations with clearly higher RGS survived the glacial cycles in more western refugia; several isolated refugia along the southern margin of the Alps have also been suggested for other alpine plants by Schönswetter et al. [96]. The observed RGS divergence in *D. sylvestris* within the Alps corresponds to a genetic discontinuity (Luqman et al., unpublished; Temunović et al., unpublished), but the exact border between the two lineages and their relation to the GS remains to be determined. In South Tyrol and adjacent Veneto (Italy) as well as East Tyrol (Austria), populations with relatively high RGS grow in close vicinity with the populations with lower RGS, suggesting that there might be a hybrid zone between the alpine and the Balkan lineage.

Multiple studies have suggested that environmental conditions may place constraints on the evolution of GS [34,37], hence genome size can either be directly associated with temperature and precipitation [97] or indirectly associated through elevation [34] or latitude [17,37,98]. However, the lack of association of RGS with environmental variables in our study on one hand, and a clear geographic trend in the RGS variation in *D. sylvestris* on the other hand, suggest that it was likely the phylogeographic rather than the environmental divergence that shaped the RGS variation in our study species.

4. Materials and Methods

4.1. Plant Material

We sampled 134 populations of *D. sylvestris* s.l. throughout the Balkan Peninsula and 28 populations from the European Alps between 2018 and 2021. At each locality, basal leaves from 3 to 12 individual plants were desiccated in silica gel for RGS measurements, and an herbarium specimen was collected. For chromosome number estimations, we collected mature seeds from several localities in July and August of 2020 and 2021. The seeds were air-dried and dry-stored in darkness at room temperature. Vouchers are deposited in the herbarium ZA (Table S1). We identified the plants and assigned them to subspecies based on identification keys in national and regional floras [44,99–105], and treated the populations that we could not assign to any of the known subspecies based on their morphology as *D. sylvestris* s.l.

4.2. Chromosome Counts

We determined chromosome numbers for five populations (Table S1). The seeds were germinated at the surface of a peat medium in plastic pots with regular watering at room temperature. Root tips were harvested at about noon and pre-treated with 0.002 M 8-hydroxyquinoline for 4 h in darkness at 4 °C. Subsequently, material was fixed in 3:1 ethanol–glacial acetic acid for 12–24 h at 4 °C. The fixed root tips were hydrolyzed in 5 M HCl at room temperature for 45 min, and then washed in distilled water. The root tips were stained in Schiff's reagent for 2 h. Finally, we squashed the stained root tips on a slide glass in a drop of 45% (*v*/*v*) acetic acid. Photomicrographs of chromosomes at mitotic metaphase were taken with Zeiss Lab. A1 AXIO microscope (Carl Zeiss Microscopy, Jena, Germany) equipped with ToupCam 5.1 MP digital camera. Snapshots were exported and studied using ImageJ software.

4.3. Flow Cytometry

Silica-gel-dried leaves were analyzed using flow cytometry (FCM) of 4′,6-diamidino-2-phenylindole (DAPI; final concentration 0.036 M) stained nuclei [106] to estimate RGS and DNA ploidy levels of sampled populations. We used *Bellis perennis* as the primary internal standard [92]. Desiccated green leaf tissue (c. 0.5 cm^2) of one to two plant individuals from the same population was chopped together using a sharp razor blade in a plastic Petri dish, with an appropriate amount of fresh reference standard and processed as described in Suda et al. [20]. The relative fluorescence intensity of 3000 nuclei was recorded using a Partec CyFlow Space flow cytometer (Sysmex Partec, Münster, Germany). We used Partec FloMax software to evaluate histograms and to calculate coefficients of variation (CV) of the standard and sample peaks. We calculated RGS as the ratio between the mean relative fluorescence of sample and standard. Samples with CV of the G1 peak >10% were re-analyzed until sufficient quality was achieved [21]. The number of measured individuals per population yielding high quality FCM histograms is given in Table S1.

4.4. Statistical Analyses

We calculated mean RGS value and standard deviation for each population from individual measurements of at least three individuals and we inferred the DNA ploidy levels [107] for all analyzed populations. We performed the Kruskal–Wallis test and Tukey post-hoc tests on population means, to evaluate statistically significant differences. The difference in monoploid RGS between diploids and tetraploids was tested for significance on a subset of 25 populations of *D. sylvestris* subsp. *sylvestris*, whereas the difference in RGS among the subspecies was tested for all diploid populations (N = 152). Due to the very small sample size (only one population known from the locus classicus), *D. sylvestris* subsp. *kozjakensis* was not included in these tests. All statistical analyses were performed using R-4.0.2 [108].

We performed principal component analysis (PCA) and discriminant analysis (DA) to explore the variability and the relative importance of characteristics/variables discriminating between the diploid and the tetraploid populations of *D. sylvestris* subsp. *sylvestris*. Both PCA and DA were performed for the morphological and the environmental dataset. The morphometric PCA and DA were performed using five morphological characteristics showing significant difference between the two ploidy levels (Table S2), whereas the environmental PCA and DA were based on seven environmental variables showing significant difference between the ploidy levels (Table S3) and without collinearity issues.

We used morphological data of the populations from the Balkan Peninsula from Terlević et al. ([60], Table S1) and tested the differences between nine diploid and seven tetraploid populations of *D. sylvestris* subsp. *sylvestris* (N = 16). We downloaded environmental data from three databases: climate data from the Chelsa database [109,110], soil properties from the SoilGrids [111], and topographic variables from the EarthEnv database [112]. The environmental space of each studied population was depicted by extracting environmental data from the points defined by longitude (N) and latitude (E; Table S1), and the differences were tested between 15 diploid and 10 tetraploid populations of *D. sylvestris* subsp. *sylvestris* (N = 25). The association between DNA-ploidy levels and environmental variables was also visualized with the package 'ggplot2'. Environmental variables were standardized to meet the assumption of homogeneity of variance and linearity. The correlation between environmental and RGS variability of 152 diploid populations in the studied area was tested employing Pearson correlation coefficients.

5. Conclusions

By analyzing the RGS variation of *Dianthus sylvestris* in the Balkan Peninsula and to a lesser extent in the Alps, our results reveal complex patterns of RGS in widespread diploid populations and spatially restricted tetraploid populations in the north-western Balkan Peninsula. The populations in the central and western parts of the Alps, as well as those at the southern distribution limit in the Balkan Peninsula, exhibit higher RGS, likely corresponding to discrete evolutionary lineages. In addition, two areas of more pronounced RGS variation at small geographic distances are revealed. One is located in the eastern Alps and the other in southern Dinaric Mountains. We suggest that the observed RGS differences are a result of evolutionary divergence due to persistence in separate glacial refugia. However, only upcoming integration of phylogenomic data will show the correlation of observed morphological (taxonomic) and GS variation with evolutionary differentiation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11111481/s1. **Table S1.** Relative genome size (RGS) and ploidy level (2*x*, DNA-diploids; 4*x*, DNA-tetraploids) of 162 populations of *Dianthus sylvestris* from the Balkan Peninsula and the Alps, including their provenance and voucher data. For each population, DNA ploidy estimates, mean RGS of a holoploid genome and standard deviation are given. Number of individuals analyzed for RGS in each population (N) and the range of CV values are also provided. Ploidy: 2*x* and 4*x*. Asterisk (*) indicates the populations for which confirmatory chromosome counts were performed. Plus (+) in the column Morphometrics indicates the morphometrically analyzed populations in the previous study by Terlević et al. (submitted) [60]. **Figure S1.** Relative genome size (RGS) in diploid populations of *Dianthus sylvestris* sorted by increasing RGS values. Population means (dots) with corresponding standard deviation (vertical lines) are presented. Population names correspond to Table S1. Colors correspond to subspecies, as in Figure 2. **Table S2.** Results of Kruskal–Wallis test between diploid (2*x*, N = 9) and tetraploid (4*x*, N = 7) populations of *Dianthus sylvestris* subsp. *sylvestris*. Morphological characteristics with significant differences between ploidy levels at $p < 0.05$ are shown in bold. **Table S3.** Results of Kruskal–Wallis test between diploid (2*x*, N = 15) and tetraploid (4*x*, N = 10) populations of *Dianthus sylvestris* subsp. *sylvestris*. Environmental variables with significant differences between ploidy levels at $p < 0.05$ are shown in bold. **Figure S2.** Boxplots showing environmental differences along the 14 environmental variables between diploid (2*x*) and tetraploid (4*x*) populations of *Dianthus sylvestris* subsp. *sylvestris*. Only variables with significant differences ($p < 0.05$), as revealed by Kruskal–Wallis test (Table S3), are shown.

Author Contributions: I.R. and A.T. conceived and designed the study; I.R., S.B., A.T. and B.F. collected plant material; A.T., I.R. and S.B. performed RGS measurements; A.T. and S.B. performed chromosome counting; A.T. analyzed the data; and A.T., B.F. and I.R. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data are contained within the article or the Supplementary Materials.

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Conflicts of Interest: The authors declare no conflict of interest.

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Table S1. Relative genome size (RGS) and ploidy level (2*x*, DNA-diploids; 4*x*, DNA-tetraploids) of 162 populations of *Dianthus sylvestris* from the Balkan Peninsula and the Alps, including their provenance and voucher data. For each population, DNA ploidy estimates, mean RGS of a holoploid genome and standard deviation are given. Number of individuals analyzed for RGS in each population (N) and the range of CV values are also provided. Ploidy: 2*x* and 4*x*. Asterisk (*) indicate the populations for which confirmatory chromosome counts were performed. Plus (+) for Morphometrics indicate the morphometrically analyzed populations in the previous study by Terlević et al. (submitted) [60].

ID	**Population name**	**Taxon**	**Country**	**Altitude (m a.s.l.)**	**Long (N)**	**Lat (E)**	**Collector**	**Collection date**	**Voucher**	**Mean RGS**	**SD RGS**	**N**	**Range of CV**	**Ploidy**	**Morpho-metrics**
D131	Pindus Theodoriana	*D. sylvestris* subsp. *alboroseus*	Greece	1023	39.42647	21.21591	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	21/06/2019	ZA-54934	0.352	0.003	5	1.99–2.82	2x	
D132	Mt Gorilla	*D. sylvestris* subsp. *alboroseus*	Greece	664	39.49754	20.54052	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	21/06/2019	ZA-54935	0.351	0.002	6	2.14–2.46	2x	+
D133	Lygeres Siolades	*D. sylvestris* subsp. *alboroseus*	Greece	744	39.76638	21.09354	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	22/06/2019	ZA-54936	0.353	0.003	5	2.15–4.74	2x	+
D134	Lygeres Aoos	*D. sylvestris* subsp. *alboroseus*	Greece	1398	39.82082	21.08762	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	22/06/2019	ZA-54937	0.357	0.003	6	2.84–4.43	2x	
D135	Kapesovo	*D. sylvestris* subsp. *alboroseus*	Greece	1135	39.88615	20.78638	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	22/06/2019	ZA-54938	0.356	0.002	5	2.84–4.09	2x	+
D137	Pindus Pyrgos	*D. sylvestris* subsp. *alboroseus*	Greece	631	40.14424	20.82399	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	22/06/2019	ZA-54940	0.348	0.006	4	2.93–3.84	2x	+
D138	Pindus Mikropapingo	*D. sylvestris* subsp. *alboroseus*	Greece	1296	39.97295	20.74076	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	23/06/2019	ZA-54941	0.350	0.003	6	2.02–2.57	2x	+
D139	Pindus Drakolimni	*D. sylvestris* subsp. *alboroseus*	Greece	2093	39.99494	20.78599	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	23/06/2019	ZA-54942	0.349	0.005	5	2.77–4.72	2x	
D140	Mt Dhembel	*D. sylvestris* subsp. *alboroseus*	Albania	1523	40.21507	20.31646	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	24/06/2019	ZA-54943	0.351	0.000	6	1.98–2.31	2x	+
D141	Gjirokaster	*D. sylvestris* subsp. *alboroseus*	Albania	400	39.94504	20.25109	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	25/06/2019	ZA-54944	0.353	0.002	6	2.54–2.59	2x	+
D142-1	Llogara village	*D. sylvestris* subsp. *alboroseus*	Albania	950	40.21436	19.57959	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	25/06/2019	ZA-54946	0.355	0.003	4	3.01–4.41	2x	
D142-2	Llogara park	*D. sylvestris* subsp. *alboroseus*	Albania	1600	40.20721	19.59952	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	25/06/2019	ZA-54945	0.365	0.010	4	4.43–5.55	2x	
D78	Mt Ostrovice	*D. sylvestris* subsp. *alboroseus*	Albania	1386	40.5741	20.47551	D. Lakušić, N. Kuzmanović, I. Janković, M. Zbiljić	11/07/2018	ZA-48633	0.342	0.004	3	2.46–4.70	2x	
D150	Rrapsh	*D. sylvestris* subsp. *bertisceus*	Albania	773	42.41509	19.50371	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	27/06/2019	ZA-54956	0.336	0.005	6	2.19–2.80	2x	+
D151	Thethi Shtegut	*D. sylvestris* subsp. *bertisceus*	Albania	1735	42.38844	19.73021	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	28/06/2019	ZA-54957	0.336	0.002	6	3.63–5.02	2x	+
D153	Rruga Thethe	*D. sylvestris* subsp. *bertisceus*	Albania	665	42.32486	19.59345	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	28/06/2019	ZA-54959	0.332	0.004	5	3.25–4.62	2x	+
D154	Cijevna	*D. sylvestris* subsp. *bertisceus*	Montenegro	139	42.39835	19.36711	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	28/06/2019	ZA-54960	0.326	0.001	5	4.70–5.09	2x	+
D158	Slano jezero	*D. sylvestris* subsp. *bertisceus*	Montenegro	900	42.75111	18.79916	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	29/06/2019	ZA-54964	0.334	0.001	5	3.55–3.60	2x	+
D169	Mt Shkelzeni	*D. sylvestris* subsp. *bertisceus*	Albania	2108	42.45568	20.11681	D. Shuka	24/07/2019	ZA-54975, ZA-54976	0.361	0.006	5	4.19–5.99	2x	+
D245	Bistra	*D. sylvestris* subsp. *bertisceus*	North Macedonia	1700	41.60719	20.75689	I. Rešetnik, M. Doboš, I. Ljubičić	20/07/2021	ZA-62635	0.344	0.007	4	4.20–5.84	2x	

D250	Grahovo road	*D. sylvestris* subsp. *bertisceus*	Montenegro	800	42.47019	18.85884	I. Rešetnik, M. Doboš, I. Ljubičić	24/07/2021	ZA-62639	0.347	0.008	4	4.52–5.63	2x	
D86	Sutjeska	*D. sylvestris* subsp. *bertisceus*	Bosnia and Herzegovina	638	43.31548	18.66821	S. Bogdanović, I. Ljubičić	11/07/2018	ZA-48662	0.330	0.003	4	2.55–5.47	2x	+
D87	Mt Maglic	*D. sylvestris* subsp. *bertisceus*	Bosnia and Herzegovina	1580	43.27309	18.71955	S. Bogdanović, I. Ljubičić	12/07/2018	ZA-48663	0.334	0.001	3	4.66–4.96	2x	+
D88	Piva river	*D. sylvestris* subsp. *bertisceus*	Montenegro	735	43.26326	18.84523	S. Bogdanović, I. Ljubičić	12/07/2018	ZA-48664	0.329	0.006	3	3.22–4.92	2x	+
D89	Pivska planina	*D. sylvestris* subsp. *bertisceus*	Montenegro	1680	43.13713	18.94089	S. Bogdanović, I. Ljubičić	13/07/2018	ZA-48665	0.331	0.006	3	2.15–4.03	2x	+
D90	Mt Durmitor	*D. sylvestris* subsp. *bertisceus*	Montenegro	2002	43.10045	19.05084	S. Bogdanović, I. Ljubičić	13/07/2018	ZA-48666	0.330	0.004	3	3.04–4.70	2x	+
D91	Berane	*D. sylvestris* subsp. *bertisceus*	Montenegro	711	42.88203	19.86147	S. Bogdanović, I. Ljubičić	13/07/2018	ZA-48612	0.328	0.002	3	3.03–4.01	2x	+
D92	Andrijevica	*D. sylvestris* subsp. *bertisceus*	Montenegro	746	42.72892	19.82458	S. Bogdanović, I. Ljubičić	13/07/2018	ZA-48667	0.325	0.003	3	3.04–3.69	2x	+
D93	Mt Prokletije Zeletin	*D. sylvestris* subsp. *bertisceus*	Montenegro	2011	42.64971	19.84412	S. Bogdanović, I. Ljubičić	14/07/2018	ZA-48668	0.330	0.007	3	2.71–3.78	2x	+
D94	Mt Prokletije Popadija W	*D. sylvestris* subsp. *bertisceus*	Montenegro	1989	42.52064	19.76142	S. Bogdanović, I. Ljubičić	14/07/2018	ZA-48669	0.333	0.009	3	2.96–3.97	2x	+
D95	Mt Prokletije Popadija SW	*D. sylvestris* subsp. *bertisceus*	Montenegro	1948	42.67997	20.01136	S. Bogdanović, I. Ljubičić	15/07/2018	ZA-48670	0.331	0.002	5	2.73–4.94	2x	+
D96	Moraca river	*D. sylvestris* subsp. *bertisceus*	Montenegro	278	42.69683	19.37431	S. Bogdanović, I. Ljubičić	15/07/2018	ZA-48613	0.332	0.007	3	4.01–4.60	2x	+
D178	Mt Kozjak	*D. sylvestris* subsp. *kozjakensis*	North Macedonia	1292	41.40914	21.67842	S. Bogdanović, I. Rešetnik	14/07/2019	ZA-54985	0.336	0.003	5	1.82–2.36	2x	+
D106	Sitnica	*D. sylvestris* subsp. *nodosus*	Bosnia and Herzegovina	915	42.56432	18.44312	S. Bogdanović, I. Ljubičić	17/7/2018, 29/6/2019	ZA-48623	0.328	0.002	5	1.79–5.00	2x	+
D124	Lastovo	*D. sylvestris* subsp. *nodosus*	Croatia	48	42.75519	16.92379	I. Rešetnik, S. Bogdanović	04/06/2019	ZA-54927	0.336	0.004	12	1.61–4.88	2x	
D127	Brac	*D. sylvestris* subsp. *nodosus*	Croatia	150	43.26608	16.69359	I. Ljubičić, A. Terlević, M. Grgurev	04/06/2019	ZA-54930	0.343	0.006	4	4.17–4.50	2x	
D128	Mosor	*D. sylvestris* subsp. *nodosus*	Croatia	810	43.52237	16.61158	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević, M. Grgurev	05/06/2019	ZA-54931	0.337	0.004	5	2.73–4.41	2x	
D160	Peljesac	*D. sylvestris* subsp. *nodosus*	Croatia	160	42.91527	17.42674	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	29/06/2019	ZA-54966	0.331	0.005	5	4.28–5.03	2x	+
D183	Smricnjak	*D. sylvestris* subsp. *nodosus*	Croatia	83	43.75222	15.87861	L. Laca, S. Bogdanović	17/06/2020	ZA-56692	0.324	0.004	5	2.43–4.04	2x	+
D192	Vrsak	*D. sylvestris* subsp. *nodosus*	Croatia	1713	43.71589	16.8893	M. Doboš	22/07/2020	ZA-56821	0.331	0.002	5	2.61–4.10	2x	+
D193	Gornja Korita	*D. sylvestris* subsp. *nodosus*	Croatia	906	43.70998	16.8192	S. Bogdanović	22/07/2020	ZA-56822	0.337	0.009	5	3.93–5.57	2x	
D2	Korcula	*D. sylvestris* subsp. *nodosus*	Croatia	250	42.94086	17.08055	I. Rešetnik, S. Bogdanović	11/05/2018	ZA-46277	0.337	0.004	4	2.89–4.75	2x	
D254	Hrgud	*D. sylvestris* subsp. *nodosus*	Bosnia and Herzegovina	1069	43.09763	18.05759	I. Rešetnik, M. Doboš, I. Ljubičić	25/07/2021	ZA-62640	0.334	0.003	3	3.98–6.29	2x	
D27	Mt Velebit Plesevica	*D. sylvestris* subsp. *nodosus*	Croatia	1211	44.70794	14.96583	I. Rešetnik, S. Bogdanović, I. Ljubičić	15/06/2018	ZA-47427	0.335	0.007	3	2.88–4.93	2x	
D275	Hvar	*D. sylvestris* subsp. *nodosus*	Croatia	610	43.14478	16.5971	S. Bogdanović	28/07/2021	ZA-62642	0.348	0.003	5	3.20–5.61	2x	
D28	Mt Velebit Alan	*D. sylvestris* subsp. *nodosus*	Croatia	1020	44.7153	14.9569	I. Rešetnik, S. Bogdanović, I. Ljubičić	15/06/2018	ZA-47428	0.334	0.002	10	2.70–4.73	2x	+

D33	Mt Velebit Baske Ostarije	*D. sylvestris* subsp. *nodosus*	Croatia	905	44.52948	15.14316	I. Rešetnik, S. Bogdanović, I. Ljubičić	15/06/2018	ZA-47433	0.332	0.005	3	2.79–4.93	2x	+
D42	Mt Velebit Brundo	*D. sylvestris* subsp. *nodosus*	Croatia	1605	44.36112	15.51313	I. Rešetnik, S. Bogdanović, I. Ljubičić	17/06/2018	ZA-47442	0.344	0.004	5	3.00–6.03	2x	
D46	Sibenik	*D. sylvestris* subsp. *nodosus*	Croatia	18	43.73736	15.87176	I. Rešetnik, S. Bogdanović, I. Ljubičić	18/06/2018	ZA-47456	0.338	0.008	6	2.80–5.16	2x	+
D47-1	Mt Biokovo low	*D. sylvestris* subsp. *nodosus*	Croatia	325	43.25955	17.07843	I. Rešetnik, S. Bogdanović, I. Ljubičić	18/06/2018	ZA-47457	0.342	0.005	6	4.22–4.91	2x	+
D47-2	Mt Biokovo high	*D. sylvestris* subsp. *nodosus*	Croatia	1116	43.28407	17.08662	I. Rešetnik, S. Bogdanović, I. Ljubičić	18/06/2018	ZA-47459	0.344	0.009	7	2.75–5.73	2x	+
D52	Mostar	*D. sylvestris* subsp. *nodosus*	Bosnia and Herzegovina	382	43.32759	17.80412	I. Rešetnik, S. Bogdanović, I. Ljubičić	19/06/2018	ZA-47463	0.331	0.009	4	2.87–4.60	2x	+
D55	Konjic	*D. sylvestris* subsp. *nodosus*	Bosnia and Herzegovina	360	43.64998	17.96701	I. Rešetnik, S. Bogdanović, I. Ljubičić	19/06/2018	ZA-47466	0.332	0.001	6	2.24–4.75	2x	+
D56	Vranic	*D. sylvestris* subsp. *nodosus*	Bosnia and Herzegovina	549	43.41747	17.41643	I. Rešetnik, S. Bogdanović, I. Ljubičić	20/06/2018	ZA-47467	0.327	0.004	4	4.00–4.65	2x	+
D57	Blidinje	*D. sylvestris* subsp. *nodosus*	Bosnia and Herzegovina	1298	43.58668	17.48789	I. Rešetnik, S. Bogdanović, I. Ljubičić	20/06/2018	ZA-47468	0.334	0.004	5	2.49–5.07	2x	+
D6	Mironja	*D. sylvestris* subsp. *nodosus*	Croatia	332	42.81569	17.84349	I. Rešetnik, S. Bogdanović	13/5/2018, 29/6/2019	ZA-46281	0.325	0.003	9	2.60–4.85	2x	+
D60	Mt Cvrsnica Cavkarice	*D. sylvestris* subsp. *nodosus*	Bosnia and Herzegovina	1556	43.57896	17.54072	I. Rešetnik, S. Bogdanović, I. Ljubičić	20/06/2018	ZA-47471	0.346	0.008	4	2.72–4.92	2x	+
D61	Trobukva	*D. sylvestris* subsp. *nodosus*	Bosnia and Herzegovina	904	43.54695	17.27952	I. Rešetnik, S. Bogdanović, I. Ljubičić	20/06/2018	ZA-47472	0.331	0.001	4	1.36–3.96	2x	+
D63	Mt Cincar Krug	*D. sylvestris* subsp. *nodosus*	Bosnia and Herzegovina	1197	43.85963	17.03066	I. Rešetnik, S. Bogdanović, I. Ljubičić	21/06/2018	ZA-47474	0.334	0.006	5	1.84–2.76	2x	+
D64	Mt Cincar Begovaca	*D. sylvestris* subsp. *nodosus*	Bosnia and Herzegovina	1397	43.88544	17.05981	I. Rešetnik, S. Bogdanović, I. Ljubičić	21/06/2018	ZA-47475	0.336	0.006	4	3.95–4.70	2x	+
D65	Livno Jurina glava	*D. sylvestris* subsp. *nodosus*	Bosnia and Herzegovina	1096	43.78286	17.16035	I. Rešetnik, S. Bogdanović, I. Ljubičić	21/06/2018	ZA-47476	0.334	0.005	5	2.13–4.49	2x	+
D66	Drvar	*D. sylvestris* subsp. *nodosus*	Bosnia and Herzegovina	716	44.34866	16.35956	I. Rešetnik, S. Bogdanović, I. Ljubičić	22/06/2018	ZA-47477	0.340	0.002	3	4.52–4.99	2x	+
D8	Omis	*D. sylvestris* subsp. *nodosus*	Croatia	63	43.45025	16.69853	I. Rešetnik, S. Bogdanović	13/5/2018, 29/6/2019	ZA-46283	0.328	0.004	6	2.44–3.13	2x	+
D80	Mt Dinara Glavas	*D. sylvestris* subsp. *nodosus*	Croatia	740	44.01427	16.41704	S. Bogdanović, I. Ljubičić	30/06/2018	ZA-48607	0.340	0.008	6	4.28–6.00	2x	+
D82	Mt Svilaja	*D. sylvestris* subsp. *nodosus*	Croatia	934	43.75119	16.52322	S. Bogdanović, I. Ljubičić	01/07/2018	ZA-48608	0.336	0.003	5	3.53–4.66	2x	+
D85	Mt Vlasic	*D. sylvestris* subsp. *nodosus*	Bosnia and Herzegovina	1100	44.25852	17.62419	S. Bogdanović, I. Ljubičić	10/07/2018	ZA-48611	0.329	0.005	3	3.72–4.65	2x	+
D12	Nacinovici	*D. sylvestris* subsp. *sylvestris*	Croatia	240	45.12395	14.20468	S. Bogdanović, I. Ljubičić	19/5/2018, 13/7/2019	ZA-46416	0.641	0.007	8	2.43–4.66	4x*	+
D130	Krcic	*D. sylvestris* subsp. *sylvestris*	Croatia	345	44.03398	16.29247	S. Bogdanović, I. Ljubičić	14/06/2019	ZA-54933	0.327	0.002	5	2.48–3.89	2x	+
D162	Ostrc	*D. sylvestris* subsp. *sylvestris*	Croatia	740	45.76377	15.6486	A. Terlević, M. Grgurev	05/07/2019	ZA-54968	0.325	0.001	5	1.85–1.91	2x	+
D165	Litija	*D. sylvestris* subsp. *sylvestris*	Slovenia	295	46.08895	14.8873	A. Terlević, I. Ljubičić	09/07/2019	ZA-54971	0.331	0.003	5	3.31–4.29	2x	
D166	Polhograjska Grmada	*D. sylvestris* subsp. *sylvestris*	Slovenia	900	46.08407	14.33474	A. Terlević, I. Ljubičić	10/07/2019	ZA-54972	0.333	0.006	4	2.18–3.34	2x	+
D17	Mt Spacatto	*D. sylvestris* subsp. *sylvestris*	Italy	300	45.64227	13.8311	I. Rešetnik, S. Bogdanović, I. Ljubičić	13/06/2018	ZA-47417	0.640	0.004	3	2.00–2.54	4x	+
D184	Lim bay	*D. sylvestris* subsp. *sylvestris*	Croatia	136	45.13314	13.73433	A. Terlević	21/06/2020	ZA-56693	0.328	0.007	5	2.96–6.55	2x	+

D19-2	Vodice tetrapl	*D. sylvestris* subsp. *sylvestris*	Croatia	660	45.47917	14.04971	I. Rešetnik, S. Bogdanović, I. Ljubičić	13/6/2018, 13/7/2019	ZA-47419	0.651	0.006	8	1.80–3.57	4x	+
D196	Brkini	*D. sylvestris* subsp. *sylvestris*	Slovenia	625	45.50694	14.16806	B. Frajman	15/08/2020	ZA-56825	0.657	0.006	5	1.85–2.80	4x	
D20	Zbevnica	*D. sylvestris* subsp. *sylvestris*	Croatia	941	45.45721	14.01632	I. Rešetnik, S. Bogdanović, I. Ljubičić	13/6/2018, 19/7/2020	ZA-47420	0.651	0.004	3	3.55–4.50	4x*	+
D205	Klecice	*D. sylvestris* subsp. *sylvestris*	Croatia	968	45.26685	15.1413	I. Ljubičić, A. Terlević	16/06/2021	ZA-62605	0.337	0.007	4	4.36–5.37	2x	
D206	Mt Calvo	*D. sylvestris* subsp. *sylvestris*	Italy	360	45.65316	13.82246	I. Rešetnik, S. Bogdanović, M. Temunović, A. Terlević	28/06/2021	ZA-62606	0.646	0.008	4	2.57–3.58	4x	
D207	Hervati	*D. sylvestris* subsp. *sylvestris*	Italy	345	45.6259	13.86941	I. Rešetnik, S. Bogdanović, M. Temunović, A. Terlević	28/06/2021	ZA-62607	0.652	0.009	6	2.70–4.30	4x	
D21	Slum	*D. sylvestris* subsp. *sylvestris*	Croatia	553	45.4234	14.01213	I. Rešetnik, S. Bogdanović, I. Ljubičić	13/6/2018, 19/7/2020	ZA-47421	0.650	0.009	4	2.26–5.01	4x*	+
D22	Mt Ucka	*D. sylvestris* subsp. *sylvestris*	Croatia	525	45.31681	14.17734	I. Rešetnik, S. Bogdanović, I. Ljubičić	13/06/2018	ZA-47422	0.656	0.010	4	2.02–4.90	4x	+
D24	Gornje Jelenje	*D. sylvestris* subsp. *sylvestris*	Croatia	862	45.36157	14.61724	I. Rešetnik, S. Bogdanović, I. Ljubičić	14/06/2018	ZA-47424	0.648	0.003	5	2.35–4.87	4x	+
D256	Velez	*D. sylvestris* subsp. *sylvestris*	Bosnia and Herzegovina	1912	43.35755	17.93495	I. Rešetnik, M. Doboš, I. Ljubičić	25/07/2021	ZA-62641	0.340	0.009	4	4.23–5.21	2x	
D34	Mt Velebit Visocica	*D. sylvestris* subsp. *sylvestris*	Croatia	1458	44.4293	15.3643	I. Rešetnik, S. Bogdanović, I. Ljubičić	16/06/2018	ZA-47434	0.334	0.004	4	3.14–4.43	2x	
D37	Mt Velebit Buljma	*D. sylvestris* subsp. *sylvestris*	Croatia	1218	44.36597	15.46143	I. Rešetnik, S. Bogdanović, I. Ljubičić	17/06/2018	ZA-47437	0.336	0.004	5	3.77–4.93	2x	+
D38	Mt Paklenica Struge	*D. sylvestris* subsp. *sylvestris*	Croatia	1368	44.37109	15.46567	I. Rešetnik, S. Bogdanović, I. Ljubičić	17/06/2018	ZA-47438	0.339	0.006	3	2.74–4.53	2x	+
D58	Mt Cvrsnica SW	*D. sylvestris* subsp. *sylvestris*	Bosnia and Herzegovina	1928	43.59503	17.54076	I. Rešetnik, S. Bogdanović, I. Ljubičić	20/06/2018	ZA-47469	0.325	0.001	3	2.97–4.59	2x	
D59	Mt Cvrsnica Siroka kosa	*D. sylvestris* subsp. *sylvestris*	Bosnia and Herzegovina	1732	43.58652	17.53378	I. Rešetnik, S. Bogdanović, I. Ljubičić	20/06/2018	ZA-47470	0.334	0.007	4	3.72–5.07	2x	+
D81	Mt Dinara Osljak	*D. sylvestris* subsp. *sylvestris*	Croatia	1418	44.03867	16.39343	S. Bogdanović, I. Ljubičić	30/06/2018	ZA-48606	0.336	0.008	3	4.39–4.93	2x	+
D83	Banja Luka	*D. sylvestris* subsp. *sylvestris*	Bosnia and Herzegovina	207	44.68547	17.1797	S. Bogdanović, I. Ljubičić	10/07/2018	ZA-48609	0.329	0.005	3	2.99–4.99	2x	+
D84	Jajce	*D. sylvestris* subsp. *sylvestris*	Bosnia and Herzegovina	380	44.3468	17.24253	S. Bogdanović, I. Ljubičić	10/07/2018	ZA-48610	0.338	0.009	3	2.12–4.87	2x	
D1	Klek village	*D. sylvestris* subsp. *tergestinus*	Croatia	80	42.94874	17.56451	I. Rešetnik, S. Bogdanović	09/05/2018	ZA-46276	0.345	0.005	3	4.58–5.00	2x	+
D11	Gornji Kamenjak	*D. sylvestris* subsp. *tergestinus*	Croatia	55	44.81097	13.90088	S. Bogdanović, I. Ljubičić	17/05/2018	ZA-46415	0.339	0.005	4	2.96–4.68	2x	+
D111	Cres	*D. sylvestris* subsp. *tergestinus*	Croatia	40	44.97721	14.445	S. Bogdanović, S. Cambria	21/10/2018	ZA-48628	0.342	0.002	4	2.05–4.25	2x	
D129	Gradina	*D. sylvestris* subsp. *tergestinus*	Croatia	186	43.77399	15.95608	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević, M. Grgurev	05/06/2019	ZA-54932	0.340	0.004	4	2.98–4.02	2x	+
D13	Krizisce	*D. sylvestris* subsp. *tergestinus*	Croatia	513	45.2676	14.59441	S. Bogdanović, I. Ljubičić	19/05/2018	ZA-46417	0.333	0.004	4	3.75–4.68	2x	+
D16	Sezana	*D. sylvestris* subsp. *tergestinus*	Italy	344	45.70153	13.83729	I. Rešetnik, S. Bogdanović, I. Ljubičić	13/06/2018	ZA-47416	0.341	0.007	4	3.31–3.82	2x	+
D18	Petrinje	*D. sylvestris* subsp. *tergestinus*	Slovenia	422	45.57284	13.90681	I. Rešetnik, S. Bogdanović, I. Ljubičić	13/06/2018	ZA-47418	0.336	0.008	5	2.66–4.99	2x	+
D185	Krk	*D. sylvestris* subsp. *tergestinus*	Croatia	220	45.02389	14.64694	I. Rešetnik	14/06/2020	ZA-56694	0.340	0.004	5	3.30–6.93	2x*	

D25	Bunica	*D. sylvestris* subsp. *tergestinus*	Croatia	8	45.02484	14.88473	I. Rešetnik, S. Bogdanović, I. Ljubičić	14/06/2018	ZA-47425	0.337	0.001	4	2.67–4.62	2x	
D29	Donji Bileni	*D. sylvestris* subsp. *tergestinus*	Croatia	274	44.71196	14.9225	I. Rešetnik, S. Bogdanović, I. Ljubičić	15/06/2018	ZA-47429	0.340	0.007	5	2.46–4.92	2x	
D32	Karlobag	*D. sylvestris* subsp. *tergestinus*	Croatia	12	44.53338	15.06519	I. Rešetnik, S. Bogdanović, I. Ljubičić	15/06/2018	ZA-47432	0.335	0.005	3	4.09–4.66	2x*	
D36	Mt Velebit Paklenica	*D. sylvestris* subsp. *tergestinus*	Croatia	397	44.31347	15.45328	I. Rešetnik, S. Bogdanović, I. Ljubičić	17/06/2018	ZA-47436	0.340	0.006	4	2.86–4.77	2x	+
D4	Dubrovnik	*D. sylvestris* subsp. *tergestinus*	Croatia	279	42.64521	18.12021	I. Rešetnik, S. Bogdanović	12/05/2018	ZA-46279	0.352	0.005	3	2.75–4.26	2x	+
D43	Vransko jezero	*D. sylvestris* subsp. *tergestinus*	Croatia	35	43.86523	15.6423	I. Rešetnik, S. Bogdanović, I. Ljubičić	18/06/2018	ZA-47453	0.341	0.008	4	2.58–4.72	2x	
D50	Mostar Vukodol	*D. sylvestris* subsp. *tergestinus*	Bosnia and Herzegovina	250	43.32542	17.79292	I. Rešetnik, S. Bogdanović, I. Ljubičić	19/06/2018	ZA-47461	0.353	0.005	5	3.32–4.18	2x	
D53	Mostar Hum	*D. sylvestris* subsp. *tergestinus*	Bosnia and Herzegovina	385	43.32755	17.80407	I. Rešetnik, S. Bogdanović, I. Ljubičić	19/06/2018	ZA-47464	0.355	0.009	5	2.11–4.87	2x	
D62	Livno	*D. sylvestris* subsp. *tergestinus*	Bosnia and Herzegovina	821	43.84762	16.9733	I. Rešetnik, S. Bogdanović, I. Ljubičić	21/06/2018	ZA-47473	0.340	0.008	3	2.81–4.33	2x	+
D7	Mravinica Doli	*D. sylvestris* subsp. *tergestinus*	Croatia	351	42.81084	17.83079	I. Rešetnik, S. Bogdanović	13/05/2018	ZA-46282	0.351	0.007	3	2.49–4.33	2x	+
D9	Malacka	*D. sylvestris* subsp. *tergestinus*	Croatia	602	43.58147	16.32579	I. Rešetnik, S. Bogdanović	13/05/2018	ZA-46284	0.339	0.003	3	4.57–5.88	2x	+
16866	Ahrntal	*D. sylvestris* s.l. - Alps	Italy	1075	46.89278	11.96194	D. Volgger, F. Faltner, B. Frajman	12/07/2021	IB-16866	0.366	0.001	3	5.99–8.21	2x	
16867	Pustertal Niederdorf	*D. sylvestris* s.l. - Alps	Italy	1850	46.70778	12.17528	D. Volgger, A. Seyer, B. Frajman	12/07/2021	IB-16867	0.352	0.010	3	2.71–3.08	2x	
16868	Karnische Alpen	*D. sylvestris* s.l. - Alps	Austria	1960	46.69444	12.49306	D. Volgger, B. Frajman	13/07/2021	IB-16868	0.326	0.005	3	4.30–5.60	2x	
16869	Dolomiti Passo Tre Croci	*D. sylvestris* s.l. - Alps	Italy	1810	46.5575	12.19944	D. Volgger, B. Frajman	13/07/2021	IB-16869	0.330	0.004	3	4.89–9.03	2x	
16870	Dolomiti Fiames	*D. sylvestris* s.l. - Alps	Italy	1410	46.59667	12.11222	D. Volgger, B. Frajman	13/07/2021	IB-16870	0.334	0.001	3	4.80–8.79	2x	
16871	Dolomiti Furkelpass	*D. sylvestris* s.l. - Alps	Italy	2140	46.71139	11.97028	B. Frajman	13/07/2021	IB-16871	0.354	0.003	3	6.76–7.30	2x	
16872	Pustertal Issing	*D. sylvestris* s.l. - Alps	Italy	870	46.81	11.84306	D. Volgger, B. Frajman	13/07/2021	IB-16872	0.359	0.004	3	6.80–9.45	2x	
16875	Pustertal Burgkofl	*D. sylvestris* s.l. - Alps	Italy	935	46.79278	11.88111	F. Faltner	12/07/2021	IB-16875	0.348	0.008	3	8.83–10.19	2x	
16927	Villgrater Berge	*D. sylvestris* s.l. - Alps	Austria	1500	46.80917	12.33083	B. Frajman, M. Doboš	02/08/2021	IB-16927	0.339	0.001	5	2.34–5.62	2x	
16972	Pordenone	*D. sylvestris* s.l. - Alps	Italy	900	46.28	12.36778	P. Schönswetter, T. Zeni	31/07/2021	IB-16972	0.346	0.006	5	3.81–5.44	2x	
16973	Prealpi Bellunesi	*D. sylvestris* s.l. - Alps	Italy	1515	46.11278	12.50778	P. Schönswetter, T. Zeni	01/08/2021	IB-16973	0.328	0.001	3	6.65–9.27	2x	
16977	Dolomiti Strudelkopf	*D. sylvestris* s.l. - Alps	Italy	2170	46.64417	12.2	A. Seyr	13/08/2021	IB-16977	0.335	0.005	6	3.82–4.90	2x	
D121	Oberes Gericht	*D. sylvestris* s.l. - Alps	Austria	1160	47.03669	10.64664	M. Falch	16/07/2018	ZA-54924	0.351	0.003	3	2.47–3.43	2x	
D122	Innsbruck S	*D. sylvestris* s.l. - Alps	Austria	880	47.24425	11.41853	M. Falch	03/09/2018	ZA-54925	0.360	0.004	3	1.87–4.49	2x	
D164	Vrsic	*D. sylvestris* s.l. - Alps	Slovenia	1954	46.43132	13.73659	A. Terlević, I. Ljubičić	09/07/2019	ZA-54970	0.333	0.000	6	2.24–2.87	2x	
D197	Achberg	*D. sylvestris* s.l. - Alps	Austria	785	47.28972	10.98667	B. Frajman	07/07/2020	ZA-56826	0.359	0.004	5	2.49–6.76	2x	

D199	Gailtal Arnoldstein	*D. sylvestris* s.l. - Alps	Austria	545	46.56528	13.69083	B. Frajman, P. Schönswetter	22/05/2020	ZA-56828	0.333	0.002	5	2.62–6.11	2x	
D67	Prutz	*D. sylvestris* s.l. - Alps	Austria	904	47.07507	10.65361	I. Rešetnik	27/06/2018	ZA-47478	0.363	0.008	3	1.94–4.48	2x	
D68	Mals	*D. sylvestris* s.l. - Alps	Italy	1039	46.67989	10.55889	I. Rešetnik	27/06/2018	ZA-47479	0.361	0.002	3	2.04–3.96	2x	
D69	Gailtal alps	*D. sylvestris* s.l. - Alps	Austria	1971	46.75922	12.88028	I. Rešetnik	29/06/2018	ZA-47480	0.335	0.005	5	1.93–2.89	2x	
D70	Zentralalpen	*D. sylvestris* s.l. - Alps	Austria	2030	47.01306	12.25111	I. Rešetnik	30/06/2018	ZA-47481	0.333	0.004	3	2.48–2.90	2x	
D71	Innsbruck Nordkette	*D. sylvestris* s.l. - Alps	Austria	1906	47.30494	11.35861	I. Rešetnik	08/07/2018	ZA-47482	0.358	0.005	3	2.51–4.57	2x	
Lago di Fedaia	Lago di Fedaia	*D. sylvestris* s.l. - Alps	Italy	2220	46.46606	11.85929	H. Luqman	04/08/2016	ZT-Lago di Fedaia	0.359	0.000	3	6.69–9.79	2x	
Mt Caslano	Mt Caslano	*D. sylvestris* s.l. - Alps	Switzerland	332	45.96053	8.88331	H. Luqman	26/06/2017	ZT-Mt Caslano	0.355	0.005	3	6.86–7.55	2x	
Mustair Low	Mustair Low	*D. sylvestris* s.l. - Alps	Switzerland	1300	46.63216	10.44699	H. Luqman	03/08/2017	ZT-Mustair Low	0.366	0.003	3	4.89–5.81	2x	
Rifugio Graziani	Rifugio Graziani	*D. sylvestris* s.l. - Alps	Italy	1566	45.79879	10.89384	H. Luqman	15/07/2017	ZT-Rifugio Graziani	0.353	0.005	3	3.81–4.64	2x	
Schwarzenmatt	Schwarzenmatt	*D. sylvestris* s.l. - Alps	Switzerland	1220	46.63409	7.349496	H. Luqman	23/07/2016	ZT-Schwarzenmatt	0.369	0.004	3	5.28–7.11	2x	
Thumel	Thumel	*D. sylvestris* s.l. - Alps	Italy	1940	45.53495	7.10119	H. Luqman	26/08/2017	ZT-Thumel	0.367	0.008	3	3.98–4.75	2x	
D102	Obzovica	*D. sylvestris* s.l. - Balkan	Montenegro	931	42.30581	18.93189	S. Bogdanović, I. Ljubičić	16/07/2018	ZA-48619	0.335	0.001	4	2.82–4.14	2x	+
D103	Mt Lovcen	*D. sylvestris* s.l. - Balkan	Montenegro	1681	42.4003	18.83807	S. Bogdanović, I. Ljubičić	16/07/2018	ZA-48620	0.337	0.007	4	3.95–4.88	2x	+
D108	Gramsh	*D. sylvestris* s.l. - Balkan	Albania	969	40.86694	20.38444	B. Frajman, P. Schönswetter	12/07/2018	ZA-48625	0.336	0.002	4	4.19–5.26	2x	
D109	Maja e Ostrovice	*D. sylvestris* s.l. - Balkan	Albania	1406	40.57972	20.47722	B. Frajman, P. Schönswetter	13/07/2018	ZA-48626	0.336	0.007	3	2.92–4.93	2x	
D110	Mt Jablanica	*D. sylvestris* s.l. - Balkan	North Macedonia	1759	41.27278	20.535	B. Frajman, P. Schönswetter	15/07/2018	ZA-48627	0.342	0.003	3	2.94–4.92	2x	
D119	Mt Valamara	*D. sylvestris* s.l. - Balkan	Albania	1670	40.82167	20.50167	M. Duchon	16/07/2018	ZA-54922	0.351	0.008	3	1.70–4.23	2x	
D145	Mat	*D. sylvestris* s.l. - Balkan	Albania	145	41.67954	19.84739	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	26/06/2019	ZA-54947	0.338	0.007	5	3.73–4.76	2x	+
D147	Shkoder	*D. sylvestris* s.l. - Balkan	Albania	129	42.07219	19.56423	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	27/06/2019	ZA-54953	0.330	0.002	8	3.16–4.50	2x	+
D170	Mt Kopaonik	*D. sylvestris* s.l. - Balkan	Serbia	1787	43.26151	20.83653	S. Bogdanović, M. Doboš, M. Grgurev, I. Rešetnik	10/07/2019	ZA-54977	0.334	0.001	6	2.14–2.48	2x	+
D171	Ljuboten peak	*D. sylvestris* s.l. - Balkan	North Macedonia	2255	42.19948	21.12385	S. Bogdanović, M. Doboš, M. Grgurev, I. Rešetnik	11/07/2019	ZA-54978	0.335	0.005	5	1.83–4.03	2x	+
D172	Ljuboten peak scardicus	*D. sylvestris* s.l. - Balkan	North Macedonia	2255	42.19948	21.12385	S. Bogdanović, M. Doboš, M. Grgurev, I. Rešetnik	11/07/2019	ZA-54979	0.336	0.004	5	2.31–4.65	2x	+
D173	Mt Bistra	*D. sylvestris* s.l. - Balkan	North Macedonia	1687	41.6293	20.68481	S. Bogdanović, M. Doboš, M. Grgurev, I. Rešetnik	12/07/2019	ZA-54980	0.336	0.001	6	1.86–2.17	2x	+
D174	Mt Galicica	*D. sylvestris* s.l. - Balkan	North Macedonia	1510	40.96566	20.81879	S. Bogdanović, M. Doboš, M. Grgurev, I. Rešetnik	12/07/2019	ZA-54981	0.339	0.000	5	2.37–4.61	2x	+
D175	Mt Korab Nistrovski	*D. sylvestris* s.l. - Balkan	North Macedonia	2112	41.7919	20.59291	S. Bogdanović, M. Doboš, M. Grgurev, I. Rešetnik	13/07/2019	ZA-54982	0.337	0.004	5	2.98–4.02	2x	+
D176	Mt Korab Radika	*D. sylvestris* s.l. - Balkan	North Macedonia	1379	41.79959	20.62801	S. Bogdanović, M. Doboš, M. Grgurev, I. Rešetnik	13/07/2019	ZA-54983	0.337	0.001	5	2.39–2.41	2x	+

D179	Mt Jakupica Solunska glava	*D. sylvestris* s.l. - Balkan	North Macedonia	2274	41.69279	21.40381	S. Bogdanović, M. Grgurev, I. Rešetnik	17/07/2019	ZA-54986	0.347	0.005	5	2.02–3.73	2x	+
D180	Mt Jakupica Ismailica	*D. sylvestris* s.l. - Balkan	North Macedonia	1235	41.68044	21.43242	S. Bogdanović, M. Grgurev, I. Rešetnik	17/07/2019	ZA-54987	0.345	0.006	5	1.75–2.61	2x	+
D182	Gjergjevica	*D. sylvestris* s.l. - Balkan	Albania	1300	40.58626	20.57514	L. Shuka	27/07/2019	ZA-54989, ZA-54990	0.376	0.004	4	4.73–6.00	2x	+
D204	Voskopoje	*D. sylvestris* s.l. - Balkan	Albania	1165	40.58247	20.56072	D. Lakušić, N. Kuzmanović, I. Janković, M. Zbiljić	11/07/2018	ZA-58876	0.355	0.004	3	5.63–8.89	2x	
D246	Sar Planina	*D. sylvestris* s.l. - Balkan	Albania	2119	42.09306	20.8931	I. Rešetnik, M. Doboš, I. Ljubičić	21/07/2021	ZA-62636	0.338	0.006	3	3.26–4.52	2x	
D248	Mali Gropa	*D. sylvestris* s.l. - Balkan	Albania	1616	41.37702	20.06664	I. Rešetnik, M. Doboš, I. Ljubičić	22/07/2021	ZA-62637	0.348	0.003	4	3.85–5.21	2x	
D76	Maljen	*D. sylvestris* s.l. - Balkan	Serbia	640	44.04424	20.01645	S. Đurović, G. Tomović	15/07/2018	ZA-48631	0.328	0.006	4	2.95–4.37	2x	
D77	Mt Sar Planina	*D. sylvestris* s.l. - Balkan	Serbia	1755	42.2335	20.91537	D. Lakušić, N. Kuzmanović, I. Janković, M. Zbiljić	14/07/2018	ZA-48632	0.340	0.004	3	2.23–4.34	2x	
D79	Mt Kopaonik Pancicev vrh	*D. sylvestris* s.l. - Balkan	Serbia	1660	43.26149	20.83973	S. Đurović, G. Tomović	13/07/2018	ZA-48634	0.335	0.004	3	3.69–3.95	2x	

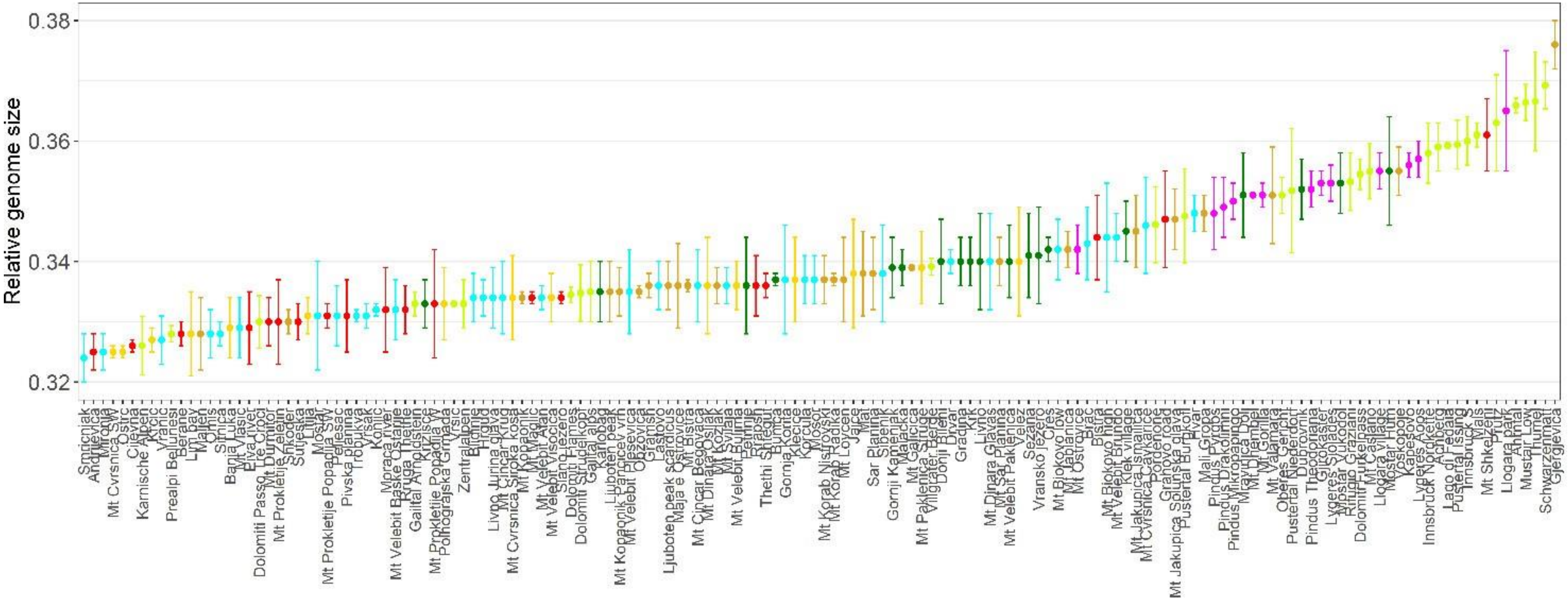

Figure S1. Relative genome size (RGS) in diploid populations of *Dianthus sylvestris* sorted by increasing RGS values. Population means (dots) with corresponding standard deviation (vertical lines)are presented. Population names correspond to Table S1. Colors correspond to subspecies, as in Figure 2.

Table S2. Results of Kruskal Wallis test between diploid (2*x*, N = 9) and tetraploid (4*x*, N = 7) populations of *Dianthus sylvestris* subsp. *sylvestris*. Morphological characters with significant differences between ploidy levels at $p < 0.05$ are shown in bold.

Character	**Abbreviation**	**statistic**	***p* value**
Length of the woody part of the stem below basal leaves	BL	0.025779	0.872442
Basal leaf length	BLL	4.495013	**0.033994**
Calyx length	CL	0.02521	0.873845
Length of the cauline leaf from the second stem node	CLL	6.463286	**0.011013**
Calyx teeth length	CTL	1.235294	0.26638
Number of epicalyx scales	ESN	1.153723	0.282771
Height of first branching	FBH	7.868347	**0.005031**
Number of flowers per stem	FPS	2.072495	0.149976
Inflorescence length	IL	2.876809	0.089864
Inner epicalyx scale apex length	ISAL	0.809524	0.368261
Inner epicalyx scale length	ISL	1.235294	0.26638
Inner epicalyx scale width	ISW	2.691877	0.100861
Length of the first stem internode	LIL	0.002818	0.957667
Plant height	PH	5.9359	**0.014835**
Petal length	PL	0.226891	0.633839
Petal limb length	PLL	0.02521	0.873845
Petal limb width	PLW	0.02521	0.873845
Petal teeth length	PTL	0.630252	0.427263
Maximum number of petal teeth	PTMAX	0.912933	0.339337
Minimum number of petal teeth	PTMIN	2.707805	0.099858
Ratio Calyx teeth length / Calyx length	RCALYX	2.699817	0.10036
Ratio Petal teeth length / Petal limb length	RDENT	0.280938	0.596087
Ratio Calyx length / Claw length	RFL	1.011204	0.314614
Ratio Inner epicalyx scale length without apex / Inner epicalyx scale width	RIS	1.481793	0.223494
Ratio Inner epicalyx scale apex length / Inner epicalyx scale length	RISA	0.070028	0.791296
Ratio Petal limb length / Petal limb width	RLIMB	1.122098	0.289467
Ratio Petal limb length / Petal length	RPETAL	0.718143	0.396754
Length of the last stem internode below branching	UIL	4.715618	**0.02989**

Table S3. Results of Kruskal Wallis test between diploid (2*x*, N = 15) and tetraploid (4*x*, N = 10) populations of *Dianthus sylvestris* subsp. *sylvestris*. Environmental variables with significant differences between ploidy levels at $p < 0.05$ are shown in bold.

short name	**long name**	**unit**	**statistic**	***p* value**
bio01	mean annual air temperature	°C/10	5.060548	**0.024477**
bio02	mean diurnal air temperature range	°C/10	4.601475	**0.031944**
bio03	isothermality	°C/10	5.829034	**0.015764**
bio04	temperature seasonality	°C/10	0.249231	0.617617
bio05	mean daily maximum air temperature of the warmest month	°C/10	0.943033	0.331499
bio06	mean daily minimum air temperature of the coldest month	°C/10	9.484988	**0.002072**
bio07	annual range of air temperature	°C/10	3.673611	0.05528
bio08	mean daily mean air temperatures of the wettest quarter	°C/10	1.492675	0.221802
bio09	mean daily mean air temperatures of the driest quarter	°C/10	0.249327	0.61755
bio10	mean daily mean air temperatures of the warmest quarter	°C/10	4.681801	**0.030484**
bio11	mean daily mean air temperatures of the coldest quarter	°C/10	6.533385	**0.010587**
bio12	annual precipitation amount	kg m^{-2}	0.062332	0.802848
bio13	precipitation amount of the wettest month	kg m^{-2}	0.197151	0.657031
bio14	precipitation amount of the driest month	kg m^{-2}	5.198299	**0.022609**
bio15	precipitation seasonality	kg m^{-2}	2.972931	0.084668
bio16	mean monthly precipitation amount of the wettest quarter	kg m^{-2}	0.249231	0.617617
bio17	mean monthly precipitation amount of the driest quarter	kg m^{-2}	4.102386	**0.042823**
bio18	mean monthly precipitation amount of the warmest quarter	kg m^{-2}	0.603309	0.437318
bio19	mean monthly precipitation amount of the coldest quarter	kg m^{-2}	0.012308	0.911664
dtm_east	eastness	index	8.972308	**0.002741**
dtm_north	northness	index	0.110769	0.73927
dtm_slope	slope	°	4.68	**0.030516**
nfd	number of frost days	No days	9.833654	**0.001713**
sdays	number of snow days	No days	7.240477	**0.007128**
soil_clay	clay content	%	6.484245	**0.010883**
soil_org_carb	soil organic carbon content	x 5 g kg^{-1}	1.563563	0.211144
soil_ph_h2o	soil pH x 10 in H2O	pH	1.238427	0.265774
soil_sand	sand content	%	1.236239	0.266197
soil_water	soil water content at 33kPa		0.625926	0.428854
srad	surface solar radiation downwards	kJ m^{-2}	4.68	**0.030516**

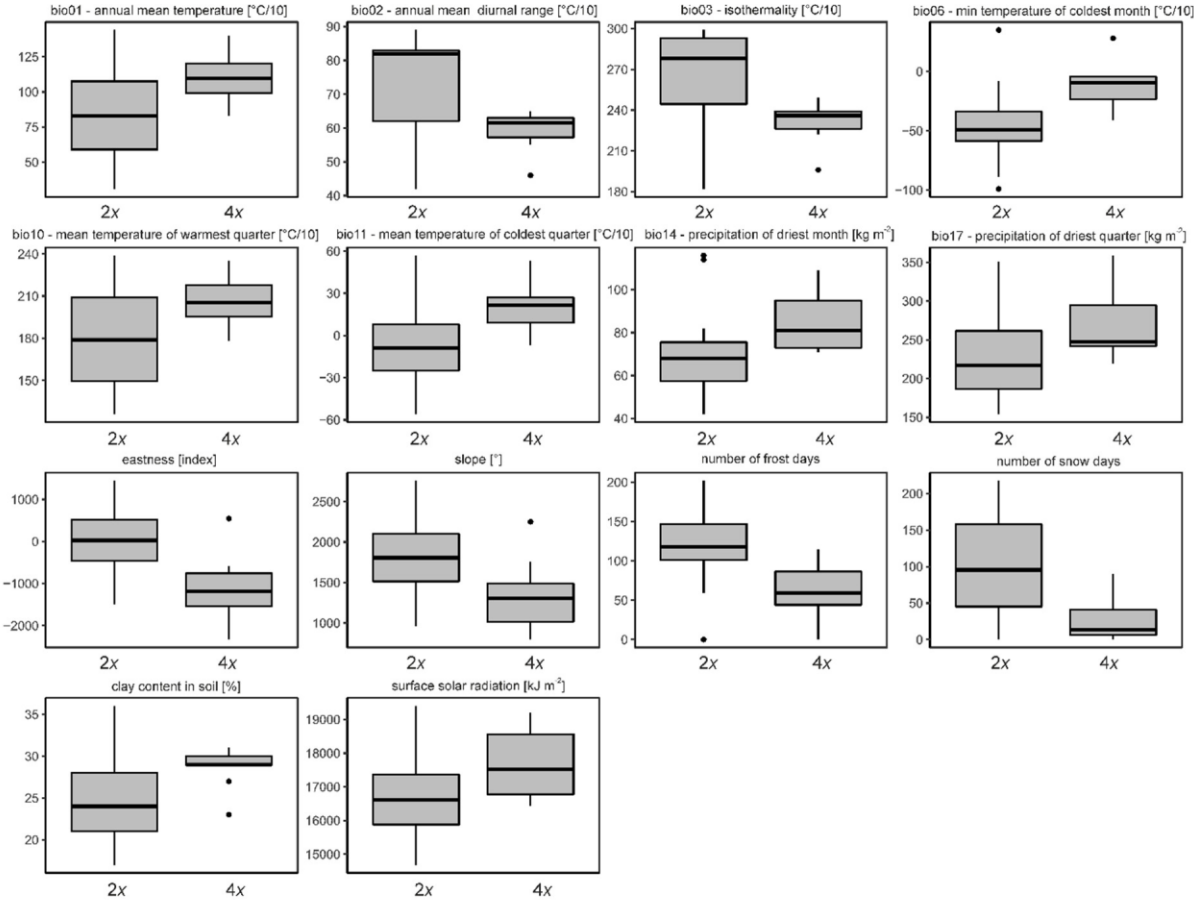

Figure S2. Boxplots showing environmental differences along the 14 environmental variables between diploid (2*x*) and tetraploid (4*x*) populations of *Dianthus sylvestris* subsp. *sylvestris*. Only variables with significant differences ($p < 0.05$), as revealed by Kruskal Wallis test (Table S3), are shown.

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AQ1-AQ4

Morphological and environmental variability of *Dianthus sylvestris* (Caryophyllaceae) in the Balkan Peninsula

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Species with wide ranges often exhibit morphological variability that is mostly shaped by ecological and evolutionary processes, and the resulting diversity is frequently reflected in intraspecific taxonomy. *Dianthus sylvestris* in the Balkan Peninsula encompasses six subspecies (*D. sylvestris* subspp. *alboroseus*, *bertisceus*, *kozjakensis*, *nodosus*, *sylvestris* and *tergestinus*), the identification of which is problematic due to a great morphological variability and complex patterns of environmental variation. To explore morphological variation of described subspecies and to investigate how their morphology varies in relation to environmental variations across their geographical distribution, here we evaluated 97 populations of *D. sylvestris* for variability in 25 morphological characters and 20 environmental variables. Number and shape of epicalyx scales, calyx length and petal denticulation captured the greatest extent of morphological variability between populations. Morphometric analyses revealed a continuous variability in quantitative morphological characters and an absence of clear morphological differentiation between the investigated subspecies. However, two main morphologically distinct entities, the north-western Balkan populations and the south-eastern Balkan populations, were identified and they were split approximately along the Neretva River valley. In addition, our results indicate that environment, rather than geography, plays a potentially more important role in shaping the morphological variability of this taxon. Overall, our results do not support the recognition of the described subspecies or the currently accepted taxonomic concept, instead pointing to a continuous morphological variability of *D. sylvestris* in the Balkan Peninsula.

ADDITIONAL KEYWORDS: cline – geographical variation – morphometrics – redundancy analysis.

INTRODUCTION

Plant morphological characteristics are influenced by biotic and abiotic factors, and spatio-temporal variation in environmental variables (such as temperature, precipitation, soil) can have profound effects on the adaptation of phenotypic traits to local environments. Morphological and ecological characteristics are both often distributed continuously across geographical gradients (Endler, 1977) and studies across elevation gradients provide evidence of how environmental variation can be one of the drivers behind morphological variability. For example, in many species, reduction in plant height and decrease in specific leaf area with increasing elevation are observed (Scheepens, Frei & Stöcklin, 2010; Wright *et al.*, 2017; Halbritter *et al.*, 2018), and the use of different strategies for pollination and diaspore dispersal along elevation gradients has also been recorded (Pellissier *et al.*, 2010).

Species occupying large areas and diverse habitats exhibit morphological variability that is often reflected in taxonomy, and groups of populations differing from

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one another in minor morphological characters, usually occupying distinct geographical areas and being ecologically isolated or phylogenetically separated, are often defined as subspecies (Meikle, 1957; Hamilton & Reichard, 1992). The delimitation of taxonomic categories is not straightforward and requires robust evidence from integrative approaches. However, the main criteria used to circumscribe organisms traditionally are based on quantitative and qualitative morphological characters. Comprehensive evaluation of morphological variability can show that differences among taxa could be related to the combined effect of ecological variables and might not have taxonomic value, as seen in the *Globularia cordifolia* L. species complex (Innangi *et al.*, 2020), or that similar environmental factors can lead to converging leaf morphologies, as observed in *Ternstroemia lineata* DC. species complex (Alcántara-Ayala *et al.*, 2020). Therefore, morphological distinctiveness may not always be a reliable guide to specific or intraspecific classification (Caković *et al.*, 2015; Španiel, Zozomová-Lihová & Marhold, 2017; Buitrago Aristizábal, Oliveira Gouvêa De Figueiredo & André, 2020), and long-standing taxonomic uncertainties may hamper the understanding of species evolutionary history, floristic investigations and conservation actions (Lega *et al.*, 2012; Theodoridis, Nogués-Bravo & Conti, 2019; Ji *et al.*, 2020).

Dianthus L. (carnations) is one of the largest genera of Caryophyllaceae with > 300 species native to Eurasia and Africa (Pax & Hoffmann, 1934; Bittrich, 1993; Fassou *et al.*, 2022). Most species have small and geographically restricted distributions, and there are > 70 endemic species in Europe, with the Mediterranean region as the centre of diversity (Bittrich, 1993). The outstanding diversity of the genus originated from a rapid radiation with the fastest rate of speciation known in flowering plants, the onset of which was dated to late Pliocene and probably continued through the Pleistocene (Valente, Savolainen & Vargas, 2010). Rapid diversification is reflected in a complex record of names, diverse taxonomic concepts and a lack of reliable distributional data of individual species, rendering *Dianthus* one of the taxonomically most challenging genera in the European flora (Tutin & Walters, 1993; Brullo & Guarino, 2019).

A detected increase in diversification rates of carnations coincided with increased aridity and seasonality in the Pleistocene, suggesting a link between climate and biodiversity (Valente *et al.*, 2010). Studies associating environmental variations with intraspecific differentiation in *Dianthus* indicated high morphological, environmental and genetic overlaps hampering straightforward intraspecific classification. For example, testing the ecological differentiation of subspecies of *D. superbus* L. based on floral volatile compounds under selection of habitat-specific pollinators showed a lack of phylogenetic support for ecological subspecies, leading the authors to refer to them as ecotypes (Hardion *et al.*, 2020). In the *D. pungens* L. complex a pronounced morpho-environmental overlap, i.e. high redundancy effect, may account for the difficulty of distinguishing subspecies in this taxonomic group, and this was postulated to be a consequence of its recent origin and intense glacial-interglacial fluctuations across the Plio-Pleistocene period (Castro *et al.*, 2021).

Dianthus sylvestris Wulfen is considered as one of the most challenging groups in the genus, and the statement by Williams (1893) in the 'Monograph of the genus *Dianthus*' that *D. sylvestris* is a 'species with a polymorphic tendency in which it is very difficult to satisfactorily circumscribe the limits of the specific type' is still true today. *Dianthus sylvestris* is distributed in the Mediterranean area (Balkan and Apennine Peninsulas, Sardinia, Sicily, south-eastern France and Corsica, south-eastern Spain, Algeria and Morocco), the Swiss Jura and the Alps (Bernal, Laínz & Munoz Garmendia, 1990; Tutin & Walters, 1993; Bacchetta *et al.*, 2010; Marhold, 2011; Meyer, 2011; Dimopoulos *et al.*, 2013; Tison & Foucault, 2014; Bornand *et al.*, 2018; Julve, 2018). A recent study showed that *D. sylvestris* comprises three evolutionary lineages, the Alpine, the Apennine and the Balkan lineage, and that they diverged *c.* 200–115 Kya, i.e. during the penultimate glacial-interglacial period (Luqman *et al.*, 2022). To capture the observed morphological diversity of populations on the central Apennine Peninsula, Sardinia and Sicily, Bacchetta *et al.* (2010) recognized up to 17 taxa at specific and intraspecific levels, six of which were described as new to science. The nomenclature of these taxa has been discussed, and the name *D. virgineus* L., being the oldest available name for this group of taxa, has been recently proposed by Domina *et al.* (2021a, b).

The present study focuses on *D. sylvestris* in the Balkan Peninsula. According to Greuter, Burdet & Long (1984), Marhold (2011) and Meyer (2011), seven subspecies are recognized from the Balkan Peninsula: *D. sylvestris* subsp. *alboroseus* F.K.Mey, *D. sylvestris* subsp. *bertisceus* Rech.f., *D. sylvestris* subsp. *kozjakensis* Micevski, *D. sylvestris* subsp. *longicaulis* (Ten.) Greuter & Burdet, *D. sylvestris* subsp. *nodosus* (Tausch) Hayek, *D. sylvestris* subsp. *sylvestris* and *D. sylvestris* subsp. *tergestinus* (Rchb.) Hayek. Numerous other intraspecific taxa (subspecies, varieties and forms) that are now neglected or treated as synonyms have been described and proposed by some authors (Beck-Mannagetta, 1909; Trinajstić, 1979b; Micevski, 1990). During the last two centuries, many authors have tried to develop a workable classification of *D. sylvestris* in the Balkan Peninsula (Schlosser &

Vukotinović, 1869; Borbás, 1876; Beck-Mannagetta, 1909; Hayek, 1924; Gjurašin, 1933; Trinajstić, 1979a), but none of these systems seemed to provide an unambiguous identification key.

The distinction between subspecies of *D. sylvestris* is based on geographical distribution and slight morphological differentiation, whereas their ecological preferences have not yet been investigated. In most studies, habitat descriptions are given in the broad sense as rocky and sunny stands, rocky slopes, mountain and xeric meadows, garrigues and forest fringes (Bacchetta *et al.*, 2010). By studying genetic and phenotypic differentiation between populations of *D. sylvestris* growing on serpentine and limestone soils on the Apennines, Gammella (2016) detected a high amount of gene flow between serpentine and limestone populations. Despite the lack of genetic differentiation, several morphological characters were statistically different and persisted independently from the original soil type, suggesting that these differences could have genetic basis (Gammella, 2016). Aside from this study, little is known about the joint effect of abiotic drivers on the distribution of subspecies of *D. sylvestris*.

By incorporating environmental variation, *D. sylvestris* represents a promising model for exploring the population differentiation regarding the relationships between morphological variability and environmental conditions throughout its distribution range. Hence, the aims of our study are to: (1) explore in detail the morphological differentiation of subspecies of *D. sylvestris*, following the current taxonomic concept, and to identify the most discriminatory morphological characters and (2) analyse how morphology varies in relation to environmental variations across the geographical distribution of *D. sylvestris*, to detect whether environment has influenced the morphological differentiation. To address these questions, a comprehensive morphological and environmental dataset was created on the basis of populations from across the morphological and geographical range of these subspecies in the Balkan Peninsula.

MATERIAL AND METHODS

PLANT MATERIAL

Dianthus sylvestris is a perennial species with shortly branched woody stocks and dense terminal leaf rosettes from which branches with linear and acute leaves and loose cymose inflorescences with one to 15 flowers grow. The flowers are characterized by two to ten epicalyx scales, abruptly contracted into a short apex and glabrous petals with limb entire to denticulate at the apex (Tutin & Walters, 1993; Bacchetta *et al.*, 2010). The sampling was focused on the area of the Balkan Peninsula where *D. sylvestris* populations have been reported. Information on their distribution was collected from the following sources: (1) herbaria (BEO, BEOU, BP, CAT, CNHM, G, JE, LJU, NHMR, P, PAD, PRC, SARA, TIR, ZA, ZAGR, ZAHO, W and WU; acronyms according to Thiers, 2021), (2) published literature sources, including regional floras of the Balkan countries and the Flora Croatica Database (Beck-Mannagetta, 1909; Josifović, 1970; Trinajstić, 1979a; Strid, 1986; Micevski, 1990, 1993; Martinčič, 2007; Meyer, 2011; Dimopoulos *et al.*, 2013; Stešević & Caković, 2013; Vangjeli, 2015; Nikolić, 2020) and (3) the authors' field observations. The plants were identified and assigned to subspecies on the basis of their morphology and geographical distribution, whereas populations that we could not assign to any of the known subspecies were treated as *D. sylvestris s.l.* The names applied to these populations follow Marhold (2011). On the basis of the used identification keys and regional floras (Micevski, 1993; Tutin & Walters, 1993; Martinčič, 2007; Stešević & Caković, 2013; Dimopoulos *et al.*, 2013; Vangjeli, 2015; Barina *et al.*, 2018; Nikolić, 2020), none of the sampled populations was assigned to *D. sylvestris* subsp. *longicaulis*, although this subspecies was recorded for the Balkan Peninsula (Marhold, 2011). Altogether, 183 populations with 774 individuals were sampled from 2018 to 2020, and a subset of 97 populations was used for the detailed morphometric measurements and analysis. Voucher data are presented in the Supporting Information, Table S1, and the geographical origin of the sampled populations is shown in Figure 1. All voucher specimens are deposited at ZA.

MORPHOLOGICAL VARIABILITY

The morphological analyses were based on 461 individuals from 97 populations and, depending on the population size, usually five flowering plants were collected per population (Fig. 1, Table S1 in the Supporting Information). The analysed morphological data included 8–27 populations from each of the subspecies of *D. sylvestris*, including populations from the type localities whenever possible, except *D. sylvestris* subsp. *kozjakensis* from which a single population from the type locality was available. Four qualitative (two possible states; Fig. S1, Table S2 in the Supporting Information) and 21 quantitative morphological characters were examined for each individual, and seven ratios were derived (Table S3, Supporting Information). Characters were chosen on the basis of features used in regional floras to distinguish subspecies of *D. sylvestris* (Micevski, 1993; Tutin & Walters, 1993; Martinčič, 2007; Vangjeli, 2015), or used as diagnostic characters for subspecies (Hayek, 1924; Rechinger fil., 1935; Micevski, 1990; Meyer, 2011).

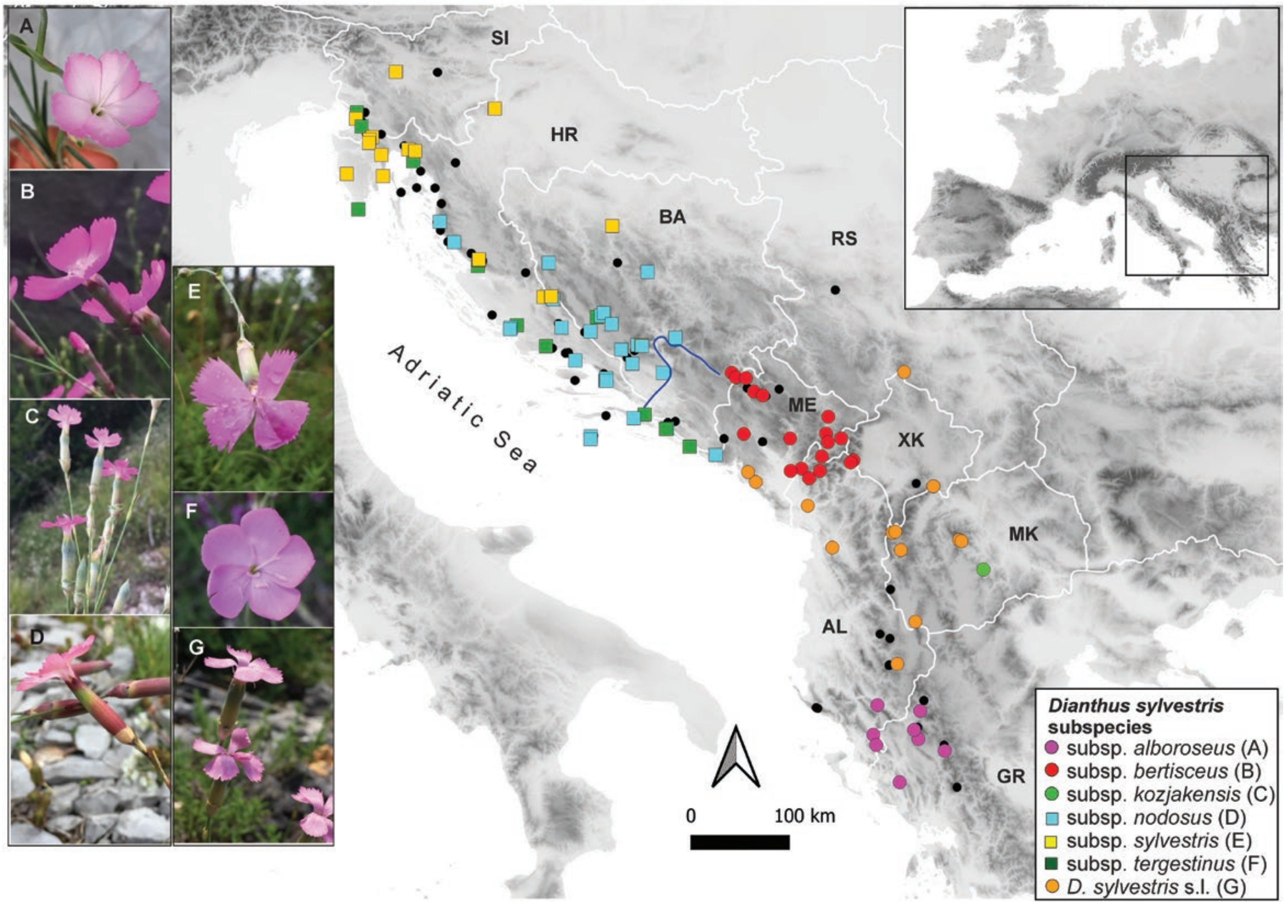

Figure 1. Localities of sampled populations of *Dianthus sylvestris* in the Balkan Peninsula used for characterizing the environmental variability (n = 183 populations, black dots) and a subset for morphometric analyses (n = 97 populations; for details see Table S1 in the Supporting Information). Squares represent populations from the north-western group according to morphometric PCA, whereas circles represent populations from the south-eastern group. The Neretva River is highlighted by a dark blue line.

Floral parts of one well-developed flower per plant were attached to transparent foil with adhesive tape, scanned on inverted Epson Expression 11000XL Pro A3 scanner and measured using the ImageJ program (Rueden *et al.*, 2017). In some specimens, one or a few structures were not developed, and the missing character states were replaced by imputation with the 'mice' package (Buuren & Groothuis-Oudshoorn, 2011) and the built-in 'pmm' univariate imputation method, which uses predictive mean matching, i.e. information from other variables in the dataset to predict and impute the missing values. All morphometric analyses were conducted in R v.4.0.2 (R Core Team, 2020).

Each of the four qualitative morphological characters had two possible states, and their total counts per subspecies were obtained to test whether differences of observed counts between subspecies are statistically significant. Chi-square tests were performed on individual plants, using the function 'chisq.test'. Quantitative morphological characters were examined using both univariate and multivariate methods. The Kruskal–Wallis test and Dunn's *post hoc* test with Bonferroni adjustment were performed on population averages for all quantitative morphological characters to evaluate statistically significant differences among subspecies. To visualize the variability between subspecies, boxplots of quantitative morphological characters were generated from population averages. Because of the small sample size (only one population known from the *locus classicus*), *D. sylvestris* subsp. *kozjakensis* was not included in the Chi-square, Kruskal–Wallis and Dunn's *post hoc* tests.

Before multivariate analyses, correlation among quantitative characters was tested using Spearman correlation coefficients ('corr.test' function of the 'psych' R package; Revelle, 2022). Correlation coefficients were based on the matrix including all the studied populations to eliminate pairs of highly correlated characters that may distort downstream analyses. In the case of two highly correlated characters ($r \geq 0.85|$), we decided to keep the character that displayed the higher statistical significance (smaller P value) among the subspecies according to the Kruskal–Wallis test. Principal component analysis (PCA) was performed based on population averages of selected 13 quantitative (Fig. S2, Supporting Information) and two qualitative morphological characters, turned into dummy variables (IND and EPI; a total of 15 morphological characters) to display the morphological variability between populations of subspecies of *D. sylvestris*. PCA was carried out on the correlation matrix using a set of R functions MorphoTools created by Koutecký (2015).

ENVIRONMENTAL VARIABILITY

We initially obtained 183 occurrences of *D. sylvestris* for characterizing its environmental variability, which included 97 populations used for the morphometric and redundancy analysis (RDA) analyses (Fig. 1). To characterize the environmental variability of *D. sylvestris* populations, we used environmental data from three databases: climate data from the Chelsa database (Karger *et al.*, 2017, 2020); soil properties from the SoilGrids (Hengl *et al.*, 2017); and topographic variables from the EarthEnv database (Amatulli *et al.*, 2018, 2019). An initial set of 43 environmental variables were evaluated for collinearity with the 'vifstep' function of the 'usdm' R package (Naimi *et al.*, 2014), and the number of variables was reduced to 20 (Table S4, Supporting Information). The 'vifstep' function calculates the variance inflation factor (VIF) for all variables, excludes one with the highest VIF (greater than the threshold set at 10) and repeats the procedure until no variables with VIF greater than threshold remain. The multivariate environmental space was visualized by the first two axes of the PCA on the basis of values of 20 environmental variables.

RELATIONSHIP OF MORPHOLOGICAL VARIABILITY WITH ENVIRONMENTAL VARIABLES

To explore the relationship between morphological and environmental variability we used RDA (Legendre & Legendre, 2012) implemented in the 'vegan' R package v.2.5–6 (Oksanen *et al.*, 2018). The 20 environmental variables previously selected for characterizing the environmental variability of populations of *D. sylvestris* (standardized to a zero mean and unit variance), and the geographical coordinates of populations (latitude and longitude) were used as explanatory matrices, and Hellinger-transformed population averages of the morphological characters measurements were used as a response matrix.

To disentangle the unique effects of environment and geography and their joint effects on the variation of morphological characters, we performed three RDA models: (1) the full RDA model with both environmental and geographical variables as predictors; (2) partial RDA in which environmental effects were conditioned on the effects of geography (to obtain the unique effect of the environment) and (3) partial RDA in which geography was conditioned on the effects of environment (to obtain the unique effect of the geography). The significance of the RDA models was assessed using ANOVA with 999 permutations as implemented in 'vegan'. Finally, to identify which environmental and/or geographical variables best explain the overall morphological variability of our populations, we applied a forward selection procedure with the double-stopping criterion (Blanchet, Legendre, & Borcard, 2008) from the 'packfor' R package (Dray, 2013). The variables selected by this procedure as significant were used for performing an additional RDA model (reduced RDA model). We also conducted linear Spearman correlation analyses between the 15 morphological characters used in PCA and the 20 selected environmental variables, with Bonferroni adjustment for multiple tests. Latitude and longitude, representing geographical variables, were also included to detect patterns of morphological variability through latitudinal and longitudinal gradients.

RESULTS

MORPHOLOGICAL VARIABILITY

Qualitative morphological characters were analysed on individual plants (Fig. S1, Supporting Information) and the statistically significant differences between the subspecies are shown in Table S2 (Supporting Information). *Dianthus sylvestris* subsp. *bertisceus* showed a statistically significant difference from other subspecies in the arrangement of epicalyx scales (EPI), whereas petal denticulation (DEN) and stem indumentum coverage (IND) had the highest Chi-square values for *D. sylvestris* subsp. *tergestinus* and subsp. *nodosus*, respectively. The range of values of quantitative morphometric characters mostly overlapped between subspecies (Fig. S2, Supporting Information). However, according to the Kruskal–Wallis test (Table S3, Supporting Information), 23 out of 28 investigated characters proved to be significantly different ($P < 0.01$) between the subspecies. Dunn's pairwise *post hoc* tests revealed that, for these 23 characters, at least one pair of subspecies showed a significant difference. Shape of epicalyx scale (RIS, RISA, ISAL, ISW), ratio of petal teeth length and petal limb length (RDENT) and ratio of calyx teeth

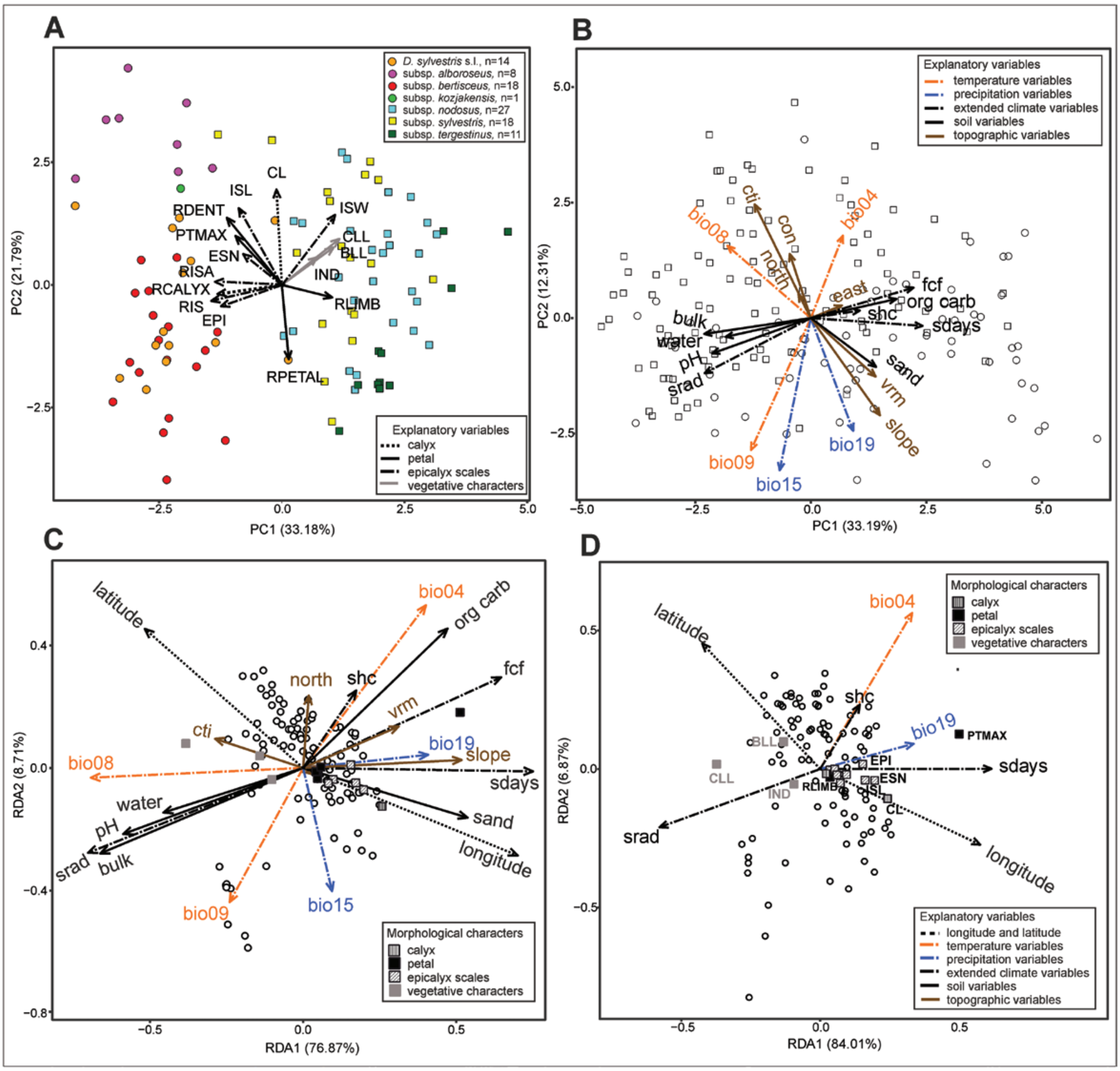

Figure 2. A, Principal component analysis (PCA) based on 15 morphological characters examined on 97 populations of *Dianthus sylvestris,* represented by the first two principal components; taxonomic identification at the intraspecific level, based on regional keys and floras, is projected using differently coloured symbols. B, Environmental variability of 183 populations of *D. sylvestris* along the first two axes of the PCA based on 20 environmental variables. Symbols are according to Figure 1. C, Redundancy analysis (RDA) plot of the full RDA model including 20 environmental and two geographical variables (explanatory matrices) in relation to the variability of 15 measured morphological characters (response matrix) examined on 97 populations. D, RDA plot of the reduced RDA model including only the seven variables selected by the forward selection procedure.

length and calyx length (RCALYX) were the six most statistically significant characters (Table S3, Supporting Information).

The PCA was performed on a selection of 15 morphological characters, including two qualitative, five continuous, six ratios and two meristic (Fig. 2A, Fig. S2, Supporting Information). When PCA was performed on averaged measurement values of populations, the first principal component (PC1) explained 33.18% of total variation, the second principal component (PC2) explained 21.79% and the third explained 8.97% (PC3). Characters showing the highest correlation with the first axis (0.73–0.80) in the coefficient matrix were RIS, RISA and RCALYX (listed in decreasing component score value). Characters that showed the highest correlation with the second axis (component scores ranging from 0.69 to 0.88) were CL, ISL and RPETAL. When the populations were considered in two-dimensional morpho-space, a separation of southern Balkan populations and northern Balkan populations along PC1 and a vast amount of overlap along PC2 were detected (Fig. 2A). The gradual change in epicalyx scale shape can be seen in Supplementary Figure S3 (in the Supporting Information). The morphometric PCA plot (Fig. 2A), showed that RIS and RISA had the highest correlation with PC1, separating populations from the northern Balkan Peninsula with more elliptical scales and populations from the southern Balkan Peninsula with scales longer than wide.

ENVIRONMENTAL VARIABILITY

The first principal component (PC1, Fig. 2B) explained 33.19% of the environmental variability in *D. sylvestris s.l*. It mainly reflected the number of snow days per year (sdays), soil bulk density (soil bulk, indicating the size, shape and arrangement of particles and voids) and solar radiation in the study area (Supporting Information, Table S4; 44, B). PC2 explained 12.31% of the environmental variability corresponding to precipitation seasonality (bio15), the mean daily air temperatures of the driest quarter (bio09), the compound topographic index (cti, a metric of potential ground wetness that is considered steady state) and the mean monthly precipitation of the coldest quarter (bio19; Table S4, Supporting Information; Fig. 2B).

RELATIONSHIP BETWEEN MORPHOLOGICAL VARIABILITY, ENVIRONMENT AND GEOGRAPHY

The full RDA model with 20 environmental and two geographical variables as predictors was significant ($F = 5.015$, $P = 0.001$) and explained 47.9% of the morphological variation (R^2_{adj}, Table 1). Only the first RDA axis was significant ($F = 92.84$, $P < 0.001$), and the first two RDA axes accounted for 76.87 and 8.71% of the explained variance (Fig. 2C). Environmental variation independent of geography explained 39.3% of the morphological variation, 8.7% of the variance was explained only by geography holding the environment constant (unique effect of geography, Table 1) and the interaction between environmental and geographical variables accounted for 17.8% of the variance. The most important environmental and geographical variables explaining the morphological variation of our populations were the number of sdays, longitude and temperature seasonality (bio04), as selected by the forward selection procedure (Table S5, Supporting Information). The first two axes of the reduced RDA model based on seven selected variables from Table S5 (Supporting Information) explained 84.01 and 6.87% of the morphological variation (Fig. 2D). None of the considered soil or topographic variables seemed to significantly affect the overall morphological variation in investigated populations of *D. sylvestris* (Fig. 2C, D).

We conducted linear Spearman correlations between 15 morphological characters used in PCA, 20 environmental and two geographical variables. Longitude, latitude, temperatures of the wettest quarter (bio08) and number of sdays were significantly correlated ($r > |0.60|$, $P < 0.05$) with four morphological characters (RIS, RISA, RCALYX, cauline leaf length (CLL); Fig. S4, Supporting Information) after applying a Bonferroni correction for multiple comparisons.

DISCUSSION

MORPHOLOGICAL CLINE HINDERS TAXONOMICAL DELINEATION

The first extensive morphometric study of *D. sylvestris* in the Balkan Peninsula presented here showed extensive overlap in most of the morphological characters of investigated subspecies and a lack of support for current taxonomic intraspecific delineation. Our results indicate two morphologically distinct entities of *D. sylvestris* as seen in PCA ordination space (Fig. 2A). The split between these two groups corresponds to the split between allopatric north-western Balkan populations (including *D. sylvestris* subspp. *nodosus*, *sylvestris* and *tergestinus*) and south-eastern Balkan populations (including *D. sylvestris* subspp. *alboroseus, bertisceus*, *kozjakensis*

Table 1. Summary of RDA and partial RDA (pRDA) analyses. Significant *P* values are shown in bold

Model	Predictors/effect	R^2	R^2_{adj}	*F*	*P*
RDA_{full}	Environment + Geography	0.599	0.479	5.015	**0.001**
$pRDA_{env}$	Environment | Geography	0.319	0.215	2.9399	**0.001**
$pRDA_{geo}$	Geography | Environment	0.079	0.087	7.3123	**0.001**
RDA_{env}	Environment	0.519	0.393	4.104	**0.001**

and *D. sylvestris s.l.*) roughly along the Neretva valley in southern Dalmatia (Croatia, Fig. 1). Although the observed split occurs south of Neretva in the Boka Kotorska region, the populations between Neretva and Boka Kotorska are morphologically intermediate and resemble *D. sylvestris* subsp. *nodosus* and *D. sylvestris* subsp. *bertisceus*. The area adjacent to the present Neretva River valley has been identified as a lineage border in a number of genetic and morphometric studies (Surina, Schönswetter & Schneeweiss, 2011; Mereďa *et al.*, 2011; Falch, Schönswetter & Frajman, 2019; Đurović *et al.*, 2021; Reich *et al.*, 2022). Similar patterns of morphologically hybrid populations in southern Croatia were found in the *Cardamine maritima* DC. group (Kučera, Marhold & Lihová, 2010), whereas molecular and morphological data of the *Campanula pyramidalis* L. complex suggested that individuals occurring south of the Neretva River valley form a distinct entity and only partly overlap with individuals from the northern Adriatic (Lakušić *et al.*, 2013). Likewise, morphometric analysis of the *Alyssum montanum* L. - *A. repens* Baumg. complex revealed this area in southern Croatia to be the border between distribution areas of the closely related *A. austrodalmaticum* Trinajstić and *A. montenegrinum* (Bald.) Španiel, Lihová & Marhold, which are morphologically divergent in several characteristics (Španiel *et al.*, 2017). Lakušić *et al.* (2013) posited that this pattern of splits observed in several plant taxa may mark a border between strongly different ecological conditions due to the Adriatic Sea oscillation in a north–south direction at the time of the last glacial maximum. On the other hand, a recent study of three taxa of *Cerastium* L. from the Dinaric Alps suggested that the deep Neretva valley may have acted as a physical barrier for some plant taxa, rather than an ecological one (Đurović *et al.*, 2021).

Several morphological characters reported in floras (Tutin & Walters, 1993) as diagnostic for subspecies of *D. sylvestris* were considerably variable in our investigated populations. As seen from the results in Table S3 (Supporting Information), most significantly different morphological characters describe the flower, i.e. the reproductive parts of the plant, whereas vegetative characters were less informative. However, in the study by Terlević *et al.* (2022) differences in vegetative characters were found between diploid and tetraploid plants of *D. sylvestris* subsp. *sylvestris*, with tetraploid plants being taller and having longer internodes and leaves.

Mayer & Trpin (1965) identified stem indumentum coverage, number and shape of epicalyx scales, length of calyx, shape of calyx teeth and shape of petals as the most important morphological characters used for taxonomic treatment. They are used in identification keys to distinguish typical *D. sylvestris* from *D. sylvestris* subsp. *nodosus* (Mayer & Trpin, 1965), but the results of our morphometric analyses showed that character values often overlap, and plant identification turned out to be difficult and ambiguous. For example, the proportion of individual plants of *D. sylvestris* subsp. *nodosus* having puberulent stem indumentum is equal to the proportion of plants having glabrous stems (Fig. S1, Supporting Information), thus revealing variability of indumentum coverage within a population and thus questioning the taxonomic usefulness of this character. Although the sizes and shapes of calyx (CL, RCALYX), epicalyx scales (ISL, RIS, RISA) and petals (RPETAL; Fig. 2A) captured the greatest extent of morphological variability among populations, they should not be considered as taxonomically reliable, as noted previously by Mayer & Trpin (1965). Thus, *D. sylvestris* subsp. *nodosus* and subsp. *sylvestris* have similar morphologies and cannot be clearly differentiated. *Dianthus sylvestris* subsp. *bertisceus* can be distinguished from other Balkan subspecies by its divaricated epicalyx scales. However, this character is not discriminatory for this subspecies when populations from the European Alps are considered, as they also have divaricated epicalyx scales (authors' personal observations).

The lack of petal denticulation and usually only one pair of epicalyx scales are the morphological characters frequently used in identification keys to distinguish *D. sylvestris* subsp. *tergestinus* from other subspecies that usually have denticulate petals and two pairs of epicalyx scales (Trinajstić, 1979a; Tutin & Walters, 1993; Vangjeli, 2015). Thus, our results partially confirm that these two diagnostic characters can be used for delimitation of *D. sylvestris* subsp. *tergestinus*. Trinajstić (1979a, b) treated *D. sylvestris* subsp. *tergestinus* as a species and he singled out the populations from the island of Lastovo (Croatia) as a separate endemic taxon *D. tergestinus* (Rchb.) Kerner subsp. *lastovoënsis* Trinajstić. Unlike typical *D. tergestinus*, *D. tergestinus* subsp. *lastovoënsis* has four epicalyx scales (Trinajstić, 1979a), and the plants we collected in early June 2019 from the *locus classicus* had puberulent lower internodes and had just started flowering. These characters led us to identify those plants as *D. sylvestris* subsp. *nodosus*. The populations from Lastovo morphologically resemble *D. sylvestris* subsp. *nodosus* by having slightly smaller petals and two pairs of epicalyx scales, whereas they share the similarity with *D. sylvestris* subsp. *tergestinus* by having entire petal limbs, partly justifying their inclusion under *D. tergestinus* by Trinajstić (1979a).

Although it has not been emphasized in any floras or keys, during our detailed field sampling we observed the flowering time shift between *D. sylvestris* subsp. *tergestinus* on the one hand and *D. sylvestris* subspp. *sylvestris* and *nodosus* on the other. All populations of

D. sylvestris subsp. *tergestinus* flowered first from late April to late May and had no overlap in flowering time with populations of *D. sylvestris* subsp. *sylvestris* or subsp. *nodosus* which flowered from late June to late July. Although the variance in flowering time appears to be conserved among plants such that closely related species tend to flower at similar times (Davies *et al.*, 2013), the slightly different flowering time of *D. sylvestris* subsp. *tergestinus* may therefore be indicative of its phylogenetic divergence from *D. sylvestris*. Furthermore, the identification of *D. sylvestris* subsp. *tergestinus* based on the flowering time has been proven to be correct on the basis of preliminary genetic results in which plants identified as *D. sylvestris* subsp. *tergestinus* form a separate lineage (Temunović *et al.*, unpubl. data). Our results thus indicate that *D. sylvestris* subsp. *tergestinus* is a sympatric subspecies with *D. sylvestris* subsp. *sylvestris* and subsp. *nodosus*, differentiated by its flowering time.

MORPHOLOGICAL VARIABILITY CORRELATES WITH ENVIRONMENTAL VARIABILITY

Bacchetta *et al.* (2010) provided notes on ecology for species/populations belonging to *D. sylvestris* in central and southern Italy, Sicily and Sardinia, but there are no such data describing ecological preferences of subspecies/populations in the Balkan Peninsula. To fill this knowledge gap, we examined the environmental preferences of the Balkan populations and investigated the relationship between morphological variability, environment and geography. Our RDA results indicate that environment plays a potentially more important role in the morphological variability in *D. sylvestris* than geography (Table 1). Number of sdays and temperature seasonality (bio04) turned out to be the two environmental variables that explained most morphological variability (Fig. 2D, Table S5, Supporting Information). *Dianthus sylvestris* occurs from Mediterranean rocky grasslands to high-Alpine habitats, including changes in elevation and its associated shifts in these two variables. Our results showed shorter leaves, and consequently smaller plant biomass, to be common in environments with longer snow cover (Fig. S4B, Supporting Information), which is consistent with previous evidence for the smaller size of Alpine plants compared to lowland plants (Körner, 2003). Declining plant biomass with elevation might result from selection for slower growth, which provides the advantage of warmer microclimatic conditions closer to the ground and protection from wind (Körner, 2003). Additionally, time of flowering is dependent on the length of the season and thus correlated with the duration of snow cover (Halbritter *et al.*, 2018). We hypothesize that the number of sdays encompasses the morphological variability from the thermophilic *D. sylvestris* subsp. *tergestinus* at the one extreme to the Alpine *D. sylvestris* subsp. *bertisceus* on the other end. This is also reflected in phenology, with *D. sylvestris* subsp. *tergestinus* flowering first in May, whereas Alpine populations of *D. sylvestris* subsp. *bertisceus* flower later in July. Similar results were also reported in the study of Neuffer & Hurka (1986) in which populations of *Capsella bursa-pastoris* (L.) Medik. from high elevations generally flowered later than those from lower elevations. The morphological characters describing less stem indumentum (IND, glabrous or puberulent) showed correspondence with the duration of snow coverage. Pubescence in Alpine flora is quite rare (Körner, 2003), in concordance with our result of negative correlation with the number of sdays (Fig. 2C, D). The observed positive correlation with solar radiation (Fig. 2C, D) may be associated with buffering of short-term oscillations of ambient humidity and water saving strategies in drier environments (Körner, 2003) or protection against excess radiation over photosynthetic tissues (Manetas, 2003). Besides these observed correlations, we did not find any clear evidence that would support puberulent indumentum to be taxonomically relevant for any of the subspecies, and thus it may rather represent a structure influenced by environment and/or geography.

Some climatic variables follow latitudinal and/or longitudinal gradients, and hence morphological characters can show patterns of latitudinal and longitudinal variation (Alcántara-Ayala *et al.*, 2020). In our study, longitude was also a major geographical predictor associated with morphological variability (Fig. 2C, D), indicating an effect of geographical isolation. Slope, number of sdays and soil sand content increase eastwards, i.e. from the Adriatic coast towards the Dinaric Alps, whereas mean daily air temperatures of the winter-wettest quarter (bio08) decrease. Thus, the contribution of longitude to morphological variability of investigated populations of *D. sylvestris* might reflect the gradual transition from the coastal Mediterranean environment to the Alpine environment, which is in turn reflected mainly in the CLL and shape of epicalyx scales (RIS and RISA). Specifically, moving eastwards (longitude) calyces have longer teeth, epicalyx scales are less elliptical (ovate to obovate) and have longer apices and cauline and basal leaves are shorter, whereas moving northwards (latitude) calyces have shorter teeth and epicalyx scales are more elliptical (Fig. S3, Supporting Information). Similar morpho-environmental continuity along the eastern Adriatic coast can be observed in *Dianthus ciliatus* Guss. and its three subspecies, with populations identified as *D. ciliatus* subsp. *ciliatus* occurring in northern parts of the distribution area having entire petals and slightly wider scales and populations identified as *D. ciliatus* subspp. *dalmaticus* (Čelak.) Hayek and *medunensis* AQ7 occurring towards the south having denticulate petals and narrower scales (Tokić, 2022). These examples of subtle morphological modifications combined with uninterrupted distributions reveal the continuous combination of morpho-environmental strategies in spatially heterogenous and temporally dynamic landscapes occurring in young diverse groups.

CONCLUSIONS

Our morphometric analyses revealed a continuous variability in quantitative morphological characters in *D. sylvestris* and an absence of clear-cut qualitative morphological differences between the subspecies in the Balkan Peninsula. The morphological variation gradually changes with environment and geography, markedly with climate and longitude. Our results do not lend support to the recognition of most of these subspecies or to the current generally accepted taxonomic concept. As an extension to this study and as an integrative approach, molecular analyses of these populations are under way. Until we have access to these data, and due to our desire to make stable taxonomic decisions, we refrain from making comprehensive taxonomic changes at this point.

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AQ8

DATA AVAILABILITY

The data underlying this article are available in [repository name, e.g. the GenBank Nucleotide Database] at [URL], and can be accessed with [unique identifier, e.g. accession number, deposition number].

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. The number of plant individuals per state of each qualitative character and per subspecies. Abbreviated labels: *alb* – subsp. *alboroseus*, *bert* – subsp. *bertisceus*, *kozj* – subsp. *kozjakensis*, *nod* – subsp. *nodosus, s.l.* – *D. sylvestris* sensu lato, *sylv* – subsp. *sylvestris*, *terg* – subsp. *tergestinus.*

Figure S2. Variation in quantitative characters selected for performing PCA. Abbreviated labels: *alb* – subsp. *alboroseus*, *bert* – subsp. *bertisceus*, *kozj* – subsp. *kozjakensis*, *nod* – subsp. *nodosus*, *s.l.* – *D. sylvestris sensu lato*, *sylv* – subsp. *sylvestris*, *terg* – subsp. *tergestinus.* Means not significantly different at $P < 0.01$, according to Dunn's *post hoc* test, are indicated by the same letter.

Figure S3. Sampling area with insertions of one epicalyx scale from the first pair (the closest to the calyx) showing the gradual change of epicalyx scale's shape.

Figure S4. Significant correlations ($P < 0.05$) exceeding $r = |0.60|$ between morphological characters and two environmental (B and D) and two geographical variables (A and C), according to Spearman's correlation test with Bonferroni adjustment for multiple comparisons.

Table S1. Studied populations of the *Dianthus sylvestris s.l.* including number of individuals collected per population, number of measured individuals used in morphometric analyses, taxon name, voucher, locality data, collectors and collection date. The herbarium acronyms are according to Index Herbariorum.

Table S2. Chi-square test between each of the 15 pairs for each qualitative morphological character. Significant differences ($P < 0.01$) are shown in bold. Abbreviated labels: *alb* – subsp. *alboroseus*, *bert* – subsp. *bertisceus*, *nod* – subsp. *nodosus*, *s.l.* – *D. sylvestris* sensu lato, sylv – subsp. *sylvestris*, *terg* – subsp. *tergestinus.*

Table S3. Quantitative morphological characters studied in *Dianthus sylvestris s.l.* from the Balkan Peninsula. Morphological differences along 21 quantitative morphological characters and seven ratios between the *D. sylvestris* subspecies as revealed by Kruskal–Wallis test. Significant differences ($P < 0.01$) are shown in bold.

Table S4. Environmental variables used for characterizing environmental variability of *Dianthus sylvestris* populations using principal component analysis (PCA) and for RDA analysis, with their abbreviated and full names/definitions, units and types. Shown are also factor loadings of PCA of environmental data related to *D. sylvestris* populations.

Table S5. Results of a forward selection procedure with double-stopping criterion (P value and R^2_{adj}), on the RDAfull model.

SUPPLEMENTARY DATA

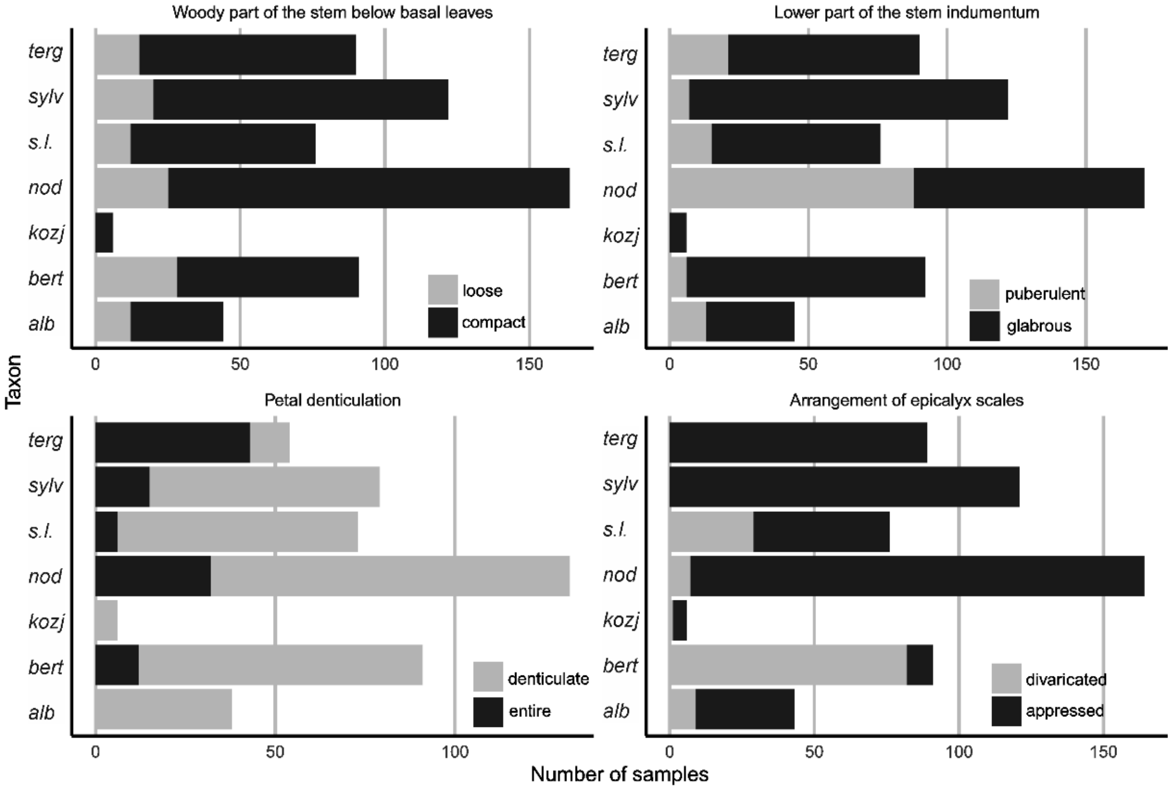

Figure S1. Barplots for the four qualitative characters examined on 97 populations of *Dianthus sylvestris* in the Balkan Peninsula, showing the number of plant individuals per character state and per subspecies. Abbreviated labels: *alb* - subsp. *alboroseus*, *bert* - subsp. *bertisceus, kozj* - subsp. *kozjakensis, nod* - subsp. *nodosus, s.l.* - *D. sylvestris* sensu lato, *sylv* - subsp. *sylvestris, terg* - subsp. *tergestinus.*

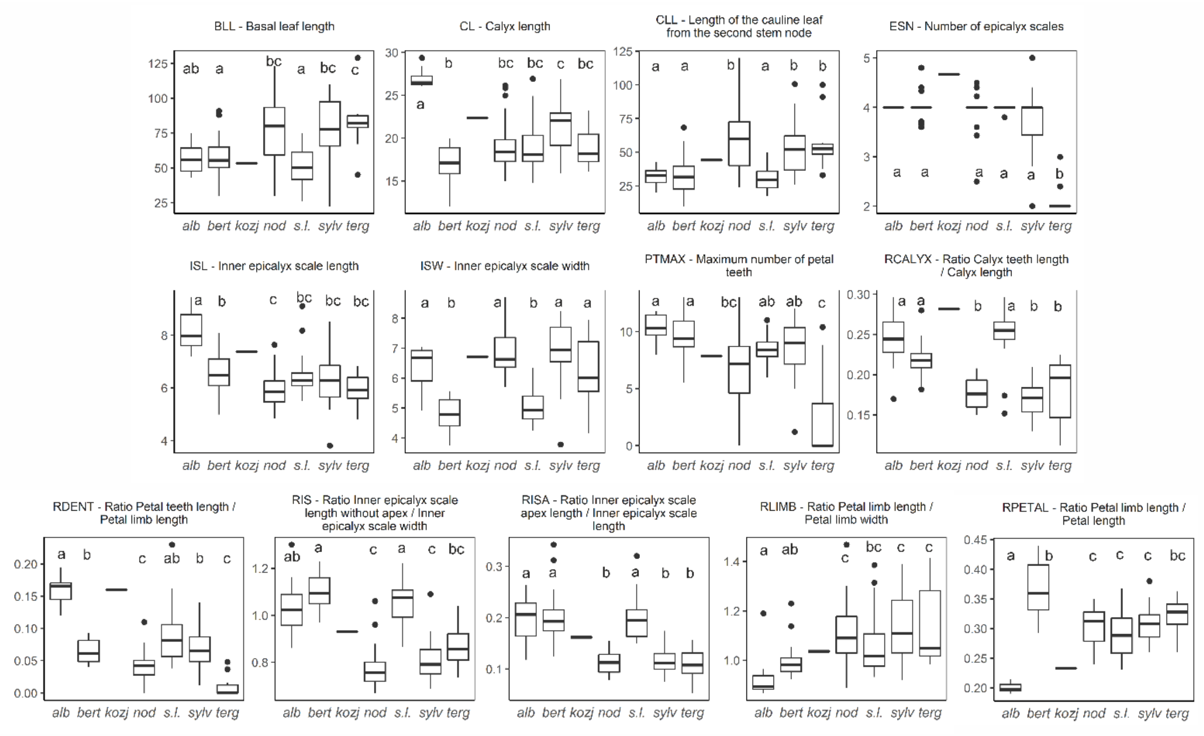

Figure S2. Variation in quantitative characters, examined on 97 populations of *Dianthus sylvestris* in the Balkan Peninsula, selected for performing PCA. Abbreviated labels: *alb* - subsp. *alboroseus, bert* - subsp. *bertisceus, kozj* - subsp. *kozjakensis, nod* - subsp. *nodosus, s.l.* - *D. sylvestris sensu lato, sylv* - subsp. *sylvestris, terg* - subsp. *tergestinus.* Means not significantly different at $p < 0.01$, according to Dunn's post-hoc test, are indicated by the same letter.

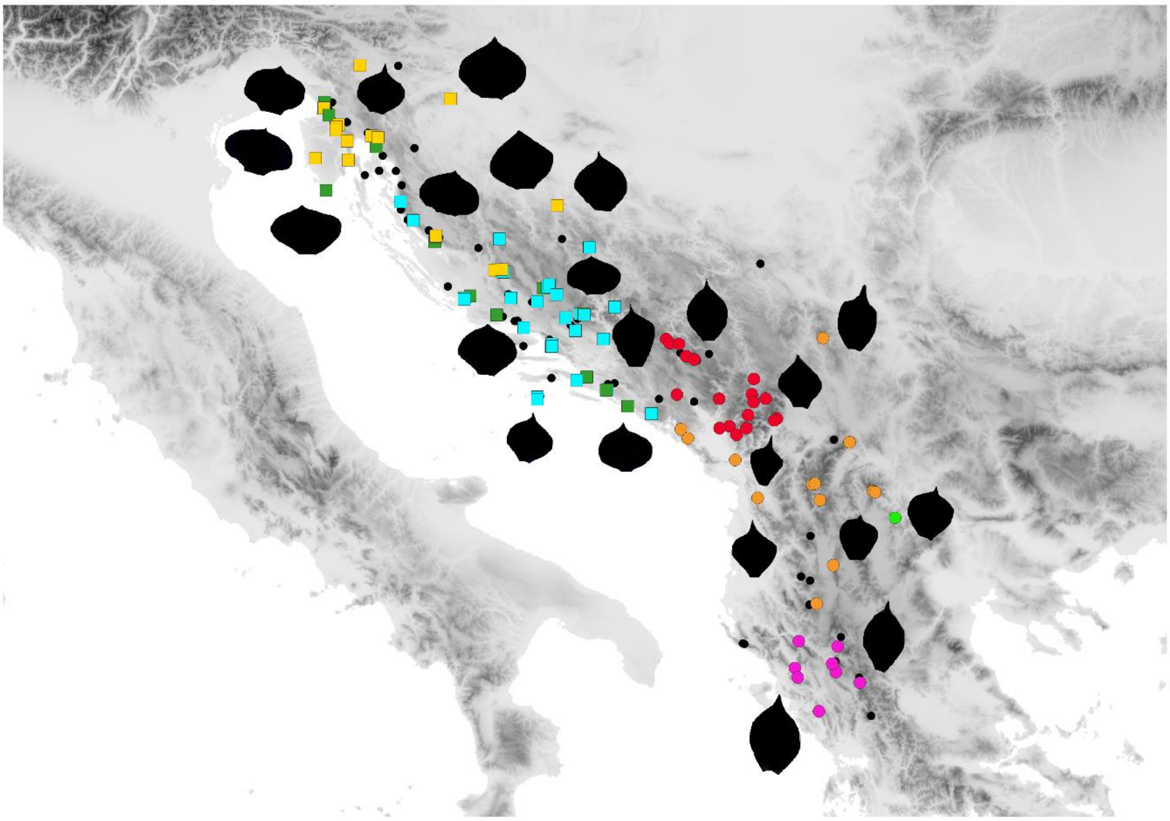

Figure S3. Sampling area with insertions of one epicalyx scale from the first pair (the closest to the calyx) showing the gradual change of epicalyx scale's shape in sampled populations of *Dianthus sylvestris*. Symbols are according to Figure 1.

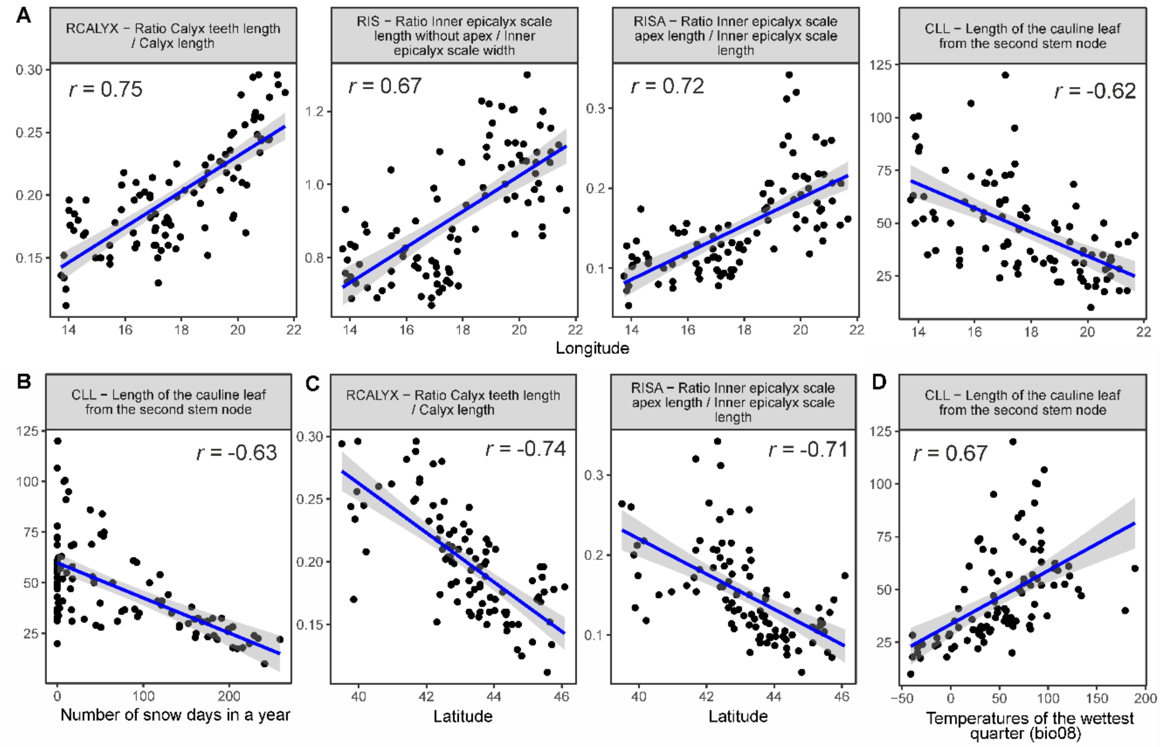

Figure S4. Significant correlations ($p < 0.05$) exceeding $r = |0.60|$ between morphological characters and two environmental (B and D) and two geographical variables (A and C), according to the Spearman correlation test with Bonferroni adjustment for multiple comparisons.

Table S1. Studied populations of the *Dianthus sylvestris* including number of individuals collected per population, number of measured individuals used in morphometric analyses, taxon name, voucher, locality data, collectors and collection date. The herbarium acronyms are according to Index Herbariorum.

ID	No of indiv.	Morpho-metrics	Taxon	Voucher	Country	Alt. (m a.s.l.)	Long. (N)	Lat. (E)	Collectors	Collection date
D76	5	-	*D. sylvestris s.l.* - Balkan	ZA 48631	Serbia	640	44.04424	20.01645	S. Đurović, G. Tomović	15/07/2018
D77	5	-	*D. sylvestris s.l.* - Balkan	ZA 48632	Serbia	1755	42.2335	20.91537	D. Lakušić, N. Kuzmanović, I. Janković, M. Zbiljić	14/07/2018
D79	5	-	*D. sylvestris s.l.* - Balkan	ZA 48634	Serbia	1660	43.26149	20.83973	S. Đurović, G. Tomović	13/07/2018
D102	5	5	*D. sylvestris s.l.* - Balkan	ZA 48619	Montenegro	931	42.30581	18.93189	S. Bogdanović, I. Ljubičić	16/07/2018
D103	5	5	*D. sylvestris s.l.* - Balkan	ZA 48620	Montenegro	1681	42.4003	18.83807	S. Bogdanović, I. Ljubičić	16/07/2018
D108	5	-	*D. sylvestris s.l.* - Balkan	ZA 48625	Albania	969	40.86694	20.38444	B. Frajman, P. Schönswetter	12/07/2018
D109	5	-	*D. sylvestris s.l.* - Balkan	ZA 48626	Albania	1406	40.57972	20.47722	B. Frajman, P. Schönswetter	13/07/2018
D110	4	-	*D. sylvestris s.l.* - Balkan	ZA 48627	North Macedonia	1759	41.27278	20.535	B. Frajman, P. Schönswetter	15/07/2018
D119	4	-	*D. sylvestris s.l.* - Balkan	ZA 54922	Albania	1670	40.82167	20.50167	M. Duchon	16/07/2018
D145	5	5	*D. sylvestris s.l.* - Balkan	ZA 54947	Albania	145	41.67954	19.84739	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	26/06/2019
D147	8	8	*D. sylvestris s.l.* - Balkan	ZA 54953	Albania	129	42.07219	19.56423	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	27/06/2019
D170	6	6	*D. sylvestris s.l.* - Balkan	ZA 54977	Serbia	1787	43.26151	20.83653	S. Bogdanović, M. Doboš, M. Grgurev, I. Rešetnik	10/07/2019
D171	5	6	*D. sylvestris s.l.* - Balkan	ZA 54978	North Macedonia	2255	42.19948	21.12385	S. Bogdanović, M. Doboš, M. Grgurev, I. Rešetnik	11/07/2019
D172	5	5	*D. sylvestris s.l.* - Balkan	ZA 54979	North Macedonia	2255	42.19948	21.12385	S. Bogdanović, M. Doboš, M. Grgurev, I. Rešetnik	11/07/2019
D173	6	6	*D. sylvestris s.l.* - Balkan	ZA 54980	North Macedonia	1687	41.6293	20.68481	S. Bogdanović, M. Doboš, M. Grgurev, I. Rešetnik	12/07/2019
D174	5	5	*D. sylvestris s.l.* - Balkan	ZA 54981	North Macedonia	1510	40.96566	20.81879	S. Bogdanović, M. Doboš, M. Grgurev, I. Rešetnik	12/07/2019
D175	6	6	*D. sylvestris s.l.* - Balkan	ZA 54982	North Macedonia	2112	41.7919	20.59291	S. Bogdanović, M. Doboš, M. Grgurev, I. Rešetnik	13/07/2019
D176	5	5	*D. sylvestris s.l.* - Balkan	ZA 54983	North Macedonia	1379	41.79959	20.62801	S. Bogdanović, M. Doboš, M. Grgurev, I. Rešetnik	13/07/2019

D179	5	5	*D. sylvestris s.l.* - Balkan	ZA 54986	North Macedonia	2274	41.69279	21.40381	S. Bogdanović, M. Grgurev, I. Rešetnik	17/07/2019
D180	5	5	*D. sylvestris s.l.* - Balkan	ZA 54987	North Macedonia	1235	41.68044	21.43242	S. Bogdanović, M. Grgurev, I. Rešetnik	17/07/2019
D182	5	1	*D. sylvestris s.l.* - Balkan	ZA 54989, ZA 54990	Albania	1430	40.58883	20.57972	L. Shuka	27/07/2019
D78	5	-	*D. sylvestris* subsp. *alboroseus*	ZA 48633	Albania	1386	40.5741	20.47551	D. Lakušić, N. Kuzmanović, I. Janković, M. Zbiljić	11/07/2018
D131	5	-	*D. sylvestris* subsp. *alboroseus*	ZA 54934	Greece	1023	39.42647	21.21591	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	21/06/2019
D132	5	5	*D. sylvestris* subsp. *alboroseus*	ZA 54935	Greece	664	39.49754	20.54052	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	21/06/2019
D133	5	5	*D. sylvestris* subsp. *alboroseus*	ZA 54936	Greece	744	39.76638	21.09354	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	22/06/2019
D134	7	-	*D. sylvestris* subsp. *alboroseus*	ZA 54937	Greece	1398	39.82082	21.08762	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	22/06/2019
D135	5	5	*D. sylvestris* subsp. *alboroseus*	ZA 54938	Greece	1135	39.88615	20.78638	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	22/06/2019
D136	5	-	*D. sylvestris* subsp. *alboroseus*	ZA 54939	Greece	765	40.23746	20.87395	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	22/06/2019
D137	5	4	*D. sylvestris* subsp. *alboroseus*	ZA 54940	Greece	631	40.14424	20.82399	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	22/06/2019
D138	6	5	*D. sylvestris* subsp. *alboroseus*	ZA 54941	Greece	1296	39.97295	20.74076	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	23/06/2019
D139	5	-	*D. sylvestris* subsp. *alboroseus*	ZA 54942	Greece	2093	39.99494	20.78599	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	23/06/2019
D140	6	5	*D. sylvestris* subsp. *alboroseus*	ZA 54943	Albania	1523	40.21507	20.31646	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	24/06/2019
D141	6	5	*D. sylvestris* subsp. *alboroseus*	ZA 54944	Albania	400	39.94504	20.25109	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	25/06/2019
D142-1	5	-	*D. sylvestris* subsp. *alboroseus*	ZA 54946	Albania	950	40.21436	19.57959	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	25/06/2019
D142-2	3	-	*D. sylvestris* subsp. *alboroseus*	ZA 54945	Albania	1600	40.20721	19.59952	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	25/06/2019
D181	5	1	*D. sylvestris* subsp. *alboroseus*	ZA 54988	Albania	700	39.84924	20.28111	D. Shuka	07/06/2019

D86	6	6	*D. sylvestris* subsp. *bertisceus*	ZA 48662	Bosnia and Herzegovina	638	43.31548	18.66821	S. Bogdanović, I. Ljubičić	11/07/2018
D87	5	6	*D. sylvestris* subsp. *bertisceus*	ZA 48663	Bosnia and Herzegovina	1580	43.27309	18.71955	S. Bogdanović, I. Ljubičić	12/07/2018
D88	5	5	*D. sylvestris* subsp. *bertisceus*	ZA 48664	Montenegro	735	43.26326	18.84523	S. Bogdanović, I. Ljubičić	12/07/2018
D89	5	5	*D. sylvestris* subsp. *bertisceus*	ZA 48665	Montenegro	1680	43.13713	18.94089	S. Bogdanović, I. Ljubičić	13/07/2018
D90	5	5	*D. sylvestris* subsp. *bertisceus*	ZA 48666	Montenegro	2002	43.10045	19.05084	S. Bogdanović, I. Ljubičić	13/07/2018
D91	5	5	*D. sylvestris* subsp. *bertisceus*	ZA 48612	Montenegro	711	42.88203	19.86147	S. Bogdanović, I. Ljubičić	13/07/2018
D92	6	6	*D. sylvestris* subsp. *bertisceus*	ZA 48667	Montenegro	746	42.72892	19.82458	S. Bogdanović, I. Ljubičić	13/07/2018
D93	6	6	*D. sylvestris* subsp. *bertisceus*	ZA 48668	Montenegro	2011	42.64971	19.84412	S. Bogdanović, I. Ljubičić	14/07/2018
D94	5	5	*D. sylvestris* subsp. *bertisceus*	ZA 48669	Montenegro	1989	42.52064	19.76142	S. Bogdanović, I. Ljubičić	14/07/2018
D95	5	5	*D. sylvestris* subsp. *bertisceus*	ZA 48670	Montenegro	1948	42.67997	20.01136	S. Bogdanović, I. Ljubičić	15/07/2018
D96	5	5	*D. sylvestris* subsp. *bertisceus*	ZA 48613	Montenegro	278	42.69683	19.37431	S. Bogdanović, I. Ljubičić	15/07/2018
D150	6	6	*D. sylvestris* subsp. *bertisceus*	ZA 54956	Albania	773	42.41509	19.50371	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	27/06/2019
D151	7	7	*D. sylvestris* subsp. *bertisceus*	ZA 54957	Albania	1735	42.38844	19.73021	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	28/06/2019
D153	5	5	*D. sylvestris* subsp. *bertisceus*	ZA 54959	Albania	665	42.32486	19.59345	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	28/06/2019
D154	5	3	*D. sylvestris* subsp. *bertisceus*	ZA 54960	Montenegro	139	42.39835	19.36711	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	28/06/2019
D157	1	-	*D. sylvestris* subsp. *bertisceus*	ZA 54963	Montenegro	600	42.67368	19.03014	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	28/06/2019
D158	5	5	*D. sylvestris* subsp. *bertisceus*	ZA 54964	Montenegro	900	42.75111	18.79916	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	29/06/2019

D168	5	1	*D. sylvestris* subsp. *bertisceus*	ZA 54974	Albania	1115	42.47372	20.15167	D. Shuka	23/07/2019
D169	5	2	*D. sylvestris* subsp. *bertisceus*	ZA 54975, ZA 54976	Albania	2253	42.45741	20.12222	D. Shuka	24/07/2019
SDM16	-	-	*D. sylvestris* subsp. *bertisceus*	-	Bosnia and Herzegovina	620	43.30424	18.64968	S. Bogdanović, I. Ljubičić	11/07/2018
SDM17	-	-	*D. sylvestris* subsp. *bertisceus*	-	Montenegro	1526	43.25179	18.72164	S. Bogdanović, I. Ljubičić	12/07/2018
SDM18	-	-	*D. sylvestris* subsp. *bertisceus*	-	Bosnia and Herzegovina	1647	43.28504	18.71911	S. Bogdanović, I. Ljubičić	12/07/2018
SDM19	-	-	*D. sylvestris* subsp. *bertisceus*	-	Montenegro	809	43.17217	18.85883	S. Bogdanović, I. Ljubičić	13/07/2018
SDM20	-	-	*D. sylvestris* subsp. *bertisceus*	-	Montenegro	1692	43.09217	19.08971	S. Bogdanović, I. Ljubičić	13/07/2018
SDM21	-	-	*D. sylvestris* subsp. *bertisceus*	-	Montenegro	1346	43.15262	19.25794	S. Bogdanović, I. Ljubičić	13/07/2018
D178	6	6	*D. sylvestris* subsp. *kozjakensis*	ZA 54985	North Macedonia	1292	41.40914	21.67842	S. Bogdanović, I. Rešetnik	14/07/2019
D2	7	-	*D. sylvestris* subsp. *nodosus*	ZA 46277	Croatia	250	42.94086	17.08055	I. Rešetnik, S. Bogdanović	11/05/2018
D6	5	7	*D. sylvestris* subsp. *nodosus*	ZA 46281	Croatia	332	42.81569	17.84349	I. Rešetnik, S. Bogdanović	13/5/2018, 29/6/2019
D8	5	4	*D. sylvestris* subsp. *nodosus*	ZA 46283	Croatia	63	43.45025	16.69853	I. Rešetnik, S. Bogdanović	13/5/2018, 29/6/2019
D27	5	-	*D. sylvestris* subsp. *nodosus*	ZA 47427	Croatia	1211	44.70794	14.96583	I. Rešetnik, S. Bogdanović, I. Ljubičić	15/06/2018
D28	10	1	*D. sylvestris* subsp. *nodosus*	ZA 47428	Croatia	1020	44.7153	14.9569	I. Rešetnik, S. Bogdanović, I. Ljubičić	15/06/2018
D33	5	1	*D. sylvestris* subsp. *nodosus*	ZA 47433	Croatia	905	44.52948	15.14316	I. Rešetnik, S. Bogdanović, I. Ljubičić	15/06/2018
D42	6	-	*D. sylvestris* subsp. *nodosus*	ZA 47442	Croatia	1605	44.36112	15.51313	I. Rešetnik, S. Bogdanović, I. Ljubičić	17/06/2018
D46	6	6	*D. sylvestris* subsp. *nodosus*	ZA 47456	Croatia	18	43.73736	15.87176	I. Rešetnik, S. Bogdanović, I. Ljubičić	18/06/2018

D47-1	4	4	*D. sylvestris* subsp. *nodosus*	ZA 47457	Croatia	325	43.25955	17.07843	I. Rešetnik, S. Bogdanović, I. Ljubičić	18/06/2018
D47-2	5	5	*D. sylvestris* subsp. *nodosus*	ZA 47459	Croatia	899	43.261	17.10161	I. Rešetnik, S. Bogdanović, I. Ljubičić	18/06/2018
D47-3	2	1	*D. sylvestris* subsp. *nodosus*	ZA 47459	Croatia	535	43.26069	17.09043	I. Rešetnik, S. Bogdanović, I. Ljubičić	18/06/2018
D52	7	7	*D. sylvestris* subsp. *nodosus*	ZA 47463	Bosnia and Herzegovina	355	43.32548	17.80283	I. Rešetnik, S. Bogdanović, I. Ljubičić	19/06/2018
D55	7	6	*D. sylvestris* subsp. *nodosus*	ZA 47466	Bosnia and Herzegovina	360	43.64998	17.96701	I. Rešetnik, S. Bogdanović, I. Ljubičić	19/06/2018
D56	5	5	*D. sylvestris* subsp. *nodosus*	ZA 47467	Bosnia and Herzegovina	549	43.41747	17.41643	I. Rešetnik, S. Bogdanović, I. Ljubičić	20/06/2018
D57	5	5	*D. sylvestris* subsp. *nodosus*	ZA 47468	Bosnia and Herzegovina	1298	43.58668	17.48789	I. Rešetnik, S. Bogdanović, I. Ljubičić	20/06/2018
D60	5	5	*D. sylvestris* subsp. *nodosus*	ZA 47471	Bosnia and Herzegovina	1556	43.57896	17.54072	I. Rešetnik, S. Bogdanović, I. Ljubičić	20/06/2018
D61	5	5	*D. sylvestris* subsp. *nodosus*	ZA 47472	Bosnia and Herzegovina	904	43.54695	17.27952	I. Rešetnik, S. Bogdanović, I. Ljubičić	20/06/2018
D63	5	5	*D. sylvestris* subsp. *nodosus*	ZA 47474	Bosnia and Herzegovina	1197	43.85963	17.03066	I. Rešetnik, S. Bogdanović, I. Ljubičić	21/06/2018
D64	6	6	*D. sylvestris* subsp. *nodosus*	ZA 47475	Bosnia and Herzegovina	1397	43.88544	17.05981	I. Rešetnik, S. Bogdanović, I. Ljubičić	21/06/2018
D65	5	5	*D. sylvestris* subsp. *nodosus*	ZA 47476	Bosnia and Herzegovina	1096	43.78286	17.16035	I. Rešetnik, S. Bogdanović, I. Ljubičić	21/06/2018
D66	6	5	*D. sylvestris* subsp. *nodosus*	ZA 47477	Bosnia and Herzegovina	716	44.34866	16.35956	I. Rešetnik, S. Bogdanović, I. Ljubičić	22/06/2018

D80	8	9	*D. sylvestris* subsp. *nodosus*	ZA 48607	Croatia	740	44.01427	16.41704	S. Bogdanović, I. Ljubičić	30/06/2018
D82	5	5	*D. sylvestris* subsp. *nodosus*	ZA 48608	Croatia	934	43.75119	16.52322	S. Bogdanović, I. Ljubičić	01/07/2018
D85	5	5	*D. sylvestris* subsp. *nodosus*	ZA 48611	Bosnia and Herzegovina	1100	44.25852	17.62419	S. Bogdanović, I. Ljubičić	10/07/2018
D106	5	5	*D. sylvestris* subsp. *nodosus*	ZA 48623	Bosnia and Herzegovina	915	42.56432	18.44312	S. Bogdanović, I. Ljubičić	17/7/2018, 29/6/2019
D124	7	-	*D. sylvestris* subsp. *nodosus*	ZA 54927	Croatia	48	42.75519	16.92379	I. Rešetnik, S. Bogdanović	04/06/2019
D125	5	1	*D. sylvestris* subsp. *nodosus*	ZA 54928	Croatia	126	42.74994	16.88776	I. Rešetnik, S. Bogdanović	04/06/2019
D126	5	5	*D. sylvestris* subsp. *nodosus*	ZA 54929	Croatia	46	42.72382	16.88531	I. Rešetnik, S. Bogdanović	04/06/2019
D127	7	-	*D. sylvestris* subsp. *nodosus*	ZA 54930	Croatia	150	43.26608	16.69359	I. Ljubičić, A. Terlević, M. Grgurev	04/06/2019
D128	5	-	*D. sylvestris* subsp. *nodosus*	ZA 54931	Croatia	810	43.52237	16.61158	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević, M. Grgurev	05/06/2019
D160	5	5	*D. sylvestris* subsp. *nodosus*	ZA 54966	Croatia	160	42.91527	17.42674	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	29/06/2019
D183	5	5	*D. sylvestris* subsp. *nodosus*	ZA 56692	Croatia	83	43.75222	15.87861	L. Laca, S. Bogdanović	17/06/2020
D192	5	5	*D. sylvestris* subsp. *nodosus*	ZA 56821	Croatia	1713	43.71589	16.8893	M. Doboš	22/07/2020
D193	5	-	*D. sylvestris* subsp. *nodosus*	ZA 56822	Croatia	906	43.70998	16.8192	S. Bogdanović	22/07/2020
SDM10	-	-	*D. sylvestris* subsp. *nodosus*	-	Croatia	1501	44.42911	15.36696	I. Rešetnik, S. Bogdanović, I. Ljubičić	16/06/2018
SDM13	-	-	*D. sylvestris* subsp. *nodosus*	-	Croatia	1440	43.32763	17.05769	I. Rešetnik, S. Bogdanović, I. Ljubičić	18/06/2018
SDM14	-	-	*D. sylvestris* subsp. *nodosus*	-	Bosnia and Herzegovina	551	43.47327	17.35342	I. Rešetnik, S. Bogdanović, I. Ljubičić	20/06/2018

SDM15	-	-	*D. sylvestris* subsp. *nodosus*	-	Bosnia and Herzegovina	734	43.53634	17.44794	I. Rešetnik, S. Bogdanović, I. Ljubičić	20/06/2018
SDM22	-	-	*D. sylvestris* subsp. *nodosus*	-	Croatia	803	44.88309	14.97183	S. Bogdanović, I. Ljubičić	06/09/2018
SDM29	-	-	*D. sylvestris* subsp. *nodosus*	-	Bosnia and Herzegovina	937	42.71159	18.55117	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	29/06/2019
SDM30	-	-	*D. sylvestris* subsp. *nodosus*	-	Bosnia and Herzegovina	500	42.88024	17.94704	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	29/06/2019
SDM31	-	-	*D. sylvestris* subsp. *nodosus*	-	Croatia	400	42.87273	17.85456	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević	29/06/2019
D12	6	2	*D. sylvestris* subsp. *sylvestris*	ZA 46416	Croatia	240	45.12395	14.20468	S. Bogdanović, I. Ljubičić	19/5/2018, 13/7/2019
D17	4	5	*D. sylvestris* subsp. *sylvestris*	ZA 47417	Italy	300	45.64227	13.8311	I. Rešetnik, S. Bogdanović, I. Ljubičić	13/06/2018
D19-1	2	-	*D. sylvestris* subsp. *sylvestris*	ZA 47419	Croatia	660	45.47917	14.04971	I. Rešetnik, S. Bogdanović, I. Ljubičić	13/06/2018
D19-2	8	5	*D. sylvestris* subsp. *sylvestris*	ZA 47419	Croatia	660	45.47917	14.04971	I. Rešetnik, S. Bogdanović, I. Ljubičić	13/6/2018, 13/7/2019
D20	5	5	*D. sylvestris* subsp. *sylvestris*	ZA 47420	Croatia	941	45.45721	14.01632	I. Rešetnik, S. Bogdanović, I. Ljubičić	13/6/2018, 19/7/2020
D21	5	9	*D. sylvestris* subsp. *sylvestris*	ZA 47421	Croatia	553	45.4234	14.01213	I. Rešetnik, S. Bogdanović, I. Ljubičić	13/6/2018, 19/7/2020
D22	5	5	*D. sylvestris* subsp. *sylvestris*	ZA 47422	Croatia	525	45.31681	14.17734	I. Rešetnik, S. Bogdanović, I. Ljubičić	13/06/2018
D23	5	5	*D. sylvestris* subsp. *sylvestris*	ZA 47423	Croatia	326	45.37468	14.5247	I. Rešetnik, S. Bogdanović, I. Ljubičić	14/06/2018
D24	5	5	*D. sylvestris* subsp. *sylvestris*	ZA 47424	Croatia	862	45.36157	14.61724	I. Rešetnik, S. Bogdanović, I. Ljubičić	14/06/2018
D34	5	-	*D. sylvestris* subsp. *sylvestris*	ZA 47434	Croatia	1458	44.4293	15.3643	I. Rešetnik, S. Bogdanović, I. Ljubičić	16/06/2018
D37	7	3	*D. sylvestris* subsp. *sylvestris*	ZA 47437	Croatia	1131	44.36475	15.46012	I. Rešetnik, S. Bogdanović, I. Ljubičić	17/06/2018
D38	5	1	*D. sylvestris* subsp. *sylvestris*	ZA 47438	Croatia	1368	44.37109	15.46567	I. Rešetnik, S. Bogdanović, I. Ljubičić	17/06/2018

D39	2	2	*D. sylvestris* subsp. *sylvestris*	ZA 47439	Croatia	1388	44.37557	15.46572	I. Rešetnik, S. Bogdanović, I. Ljubičić	17/06/2018
D58	5	-	*D. sylvestris* subsp. *sylvestris*	ZA 47469	Bosnia and Herzegovina	1928	43.59503	17.54076	I. Rešetnik, S. Bogdanović, I. Ljubičić	20/06/2018
D59	6	6	*D. sylvestris* subsp. *sylvestris*	ZA 47470	Bosnia and Herzegovina	1732	43.58652	17.53378	I. Rešetnik, S. Bogdanović, I. Ljubičić	20/06/2018
D81	4	5	*D. sylvestris* subsp. *sylvestris*	ZA 48606	Croatia	1418	44.03867	16.39343	S. Bogdanović, I. Ljubičić	30/06/2018
D83	5	1	*D. sylvestris* subsp. *sylvestris*	ZA 48609	Bosnia and Herzegovina	207	44.68547	17.1797	S. Bogdanović, I. Ljubičić	10/07/2018
D84	4	-	*D. sylvestris* subsp. *sylvestris*	ZA 48610	Bosnia and Herzegovina	380	44.3468	17.24253	S. Bogdanović, I. Ljubičić	10/07/2018
D130	5	5	*D. sylvestris* subsp. *sylvestris*	ZA 54933	Croatia	345	44.03398	16.29247	S. Bogdanović, I. Ljubičić	14/06/2019
D162	5	5	*D. sylvestris* subsp. *sylvestris*	ZA 54968	Croatia	740	45.76377	15.6486	A. Terlević, M. Grgurev	05/07/2019
D164	6	-	*D. sylvestris* subsp. *sylvestris*	ZA 54970	Slovenia	1954	46.43132	13.73659	A. Terlević, I. Ljubičić	09/07/2019
D165	5	-	*D. sylvestris* subsp. *sylvestris*	ZA 54971	Slovenia	295	46.08895	14.8873	A. Terlević, I. Ljubičić	09/07/2019
D166	5	5	*D. sylvestris* subsp. *sylvestris*	ZA 54972	Slovenia	900	46.08407	14.33474	A. Terlević, I. Ljubičić	10/07/2019
D184	5	5	*D. sylvestris* subsp. *sylvestris*	ZA 56693	Croatia	136	45.13314	13.73433	A. Terlević	21/06/2020
D196	5	-	*D. sylvestris* subsp. *sylvestris*	ZA 56825	Slovenia	625	45.50694	14.16806	B. Frajman	15/08/2020
D201	5	-	*D. sylvestris* subsp. *sylvestris*	ZA 57144	Croatia	340	45.40548	14.47115	B. Frajman	18/10/2020
SDM1	-	-	*D. sylvestris* subsp. *sylvestris*	-	Slovenia	351	45.70294	13.94686	I. Rešetnik, S. Bogdanović, I. Ljubičić	13/06/2018
SDM5	-	-	*D. sylvestris* subsp. *sylvestris*	-	Croatia	639	45.50666	13.97424	I. Rešetnik, S. Bogdanović, I. Ljubičić	13/06/2018

SDM6	-	-	*D. sylvestris* subsp. *sylvestris*	-	Croatia	567	45.42499	14.00793	I. Rešetnik, S. Bogdanović, I. Ljubičić	13/06/2018
SDM7	-	-	*D. sylvestris* subsp. *sylvestris*	-	Croatia	330	45.37942	14.54419	I. Rešetnik, S. Bogdanović, I. Ljubičić	14/06/2018
SDM9	-	-	*D. sylvestris* subsp. *sylvestris*	-	Croatia	30	44.63657	14.96594	I. Rešetnik, S. Bogdanović, I. Ljubičić	15/06/2018
SDM23	-	-	*D. sylvestris* subsp. *sylvestris*	-	Croatia	1411	43.78716	16.49008	M. Temunović	07/01/2018
SDM24	-	-	*D. sylvestris* subsp. *sylvestris*	-	Croatia	1462	43.7891	16.485	M. Temunović	07/01/2018
SDM25	-	-	*D. sylvestris* subsp. *sylvestris*	-	Croatia	1090	43.7781	16.51126	M. Temunović	07/01/2018
SDM32	-	-	*D. sylvestris* subsp. *sylvestris*	-	Slovenia	758	46.08555	14.34639	A. Terlević, I. Ljubičić	10/07/2019
SDM33	-	-	*D. sylvestris* subsp. *sylvestris*	-	Croatia	1029	45.26035	15.14543	D. Šincek	02/08/2020
SDM34	-	-	*D. sylvestris* subsp. *sylvestris*	-	Croatia	1104	45.26018	15.14457	D. Šincek	02/08/2020
D1	5	5	*D. sylvestris* subsp. *tergestinus*	ZA 46276	Croatia	80	42.94874	17.56451	I. Rešetnik, S. Bogdanović	09/05/2018
D4	5	5	*D. sylvestris* subsp. *tergestinus*	ZA 46279	Croatia	279	42.64521	18.12021	I. Rešetnik, S. Bogdanović	12/05/2018
D7	5	4	*D. sylvestris* subsp. *tergestinus*	ZA 46282	Croatia	351	42.81084	17.83079	I. Rešetnik, S. Bogdanović	13/05/2018
D9	5	5	*D. sylvestris* subsp. *tergestinus*	ZA 46284	Croatia	602	43.58147	16.32579	I. Rešetnik, S. Bogdanović	13/05/2018
D11	6	6	*D. sylvestris* subsp. *tergestinus*	ZA 46415	Croatia	55	44.81097	13.90088	S. Bogdanović, I. Ljubičić	17/05/2018
D13	5	5	*D. sylvestris* subsp. *tergestinus*	ZA 46417	Croatia	513	45.2676	14.59441	S. Bogdanović, I. Ljubičić	19/05/2018
D16	5	5	*D. sylvestris* subsp. *tergestinus*	ZA 47416	Italy	344	45.70153	13.83729	I. Rešetnik, S. Bogdanović, I. Ljubičić	13/06/2018
D18	5	5	*D. sylvestris* subsp. *tergestinus*	ZA 47418	Slovenia	422	45.57284	13.90681	I. Rešetnik, S. Bogdanović, I. Ljubičić	13/06/2018
D25	5	-	*D. sylvestris* subsp. *tergestinus*	ZA 47425	Croatia	8	45.02484	14.88473	I. Rešetnik, S. Bogdanović, I. Ljubičić	14/06/2018

D29	5	-	*D. sylvestris* subsp. *tergestinus*	ZA 47429	Croatia	274	44.71196	14.9225	I. Rešetnik, S. Bogdanović, I. Ljubičić	15/06/2018
D32	5	-	*D. sylvestris* subsp. *tergestinus*	ZA 47432	Croatia	12	44.53338	15.06519	I. Rešetnik, S. Bogdanović, I. Ljubičić	15/06/2018
D36	5	2	*D. sylvestris* subsp. *tergestinus*	ZA 47436	Croatia	397	44.31347	15.45328	I. Rešetnik, S. Bogdanović, I. Ljubičić	17/06/2018
D43	5	-	*D. sylvestris* subsp. *tergestinus*	ZA 47453	Croatia	35	43.86523	15.6423	I. Rešetnik, S. Bogdanović, I. Ljubičić	18/06/2018
D50	5	-	*D. sylvestris* subsp. *tergestinus*	ZA 47461	Bosnia and Herzegovina	250	43.32542	17.79292	I. Rešetnik, S. Bogdanović, I. Ljubičić	19/06/2018
D53	5	-	*D. sylvestris* subsp. *tergestinus*	ZA 47464	Bosnia and Herzegovina	355	43.32588	17.80257	I. Rešetnik, S. Bogdanović, I. Ljubičić	19/06/2018
D62	5	5	*D. sylvestris* subsp. *tergestinus*	ZA 47473	Bosnia and Herzegovina	821	43.84762	16.9733	I. Rešetnik, S. Bogdanović, I. Ljubičić	21/06/2018
D111	5	-	*D. sylvestris* subsp. *tergestinus*	ZA 48628	Croatia	40	44.97721	14.445	S. Bogdanović, S. Cambria	21/10/2018
D129	5	5	*D. sylvestris* subsp. *tergestinus*	ZA 54932	Croatia	186	43.77399	15.95608	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević, M. Grgurev	05/06/2019
D185	5	-	*D. sylvestris* subsp. *tergestinus*	ZA 56694	Croatia	220	45.02389	14.64694	I. Rešetnik	14/06/2020
SDM2	-	-	*D. sylvestris* subsp. *tergestinus*	-	Italy	491	45.62724	13.90065	I. Rešetnik, S. Bogdanović, I. Ljubičić	13/06/2018
SDM3	-	-	*D. sylvestris* subsp. *tergestinus*	-	Slovenia	472	45.60195	13.93049	I. Rešetnik, S. Bogdanović, I. Ljubičić	13/06/2018
SDM4	-	-	*D. sylvestris* subsp. *tergestinus*	-	Slovenia	411	45.5605	13.89178	I. Rešetnik, S. Bogdanović, I. Ljubičić	13/06/2018
SDM8	-	-	*D. sylvestris* subsp. *tergestinus*	-	Croatia	25	45.17663	14.69306	I. Rešetnik, S. Bogdanović, I. Ljubičić	14/06/2018
SDM11	-	-	*D. sylvestris* subsp. *tergestinus*	-	Croatia	513	44.33887	15.44411	I. Rešetnik, S. Bogdanović, I. Ljubičić	17/06/2018
SDM12	-	-	*D. sylvestris* subsp. *tergestinus*	-	Croatia	30	43.76309	15.84544	I. Rešetnik, S. Bogdanović, I. Ljubičić	18/06/2018
SDM26	-	-	*D. sylvestris* subsp. *tergestinus*	-	Croatia	251	43.56302	16.40964	I. Ljubičić, A. Terlević, M. Grgurev	04/06/2019

SDM27	-	-	*D. sylvestris* subsp. *tergestinus*	-	Croatia	100	43.5176	16.57563	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević, M. Grgurev	05/06/2019
SDM28	-	-	*D. sylvestris* subsp. *tergestinus*	-	Croatia	743	44.25694	16.06509	S. Bogdanović, I. Ljubičić, I. Rešetnik, A. Terlević, M. Grgurev	06/06/2019

Table S2. Chi-square test between each of the 15 pairs of *Dianthus sylvestris* subspecies, for each qualitative morphological character. Significant differences ($p < 0.01$) are shown in bold. Abbreviated labels: *alb* - subsp. *alboroseus*, *bert* - subsp. *bertisceus*, *nod* - subsp. *nodosus*, *s.l.* - *D. sylvestris sensu lato*, sylv - subsp. *sylvestris*, *terg* - subsp. *tergestinus.*

	Woody part of the stem below basal leaves (WST)		**Lower part of the stem indumentum (IND)**		**Petal denticulation (DEN)**		**Arrangement of epicalyx scales (EPI)**	
Subspecies pairs	*Chi-square* test	*p*-value	*Chi-square* test	*p*-value	*Chi-square* test	*p*-value	*Chi-square* test	*p*-value
alb - s.l.	2.30	0.1609	1.33	0.2624	3.30	0.0925	3.75	0.0725
alb - bert	0.17	0.6882	12.66	**0.0020**	5.52	0.0235	64.13	**0.0005**
alb - nod	3.43	0.0800	7.29	**0.0045**	11.35	**0.0020**	13.26	**0.0020**
alb - sylv	2.46	0.1304	16.71	**0.0010**	8.28	0.0050	26.80	**0.0005**
alb - terg	2.07	0.1654	0.49	0.5497	56.81	**0.0005**	19.99	**0.0005**
bert - s.l.	5.10	0.0280	6.65	0.0125	1.02	0.3193	50.15	**0.0005**
bert - nod	8.57	**0.0030**	52.60	**0.0005**	4.16	0.0605	189.81	**0.0005**
bert - sylv	6.17	0.0220	0.06	1.0000	1.07	0.4123	177.81	**0.0005**
bert - terg	4.97	0.0390	10.18	**0.0015**	63.55	**0.0005**	147.30	**0.0005**
nod - s.l.	0.01	1.0000	21.78	**0.0005**	7.99	0.0045	46.78	**0.0005**
nod - sylv	0.07	0.8621	67.94	**0.0005**	0.79	0.4163	5.29	0.0445
nod - terg	0.09	0.8541	19.18	**0.0005**	48.86	**0.0005**	3.91	0.1039
sylv - s.l.	0.01	1.0000	9.29	**0.0025**	3.69	0.0620	54.14	**0.0005**
sylv - terg	0.00	1.0000	13.99	**0.0005**	47.97	**0.0005**	4.88	0.0370
terg - s.l.	0.02	1.0000	0.31	0.7091	66.80	**0.0005**	41.20	**0.0005**

Table S3. Quantitative morphological characters studied in *Dianthus sylvestris* from the Balkan Peninsula. Morphological differences along 21 quantitative morphological characters and seven ratios between the *D. sylvestris* subspecies as revealed by Kruskal Wallis test. Significant differences ($p < 0.01$) are shown in bold.

Morphological character	**Abbreviation**	**Kruskal Wallis rank sum statistic**	***P*- value**
Length of the woody part of the stem below basal leaves	BL	5.27	0.383762
Basal leaf length	BLL	26.27	**< 0.01**
Calyx length	CL	32.09	**< 0.01**
Length of the cauline leaf from the second stem node	CLL	35.34	**< 0.01**
Calyx teeth length	CTL	45.10	**< 0.01**
Number of epicalyx scales	ESN	41.11	**< 0.01**
Height of first branching	FBH	21.72	**< 0.01**
Number of flowers per stem	FPS	5.22	0.389483
Inflorescence length	IL	8.61	0.125703
Inner epicalyx scale apex length	ISAL	59.96	**< 0.01**
Inner epicalyx scale length	ISL	26.95	**< 0.01**
Inner epicalyx scale width	ISW	53.41	**< 0.01**
Length of the first stem internode	LIL	23.15	**< 0.01**
Plant height	PH	16.08	0.006624
Petal length	PL	19.75	**< 0.01**
Petal limb length	PLL	26.43	**< 0.01**
Petal limb width	PLW	22.22	**< 0.01**
Petal teeth length	PTL	46.49	**< 0.01**
Maximum number of petal teeth	PTMAX	31.16	**< 0.01**
Minimum number of petal teeth	PTMIN	33.51	**< 0.01**
Ratio Calyx teeth length / Calyx length	RCALYX	49.65	**< 0.01**
Ratio Petal teeth length / Petal limb length	RDENT	54.74	**< 0.01**
Ratio Calyx length / Claw length	RFL	21.83	**< 0.01**
Ratio Inner epicalyx scale length without apex / Inner epicalyx scale width	RIS	63.16	**< 0.01**
Ratio Inner epicalyx scale apex length / Inner epicalyx scale length	RISA	61.09	**< 0.01**
Ratio Petal limb length / Petal limb width	RLIMB	26.23	**< 0.01**
Ratio Petal limb length / Petal length	RPETAL	42.67	**< 0.01**
Length of the last stem internode below branching	UIL	11.20	0.047556

Table S4. Environmental variables used for characterizing environmental variability of *Dianthus sylvestris* populations using principal component analysis (PCA) and for RDA analysis, with their abbreviated and full names/definitions, units and types. Shown are also factor loadings of PCA of environmental data related to *D. sylvestris* populations.

Abbreviation	Full name	Unit	Variable type	PC1	PC2
bio04	temperature seasonality	°C/10	climate	0.099	0.254
bio08	mean daily mean air temperatures of the wettest quarter	°C/10	climate	-0.257	0.223
bio09	mean daily mean air temperatures of the driest quarter	°C/10	climate	-0.184	-0.407
bio15	precipitation seasonality	kg m^{-2}	climate	-0.096	-0.470
bio19	mean monthly precipitation amount of the coldest quarter	kg m^{-2}	climate	0.131	-0.348
fcf	frost change frequency	No days	extended climate	0.317	0.093
sdays	number of snow days	No days	extended climate	0.345	-0.024
shc	Selyaninov's Hydrothermic Coefficient	(kg m^{-2}/10)/°C	extended climate	0.151	0.023
srad	surface solar radiation downwards	kJ m^{-2}	extended climate	-0.328	-0.170
dtm_slope	slope	°	topographic	0.213	-0.300
dtm_east	eastness	index	topographic	0.095	0.038
dtm_north	northness	index	topographic	-0.037	0.073
soil_org_carb	soil organic carbon content	5 x g kg^{-1}	soil properties	0.264	0.059
soil_bulk	soil bulk density	10 x kg m^{-3}	soil properties	-0.329	-0.049
soil_sand	sand content	%	soil properties	0.201	-0.150
soil_ph_h2o	soil pH x 10 in H2O	pH	soil properties	-0.304	-0.109
soil_water	soil water content at 33kPa		soil properties	-0.263	-0.058
dtm_con	convergence	index	topographic	-0.065	0.198
dtm_cti	compound topographic index	index	topographic	-0.173	0.351
dtm_vrm	vector ruggedness measure	index	topographic	0.200	-0.180

Table S5. Results of a forward selection procedure with double stopping criterion (*P*-value and R^2adj), on the RDAfull model.

No	variables	order	R^2	R^2Cum	$R^2_{adj}Cum$	*F*	*P*-value
1	sdays	10	0.263248	0.263248	0.255492	33.94425	0.001
2	longitude	21	0.103145	0.366392	0.352911	15.3022	0.001
3	bio04	11	0.035619	0.402012	0.382722	5.539577	0.002
4	latitude	22	0.039582	0.441594	0.417315	6.521354	0.002
5	shc	19	0.036414	0.478008	0.449327	6.348182	0.002
6	srad	9	0.022237	0.500246	0.466929	4.004706	0.006
7	bio19	15	0.014966	0.515211	0.477082	2.747505	0.034

Publication V

Genomic divergence of *Dianthus sylvestris* Wulfen s.l. on the Balkan Peninsula

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Contemporary patterns of genomic variation in plant species often reflect past demographic processes linked to climate events such as Pleistocene glaciations. *Dianthus sylvestris* s.l. comprises a number of taxa distributed along a wide altitudinal range from sea level up to alpine peaks across southern and south-eastern Europe. We here used RAD-seq genome-wide SNP data to unravel genomic variation patterns and evolutionary history of populations collected across the Balkan and Apennine Peninsulas, as well as the Alps, including all six currently described subspecies distributed exclusively in the Balkan Peninsula. Our phylogenetic and genetic structure analyses support the existence of three main geographically structured evolutionary lineages within *D. sylvestris* (Alpine, Apennine and the Balkan), and the divergence of *D. sylvestris* subsp. *tergestinus* from the rest of the Balkan populations. Further in-depth analyses of the Balkan lineage suggested hierarchically nested phylogeographic structure within the Balkan populations. Two main genetic groups of populations were revealed based on Bayesian clustering (a more homogeneous north-western (NW) group, and a more divergent south-eastern (SE) group), or five clusters (two in the NW and three in the SE) based on alternative model-free approach. We detected significant isolation-by-distance within our populations, while geographical patterns of genetic diversity showed that southernmost populations have reduced genetic diversity. Additional genetic connectivity and migration analyses confirmed a sharp break between the two main genetic groups. Species distribution modelling (SDM) combined with the majority of our genetic analyses' results suggest that contemporary *D. sylvestris* populations in the Balkans derive from two distinct evolutionary lineages which may survived the Pleistocene climatic oscillations in two main glacial refugia located in the NW and SE of the eastern Adriatic, with possible secondary contact of the two lineages in the intermediate area of Montenegro where admixed populations occur. In addition, we cannot exclude the existence of multiple smaller microrefugia for the more divergent SE group, where certain high-elevation populations may have persisted in situ over several glaciation cycles until today and may have diverged independently from the two main Balkan groups.

Keywords: genetic variation, phylogeography, migrations, RAD-seq, refugia, species distribution models

Introduction

Pleistocene climatic oscillations have largely shaped the contemporary patterns of genetic variation on Earth by shifting species distribution ranges (Hewitt 2000, 2004). European taxa survived the unfavourable climatic conditions during Pleistocene in various glacial refugia, of which thee major ones (macrorefugia *sensu* Rull 2009) correspond to the Balkan, Apennine and Iberian Peninsulas (Hewitt 1999). Plant species on the Balkan Peninsula experienced complex histories over the repeated cycles of Pleistocene climatic fluctuations (Španiel and Rešetnik 2022), which supported the emergence of a plant diversity greater than in any other comparable area in Europe (Hewitt 2011). The Balkan Peninsula has more than 60% of land above 1000 m a.s.l., resulting in highly heterogeneous topography, but also heterogeneous climate, due to large scale altitudinal variations in both temperature and precipitation (Willis 1994). Due to the high degree of habitat heterogeneity with barriers such as mountains, river canyons and valleys that triggered allopatric speciation, the Balkan Peninsula did not function as a single macrorefugium during Pleistocene glacial cycles. Instead, multiple microrefugia existed within the Peninsula (Rull 2009; Médail and Diadema 2009), known as the 'refugia-within-refugia hypothesis' (Gómez and Lunt 2007) proposed for some of the investigated taxa occurring on the Balkans (Surina *et al.* 2011; Jug-Dujaković *et al.* 2020; Reich *et al.* 2022). Eight putative microrefugia were proposed for the Balkans by Médail and Diadema (2009) – four along the eastern Adriatic coast (northern Istria, Velebit and Biokovo mountains and Montenegro) and four in Greece (Olympe and Pindos mountains, Peloponnese and Chalkidiki peninsulas). Geographical barriers contributed to the compartmentalization of the region and the creation of climatically suitable enclaves, and thus fostered the differentiation of genetic lineages both in animal (Previšić *et al.* 2009; Pavlek *et al.* 2022) and plant species (Kutnjak *et al.* 2014; Jug-Dujaković *et al.* 2020). The Dinaric Mountains have several deep and narrow canyons formed by karstic rivers which likely acted as geographical barriers to gene flow, and species distributed along the Dinaric Mountains commonly show a pattern of differentiation into two or three genetic groups (Španiel and Rešetnik 2022). For example, evidence for such allopatric diversification has been found between populations separated by the Zrmanja canyon in the Northern Dinaric Mountains (e.g., *Tanacetum cinerariifolium*; Grdiša *et al.* 2014), Neretva river valley in the Central Dinaric Mountains (e.g., *Alyssum austrodalmaticum*, Španiel *et al.* 2017; Zozomová-Lihová *et al.* 2020; *Cerastium*, Đurović *et al.* 2021), and karst rivers Drina and Sutjeska in the Southern Dinaric Mountains (e.g., *Heliosperma pusillum* group; Frajman and Oxelman 2007).

Climate fluctuations during the Pleistocene are closely related to migration dynamics and distribution shifts of plant species, which responded individually according to their environmental/habitat requirements (Stewart *et al.* 2010). For example, cold-adapted species probably shifted their distribution along altitudes, whereas thermophilic species shifted along latitudes (Surina *et al.* 2011). During cold stages (glacials) cold-adapted species likely had more continuous distribution sufficient for gene flow between different mountain ranges, and more fragmented distribution during subsequent warmer stages (i.e., interglacial contraction hypotheses, Stewart *et al.* 2010; Theodoridis *et al.* 2017). This pattern seems to be predominant for cold-adapted species and was for example observed in Dinaric high-mountain species *Cerastium dinaricum* (Kutnjak *et al.* 2014; Đurović *et al.* 2021) and *Edraianthus graminifolius* (Surina *et al.* 2011). On the other hand, thermophilous and temperate species mostly experienced range contraction and fragmentation during glacials and range expansions during interglacials (including the Holocene) which may have triggered their genetic divergence and allopatric speciation during isolation in different refugial areas (Hewitt 1996; Hardion *et al.* 2014; Zozomová-Lihová *et al.* 2020). However, recently, the pattern of interglacial expansion was also demonstrated for the first time in cold-adapted *Primula farinosa* across Europe (Theodoridis *et al.* 2017), confirming that for species with broad ecological preferences it may be difficult to assume *a-priori* their response to climatic fluctuations (Stewart *et al.* 2010).

Pleistocene ice ages left specific footprints on species genetic diversity patterns. Postglacial latitudinal recolonizations of thermophilous and temperate taxa out of the main southern refugia often resulted in a pattern of higher genetic diversity and demographic stability of southern populations and a reduction in genetic diversity towards the north (leading edge hypothesis; Hewitt 2000). Such pattern was observed for example in thermophilous *Edraianthus tenuifolius* (Surina *et al.* 2011) and *Euphorbia myrsinites* (Falch *et al.* 2019), but also in some species with a wider elevational tolerance and wider geographical distribution, like *Silene saxifraga* (Đurović *et al.* 2017). In addition, stability of southern populations which may represent relict populations that persisted in situ during Pleistocene climatic oscillations, may have resulted in their reduced within-population genetic diversity and increased divergence (rear-edge hypothesis, Hampe and Petit 2005). However, equivalent genetic diversity was observed among the populations of some thermophilic species, like *Viola suavis* s.l. (Mereďa *et al.* 2011), *Helichrysum italicum* (Ninčević *et al.* 2021) and *Salvia officinalis* (Jug-Dujaković *et al.* 2020), suggesting that they may have persisted in the Balkan macrorefugium during the cold stages in both northerly and southerly located microrefugia along the eastern Adriatic coast. Finally, increased genetic diversity of populations has been also observed to be a result of the secondary contact of previously isolated divergent lineages (Petit *et al.* 2003; Sakaguchi *et al.* 2011; Havrdová *et al.* 2015). Overall, various evolutionary and phylogeographic patterns in different species groups from the Balkan Peninsula and adjacent regions have been revealed, confirming the pattern of multiple glacial microrefugia often corresponding to major phylogeographic breaks within the Peninsula (Španiel and Rešetnik 2022).

The knowledge on demographic processes of species occupying habitats spanning wide altitudinal ranges from coastal Mediterranean environments to high alpine environments is still lacking (but see Kolář *et al.* 2016). One such ecologically divergent species is *Dianthus sylvestris* Wulfen, distributed across the Alpine, Apennine and Dinaric mountains, from Mediterranean coast to alpine peaks (Tutin and Walters 1993; Bacchetta *et al.* 2010; Gammella 2016; Brullo and Guarino 2019). It thus represents an excellent system to study the influence of habitat heterogeneity and climatic oscillations on population genetic variation, in order to increase the general knowledge about evolutionary history of ecologically divergent plant groups in response to climatic changes. *Dianthus sylvestris* is a still inconsistently treated taxonomic entity, currently in focus of several research groups who are investigating both its taxonomy and ecology (Bacchetta *et al.* 2010; Gammella 2016; Domina, Astuti, Barone, *et al.* 2021; Domina, Astuti, Bacchetta, *et al.* 2021; Terlević, Temunović, *et al.* 2022; Terlević, Bogdanović, *et al.* 2022; Luqman *et al.* 2022). Our morphometric study (Terlević, Temunović, *et al.* 2022) is focused on populations from the Balkan Peninsula and considered *D. sylvestris* as a species with six currently described subspecies (*D. sylvestris* subsp. *alboroseus* F. K. Mey, *D. sylvestris* subsp. *bertisceus* Rech. f., *D. sylvestris* subsp. *kozjakensis* Micevski, *D. sylvestris* subsp. *nodosus* (Tausch) Hayek, *D. sylvestris* subsp. *sylvestris* and *D. sylvestris* subsp. *tergestinus* (Rchb.) Hayek). Our results revealed only two morphologically distinct entities of Balkan populations with the split between the two groups corresponding to the split between allopatric north-western (NW) Balkan populations and south-eastern (SE) Balkan populations approximately along the Neretva River valley (Terlević, Temunović, *et al.* 2022), a major biogeographical border within the Balkans (Španiel and Rešetnik 2022). In addition, Luqman *et al.* (2022) recently showed that post-glacial range expansion of this species is related to spatial sorting of adaptive alleles where warm-associated genotypes increased their frequency in the emerging low-elevation habitats after the LGM and ancestral cold-associated genotypes remained at high-elevation habitats to which they are pre-adapted.

Due to the combination of both low- and high-elevation populations which are usually characterised by opposite migration dynamics, the demographic history of *D. sylvestris* s.l. is likely complex. We thus hypothesize that more thermophilous populations survived the glacial periods in several microrefugial locations with favourable conditions along the western Balkans, while the interglacials facilitated their expansion from multiple microrefugia and subsequent admixture. On the other hand, cold-adapted populations of *D. sylvestris* s.l. may have experienced decreased climatic suitability during interglacials and may be undergoing allopatric speciation from the end of the last glaciation onward due to their retreat to higher elevations in the Balkan Mountains. Knowledge of genetic diversification and population structure of *D. sylvestris* distributed across the Balkan Peninsula is a prerequisite for disentangling its evolutionary history and optimizing taxonomy. Hence, in this study we used restriction-associated DNA sequencing (RAD-seq) to 1) resolve (phylogenetic) relationships among *D. sylvestris* populations 2) explore the genetic variation of the Balkan *D. sylvestris* populations, and 3) assess putative migration patterns on the Balkan Peninsula in relation to Pleistocene climate fluctuations which may have shaped the contemporary patterns of genetic variation. Finally, we combined the genomic data with species distribution models (SDMs) generated for the present and the past (Last Glacial Maximum; LGM) period to identify putative glacial refugia and infer locations of potential ancestral Balkan populations.

Materials and methods

Study area and population sampling

We collected a total of 165 populations (992 individuals) of *D. sylvestris* s.l. across its distribution range for our genomic analyses: 132 populations from the Balkan Peninsula, and 33 populations across the European Alps and Apennine Peninsula. Voucher data are presented in Table S1, and the geographic origin of sampled populations is shown in Fig. S1 and Table S1. Leaf material of up to 10 individuals per population was sampled and immediately stored in silica gel for further DNA analyses.

DNA extraction and ddRAD-seq library production

Total cellular DNA was extracted from the dried leaf tissue of each individual plant using NucleoSpin® 8 Plant II kit (Macherey-Nagel) according to the manufacturer's instructions. Silica-dried leaf material was cut into small pieces, frozen, and ground at 30 Hz for 30 s in a mixer mill (MM301; Retsch GmbH & Co.) using three ceramic beads. The protocol according to (Westergaard *et al.* 2019) was used to prepare the ddRAD-seq library. The sequencing of the library was carried out by Novogene UK.

Genotyping

Raw data were demultiplexed using the internal barcode, using stacks v.2.41 (Catchen *et al.* 2013) with the default settings. Raw reads were mapped to the reference genome of *Dianthus carthusianorum* L. (Fior *et al.* unpublished) using the default settings of BWA mem v0.7.17 (Li and Durbin 2010) and reads with low mapping quality (Q20) were removed. We generated two datasets to first assign the Balkan populations to evolutionary lineages of *Dianthus sylvestris* (*sensu* Luqman *et al.* 2022) and subsequently to perform in-depth analyses on their genetic structure.

For the first dataset, hereafter "all-lineage dataset", we produced a SNP panel using 2 randomly chosen individuals for each of the 165 populations. We preformed SNP calling using freebayes v1.3.1 (Garrison and Marth 2012); parameters: F 0.1, min-repeat-entropy 1, use-best-n-alleles 4, E -1) and variants were subsequently filtered following the ddocent pipeline (Puritz *et al.* 2014) using vcflib v.1.0.1 (Garrison *et al.* 2021) and vcftools v0.1.16 (Danecek *et al.* 2011). Briefly, we retained SNPs with minor allele count of 3, a mapping quality of 20, mean depth of 10, missingness across individuals of 0.9 and minor allele frequency of 0.01. Complex SNPs containing indels were removed and we retained 1 SNPs per 1000 bases to account for potential linkage disequilibrium. The final SNP panel included 7,021 high-quality SNPs that were then used to genotype all individuals using the same settings of freebayes. The new VCF was filtered applying the same settings used to produce the SNP panel, except missingness across individuals of 0.95 and minor allele frequency of 0.05. This resulted in a final VCF including 853 SNPs for 322 individuals. For the second dataset, hereafter "Balkan-cline dataset", we produced a SNP panel using 2 randomly chosen individuals for each of the 108 populations assigned to the Balkan lineage in the all-lineage analyses. A panel of 65 843 SNP obtained using the same procedure described above was used to genotype all individuals, which resulted in a final VCF including 6240 SNPs for 587 individuals after filtering.

Delimitation of evolutionary lineages of Dianthus sylvestris

The assignment of the Balkan populations to evolutionary lineages of *Dianthus sylvestris* (following Luqman *et al.* 2022) was based on the "all-lineage dataset", which included 992 individuals from 165 populations and a set of 5644 SNPs. To detect genetically differentiated groups of populations we first applied the model-based Bayesian clustering method implemented in STRUCTURE 2.3.4. (Pritchard *et al.* 2000) available as a -st flag in structure-threader program (Pina-Martins *et al.* 2017), a software build to parallelize and automate the runs of STRUCTURE, fastSTRUCTURE, MavericK and ALStructure softwares. Structure-threader was ran on computer cluster Isabella using 28 cores (University Computing Centre, University of Zagreb), with the following parameters: admixture model and correlated allele frequencies, no location prior, 10000 MCMC (Monte Carlo Markov chain) iterations used for burn-in followed by 90000 MCMC iterations, K ranging from 1 to 20 with 10 runs for each K. Optimal number of genetic clusters (K) was identified based on the Evanno *et al.* (2005) method via STRUCTURE HARVESTER (Earl and vonHoldt 2012). STRUCTURE results were summarised using CLUMPAK server (Kopelman *et al.* 2015) and for the most likely K values visualised in ArcGIS 10.2.2. To investigate phylogenetic relationships among all individuals of the three lineages we carried out Maximum Likelihood (ML) analyses using IQ-TREE 2 (v. 2.2.0.3. pre-release, Minh *et al.* 2020). Alignment of SNPs in phylip format was generated using vcf2phylip.py script (available at: https://github.com/edgardomortiz/vcf2phylip; Ortiz 2019). Optimal model search was done with ModelFinder (Kalyaanamoorthy *et al.* 2017) with Felsenstein's ascertainment bias correction factor (Lewis 2001) using -m MFP+ASC. Afterwards, best-fit model according to AIC (Akaike Information Criterion) was chosen for phylogenetic tree reconstruction with alignment (.varsites.phy) depleted of invariant sites removed through IQTREE automatically. Tree search was done under a General Time Reversible model with uniform rate heterogeneity using -m GTR+F+ASC with 1000 ultra-fast bootstrapping replicates (Hoang *et al.* 2018) and random seed number generating 100 starting Maximum Parsimony trees. Finally, the optimum phylogenetic tree (.treefile) was visualised with FigTree v. 1.4.4. (Rambaut 2009). This approach was applied to both datasets described above. In addition, we calculated Nei’s genetic distance (Nei 1972) between all individuals using ‘StAMPP’ package v. 1.6.1. (Pembleton *et al.* 2013) and constructed a Neighbour-joining (NJ) tree which was visualised in FigTree v1.4.4 (Rambaut 2009).

Population genetic structure of the Balkan lineage

All population genetic structure analyses of the Balkan lineage were based on the “Balkan-cline dataset” which included 587 individuals from 108 populations and a set of 6240 SNPs. For exploratory purposes, we first run principal component analysis (PCA) on the SNP data in Adegenet R package 2.1.3 (Jombart *et al.* 2010). Population genetic structure was further assessed with two complementary clustering approaches. First, we applied a commonly used Bayesian approach implemented in STRUCTURE version 2.3.4, which relies on the assumptions of the population genetics models. We ran STRUCTURE within the *structure-threader* program, with the same analysis parameters as described above, except for the K values which ranged from 1 to 15. Second, we inferred the genetic clustering of the populations *de novo* using the discriminant analysis of principal components (DAPC) implemented in the R's Adegenet. Unlike STRUCTURE, DAPC is a model-free approach without any prior assumptions about underling population genetic processes and as such is recommended to be used in combination with model-based approaches (Dufresne *et al.* 2014). We first optimised the number of PCs to be retained based on the cross-validation method (built-in function *xvalDapc*) and then estimated the optimal number of genetic clusters based on K-means algorithm and the lowest associated Bayesian information criterion (BIC) value. DAPC was then built by maintaining the first 100 PCs and two discriminant functions.

Population genetic diversity and differentiation

We estimated genome-wide population genetic diversity parameters for each diploid population (n = 100) and for the main genetic groups inferred by STRUCTURE (excluding admixed populations with < 70% assignment to a genetic group, see results), including the mean allelic richness across all loci (Ar), observed (Ho) and expected (He) heterozygosity, calculated using 'hierfstat' package v. 0.5-10 (Goudet 2005). As polyploids would violate some of the basic population genetic assumptions, eight tetraploid populations (identified in Terlević, Bogdanović, *et al.* 2022) were excluded from all the population genetics analyses. We estimated the genetic differentiation among all populations (overall F_{ST}) and between all population pairs (pairwise F_{ST}) based on Weir and Cockerham (1984) using the *genet.dist* function in hierfstat. The resulting pairwise F_{ST} matrix was visualised using heatmaps. To test for genetic differentiation among main genetic groups inferred from STRUCTURE (see results), we carried out hierarchical analysis of molecular variance (AMOVA) in Arlequin 3.5.2.2. (Excoffier and Lischer 2010) and tested the significance with 10000 permutations. We also explored the spatial genetic structure of the Balkan populations by testing for isolation by distance (IBD; Rousset 1997) based on pairwise geographic distances (in km) and linearised pairwise F_{ST} values (F_{ST} /[1 − F_{ST}]). IBD was tested with a Mantel test using 9999 permutations in vegan (Oksanen *et al.* 2020) and the resulting plot of genetic vs. geographic distances was visualised using the package MASS.

Genetic connectivity and migration patterns

To identify putative genetic connectivity patterns among populations, diploid populations of the Balkan-cline dataset (n = 100) were considered to construct a Population Graph based on their genetic covariances (Dyer and Nason 2004) using ‘popgraph’ v. 1.5 and gstudio R packages (Dyer 2017). This population network reflects genetic connections (edges) among all sampled populations (nodes) arising from both gene flow and shared ancestry, and has been shown to be successful in capturing underlying demographic processes (Dyer *et al.* 2010). When population graph topology is plotted in geographic space and assessed in relation to IBD model, edges can be partitioned into extended (indicating long-distance gene-flow), compressed (indicating barriers to gene flow and/or restricted connectivity) and normal (in agreement with IBD expectations; Dyer *et al.* 2010). Furthermore, to assess directional relative migration rates (ranging between 0 and 1) and to detect putative asymmetries in gene flow between the genetic groups identified with DAPC, we used DivMigrate function (Sundqvist *et al.* 2016) implemented in the R package ‘diveRsity’ (Keenan *et al.* 2013). The analyses were run based on Jost’s D metric of genetic differentiation, using 1000 bootstraps, and alpha = 0.05. In addition, to identify putative number of migration events (m) and directionality of gene flow among DAPC groups, we used Treemix (Pickrell and Pritchard 2012) which was run with m ranging from 1–3, 10 iterations per m, varying ‘-k’ (number of SNPs per window) across each run – random k between 100 and 1000 in 50 SNP increments. TreeMix was ran through a pipeline based on scripts written by Vajana and Milanesi (2017) and R functions by Zecca, Labra and Grassi (2019) available on GitHub: https://github.com/carolindahms/TreeMix. Optimal m was selected using both Evanno and linear method implemented in the ‘optM’ package (Fitak 2021), and the resulting ML tree was plotted using R functions provided in the Treemix.

Species distribution modelling

To predicted potential habitat suitability for *D. sylvestris* during present-day and the Last Glacial Maximum (LGM, ~21 kya) time periods in our study area we used species distribution models (SDMs). We initially obtained 306 occurrences of *D. sylvestris* from various sources including our extensive field sampling. To reduce the sampling bias, occurrences closer than 1 km were omitted using the “thin” function in the “spThin” package 0.2.0 (Aiello-Lammens *et al.* 2015). After thinning, a total of 238 occurrences of *D. sylvestris* were retained for developing SDMs (Fig. 4A, Table S1). We retrieved climate data from the Chelsa database (Karger *et al.* 2017, 2020) and an initial set of 19 bioclimatic variables was first evaluated for collinearity by calculating variance inflation factor (VIF) with the "usdm" R package (Naimi *et al.* 2014). Variables with VIF > 10 were discarded, thus the number of predictor variables was reduced, retaining those considered to be ecologically relevant for *D. sylvestris* (Table 1). After the variable selection procedure, we built SDMs with a total of six climatic predictors obtained for the current and LGM period at a spatial resolution of ∼1 km2 (Table 1). Corresponding variables for the LGM period were obtained from the Chelsa climate PMIP3 dataset for four different global circulation models (GCMs): NCAR-CCSM4, MRI-CGCM3, MPI-ESM-P, and MIROC-ESM.

Table 1. Six selected climatic variables used as predictors for *Dianthus sylvestris* SDMs and predicting the potential present and past (last glacial maximum – LGM) habitat suitability. Shown is also mean variable importance to the ensemble model.

Variable abbreviation	Full name	Unit	Mean variable importance
bio4	temperature seasonality	°C/10	0.33
bio5	mean daily maximum air temperture of the warmest month	°C/10	0.32
bio8	mean daily mean air temperatures of the wettest quarter	°C/10	0.17
bio9	mean daily mean air temperatures of the driest quarter	°C/10	0.21
bio18	mean monthyl precipitation amount of the warmest quarter	kg m-2	0.15
bio19	mean monthly precipitation amount of the coldest quarter	kg m^{-2}	0.27

We built habitat suitability models using an ensemble modelling approach implemented in the 'biomod2' package (Thuiller *et al.* 2019) with five modelling algorithms (Maximum Entropy (Maxent), Generalised Linear Model (GLM), Generalised Additive Model (GAM), Boosted Regression Trees (GBM), and Random Forest (RF)). To assess model performance, we used the approach of data partitioning with 70% of the occurrences used for model training and the remaining 30% of occurrences used for model evaluation. In addition to species occurrence data, we also generated 10 000 random "background" points within the study area for model evaluation. For each algorithm, we ran ten replicates using cross-validation and assessed the model performance using AUC parameter (area under the receiver operating characteristic curve). Models with AUC value higher than the calculated median AUC score across all models (AUC > 0.934) were included in the final ensemble model. We then projected the resulting ensemble model onto present and LGM climate conditions, to obtain potential current and past habitat suitability maps for *D. sylvestris*. The four different GCM projections for the LGM were averaged to obtain a single “ensemble” habitat suitability map for the LGM period. We also converted continuous habitat suitability projections into binary (presence/absence) using the ensemble model cut-off value (0.286) based on the maximum sensitivity and specificity threshold.

Results

Population genetic structure and phylogenetic relationships among all populations

The analysed “all-lineage dataset”, encompassed 165 sampled populations (a total of 992 individuals) across the Balkans, Apennines and Alps (Fig. 1, Fig. S1). Model-based STRUCTURE and the associated Evanno method revealed an optimal genetic clustering of populations at K=2 (Fig. S4A) which corresponded to the split between the Alpine populations in one group, and the Apennine and Balkan populations which clustered together in the second group (Fig. 1A). At K=3 a further split was suggested within the Balkan populations separating the north-western (NW) and the south-eastern (SE) group of populations (Fig. 1B). At K=6 separation of the three lineages of *D. sylvestris,* as well as separation of *D. sylvestris* subsp. *tergestinus* became apparent, whereas additional substructure was detected within the SE Balkan group (Fig. 1C). Genetic clustering at K=6 was largely congruent with the constructed unrooted NJ tree based on Nei’s genetic distances which nicely/strongly supported the separation of the Apennine, Alpine and Balkan lineage according to Luqman *et al.* (2022), but also the separation of *D. sylvestris* subsp. *tergestinus* from the rest of the Balkan populations which were further split into two main groups with some more heterogeneity and divergence in the SE group (Fig. 1D, Fig. S2).

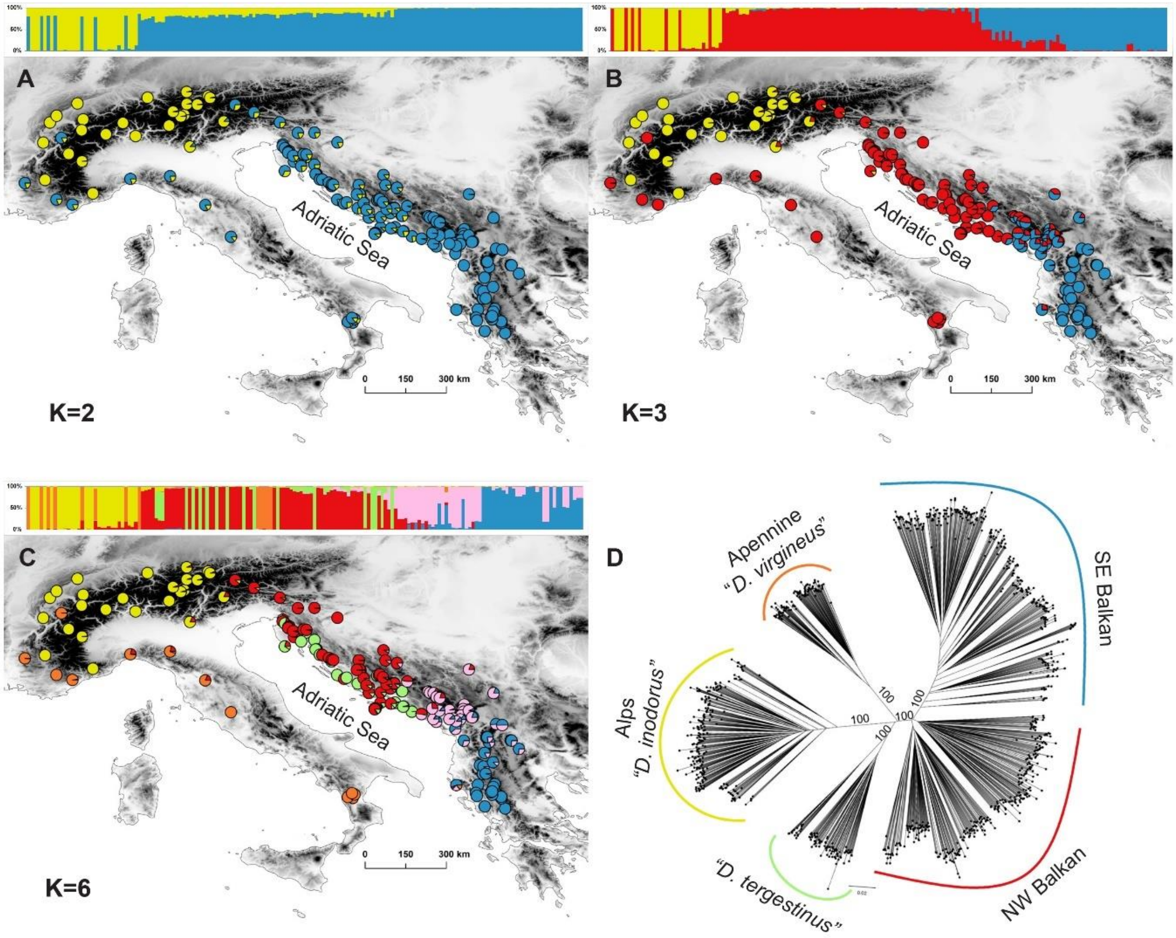

Figure 1. Population genetic structure of *Dianthus sylvestris* based on the "all-lineage dataset" (165 populations, 992 individuals and 5644 SNPs) as inferred from STRUCTURE at A) K=2, B) K=3 and C) K=6. Pie charts show the assignment probability to each of the clusters across populations and the barplots show individual assignments (individuals are ordered by longitude). D) Unrooted Neighbour-joining (NJ) tree based on Nei's genetic distance between all individuals. Bootstrap support as inferred for the Maximum Likelihood tree is shown above the branches (for details see Fig. S2).

Genetic structure of the Balkan-cline

PCA on the SNP data showed that genetic variation pattern forms a cline with some clear discontinuities visible along the first three PC axes (Fig. 2A). STRUCTURE analysis repeated on the Balkan-cline dataset revealed again two major clusters as the optimal number of ancestral groups (Fig. S4B) corresponding to the NW and SE populations, with admixed populations occurring in the area of Montenegro (Fig. 2B). This result was well supported by the ML phylogenetic tree based on the same dataset (100% BS, Fig. 2D, Fig. S3). On the other hand, DAPC results suggested that genetic variation of Balkan populations can be optimally summarised in five clusters (K=5 had the lowest BIC value, Fig. S5), two very close groups in the NW and three more divergent in the SE (Fig. 2C).

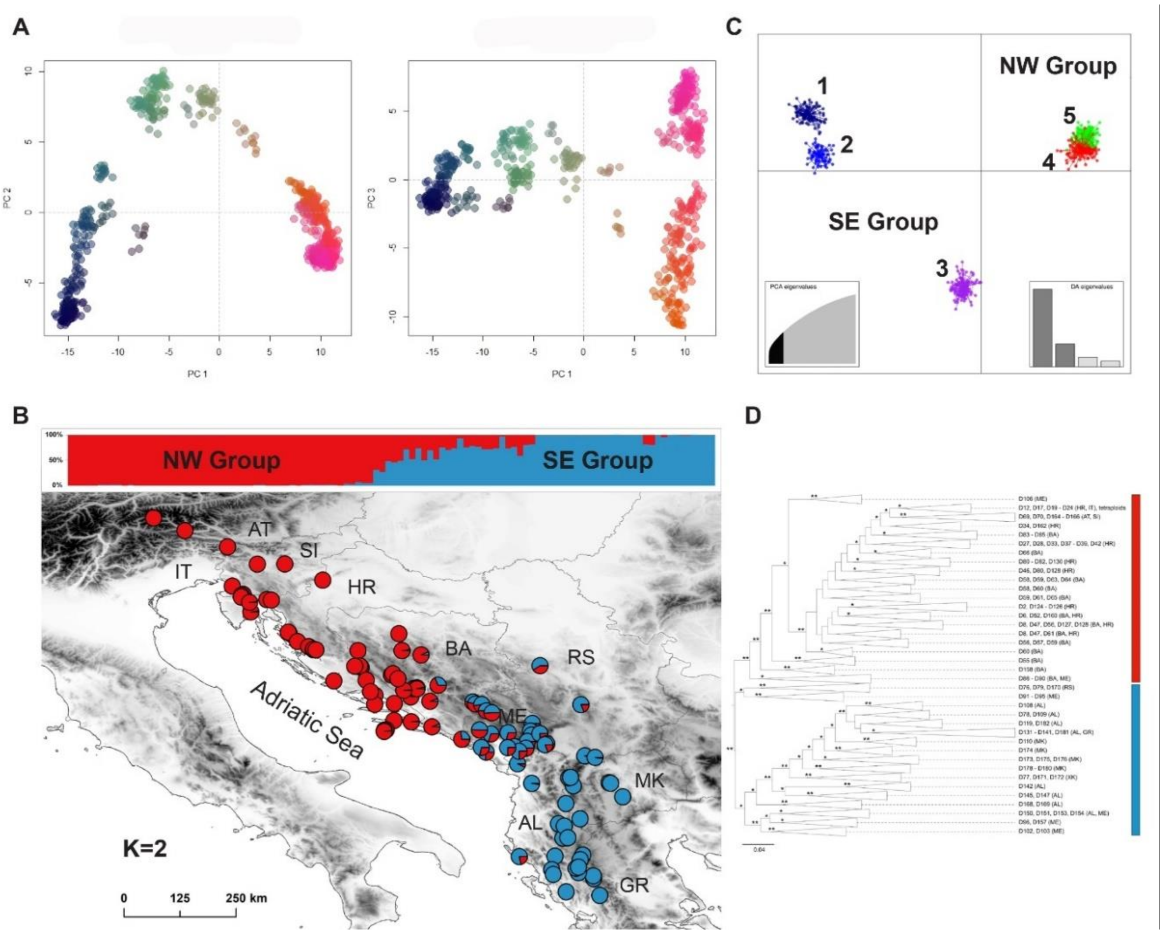

Figure 2. Population genetic structure of *Dianthus sylvestris* based on the "Balkan-cline dataset" (108 populations, 587 Individuals and 6240 SNPs). A) PCA scatterplot of individual genotypes on the RGB scale, where the genetic variation is represented in two complementary ways: by the distances (further away = more genetically different), and by the colours (more different colours = more genetically different). Five groups on the gradient can be seen, representing discontinuities in the cline. B) Optimal population genetic structure and individual assignments/ancestry at K=2 inferred by STRUCTURE showing two major genetic groups of *Dianthus sylvestris*: north-western (NW) group and south-eastern (SE) group. Individuals are ordered by longitude. C) Scatterplot of the five genetic clusters identified by DAPC. Bottom boxes indicate the number of retained principal components (100) and retained discriminant analysis eigenvalues. D) Phylogenetic Maximum Likelihood tree obtained via IQ-TREE showing strong support for the two main genetic groups identified with STRUCTURE (** indicates 100%, and * indicates 90–99% bootstrap support, for details see Fig. S3).

Population genetic diversity and differentiation of the Balkan-cline

Allelic richness per population (Ar) ranged from 1.186 to 1.277 (mean Ar = 1.218). Highest heterozygosity was observed in population D23 from Croatia (Ho = 0.359), and the lowest in population D141 from Albania (Ho = 0.189, Table S2). He was also highest in population D23 from Croatia (He = 0.266), followed by several populations from Bosnia and Herzegovina (D61, D64 and D59). Lowest He was observed in population D39 from Croatia (He = 0.168), followed by population D142 from Albania (Fig. 3A, Table S2). The observed heterozygosity (Ho) was significantly lower than expected heterozygosity (He, Fig. S6). Estimated genetic diversity parameters (Ar, Ho and He) showed very similar spatial pattern indicating a cline where southern-most populations had in general lower/reduced genetic diversity (Fig. 3A). This pattern was most pronounced for Ar which most significantly declined with latitude ($R^2 = 0.134$, p < 0.001, Table S3).

Genetic diversity values (Ar, Ho and He) were similar between the two genetic groups identified with STRUCTURE and had almost identical He values (He NW = 0.245; He SE = 0.243, Table 2). Overall, Balkan populations showed significant genetic differentiation based on global $F_{\mathrm{ST}} = 0.166$, $p < 0.001$) while pairwise F_{ST} values ranged from 0.005 (between populations D63 and D64) to 0.355 (between populations D39 and D132) (Fig. S7, Table S2). The hierarchical AMOVA results revealed significant genetic differentiation between the two main genetic groups/clusters identified with STRUCTURE (F_{CT} between groups = 0.13, $p < 0.0001$) as well as among populations where 13% of genetic variation was found to be among groups, 9.15% among populations within groups and 77.79% within populations (Table 3).

Table 2. Genetic diversity of the two main Balkan *Dianthus sylvestris* groups inferred from STRUCTURE.

Group	Ar	Ho	He
K1 SE	1.870	0.217	0.243
K2 NW	1.894	0.228	0.245

Table 3. Hierarchical Analysis of Molecular Variance (AMOVA) for the Balkan *Dianthus sylvestris* populations based on two genetic groups inferred from STRUCTURE.

Source of variation	Sum of squares	Variance components	% Variation	*F* statistics
Among groups	52591.41	101.330	13.07	$F_{CT} = 0.131$***
Among populations within two groups	124325.06	70.955	9.15	$F_{SC} = 0.105$***
Within populations	553827.42	603.298	77.79	$F_{ST} = 0.222$***

Significance after 10000 permutations ***$p < 0.0001$

Genetic connectivity and migration patterns

The isolation by distance pattern was significant and revealed that genetic differences increase with geographic distance (R^2=0.6, p=0.001, Fig. 3B). The Population graph analysis identified two major disconnected population networks and several smaller disconnected subnetworks (Fig. 3C). Higher connectivity was apparent among the NW group of populations where several populations were connected with extended edges (Fig. 3C) connecting populations that are genetically more similar than expected based on their geographic distance, thus suggesting long-distance gene flow. The southernmost group of populations from Greece, Albania and Macedonia formed a separated population network where the majority of populations were connected with normal edges and only one compressed edge was suggested indicating barriers to gene flow (Fig. 3C). The main difference in comparison to STRUCTURE results was in the area of Montenegro where admixed populations were identified with STRUCTURE at K=2, while popgraph analysis revealed several small subnetworks consisting of 2 up to 7 populations that were entirely disconnected from the two main population networks (Fig. 3C).

Relative migration rates were estimated between five genetic clusters inferred from DAPC (Fig. 3E-F). The highest levels of relative symmetric gene flow (> 0.9) were detected between the two NW Balkan DAPC groups (group 4 and 5, Fig. 3E-F). Migration rates were half lower between the two southern-most Balkan DAPC groups (groups 1 and 2), followed by migration rates between DAPC group 2 and 3. Relatively lower migration rates ($m < 0.33$) were found between the northern and southern groups. Only one significant asymmetric migration was revealed (1000 bootstraps, α=0.05), indicating gene flow from the southern-most DAPC group 1 (Greece and Albania) up to the area of Montenegro (DAPC group 3), but not further north (Fig. 3E-F). Treemix model uncovered relatively similar migration pattern compared to

DivMigrate analysis. Model with only one migration event (m = 1) fitted our data best according to the OptM function (accounting for > 99.99% of the variance in the data) and indicated single migration step from DAPC group 2 (Albania, North Macedonia) toward group 3 (Fig. 3E-F).

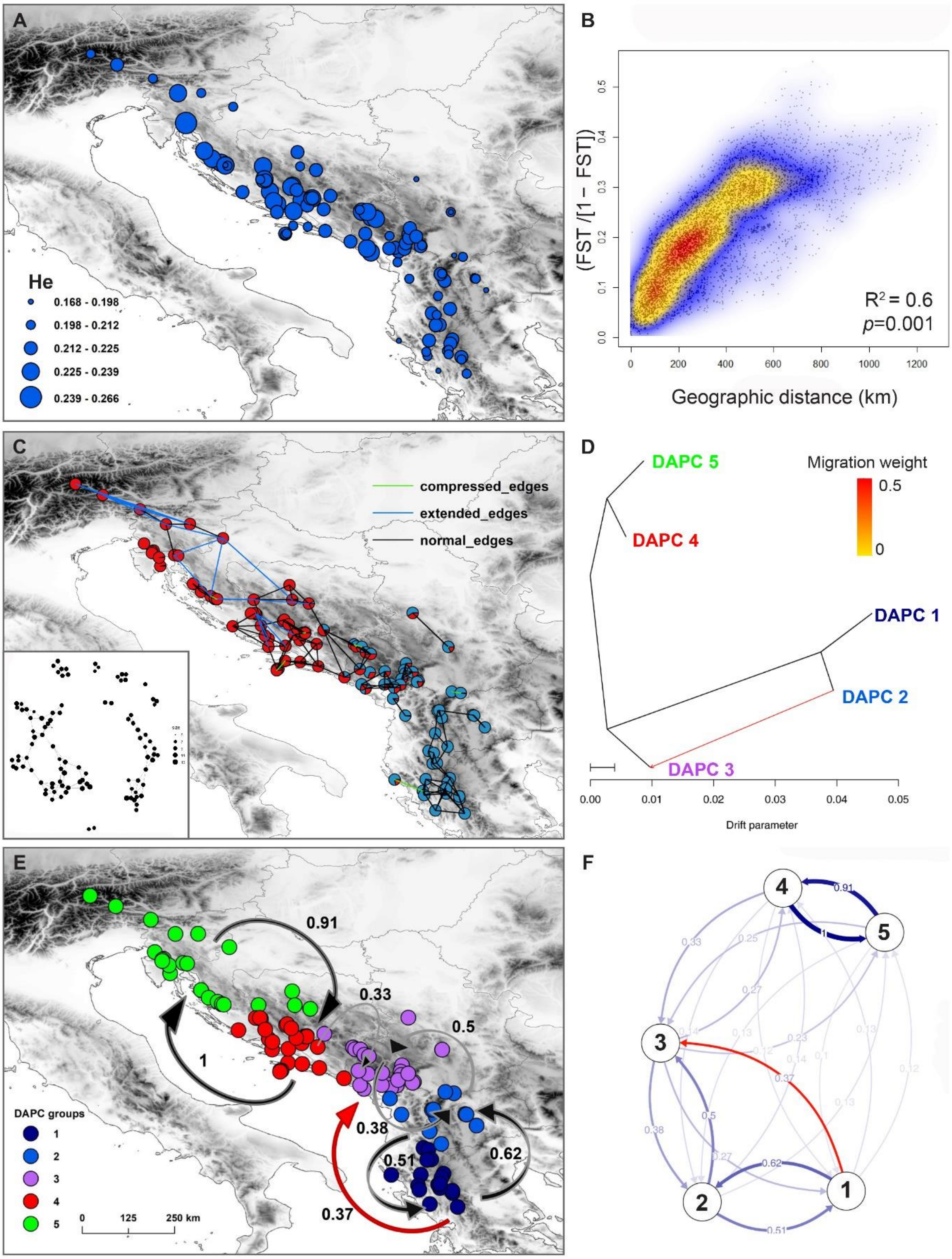

Figure 3. Genetic diversity, connectivity and migration patterns among *Dianthus sylvestris* populations of the Balkan-cline. A) Geographical pattern of genetic diversity depicted by Expected heterozygosity (He). B) Significant pattern of Isolation by distance (IBD) visualised with a density function. C) Population graph showing the genetic connectivity among populations based on their genetic covariances. Graph topology is mapped in geographic space and assessed in relation to the IBD model. Partitioning of the edges based on IBD: extended edges in blue indicate long-distance gene flow, compressed edges in green suggest geographical or ecological barriers to gene flow, normal edges are concordat with IBD expectations. Populations on the map are coloured according to K=2 inferred from STRUCTURE. In the inset, popgraph is visualised using the Fruchterman-Reingold algorithm where node size reflects within-population genetic variation. A-C panels are based on 100 diploid populations. D) Maximum Likelihood tree based on five genetic clusters identified with DAPC obtained with TreeMix showing one significant migration event from group DAPC 2 to DAPC 3 indicated with an arrow and coloured according to the migration weight. E-F) Directional relative migration between five genetic clusters identified with DAPC estimated by DivMigrate. Gene flow magnitude is indicated by arrow thickness. In E, DAPC clusters are plotted on the map and denoted by the same colours as in Figure 2C. Only relative migration rates above 0.33 are shown on the map indicating the highest gene flow among NW populations and as twice as lower among SW groups of populations. In F, all relative migrations rates based on Jost’s D metric are shown in the network. Only one significant asymmetric migration was detected from DAPC group 1 towards group 3 (admixed populations at K=2 inferred from STRUCTURE) indicated by the red arrow.

Species distribution modelling

We used SDMs to predict suitable areas for *D. sylvestris* in our study area during present and particularly during LGM period as they could point to locations of putative glacial refugia for this species. Our ensemble prediction of current habitat suitability corresponded well to the known distribution of *D. sylvestris* in the Balkans (Fig. 4A). LGM projections indicated that habitat suitability in the study area was lower than today (up to 0.58), however potential distribution of *D. sylvestris* was relatively similar to predicted current one or may have been even wider (Fig. 4B). Climatically suitable areas for species survival were predicted along the whole eastern Adriatic coast, with the highest LGM habitat suitability predicted in the south of the Balkan peninsula (ME and AL; Fig. 4C). After converting the continuous habitat suitability map for the LGM period into suitability classes, suitable areas (those above the threshold of 0.286) were clearly divided into class of higher suitability (corresponding roughly to the SE Adriatic) and a class of lower suitability (corresponding roughly to the NW Adriatic) (Fig. 4D-E). When we overlapped this resulting map of LGM habitat suitability with the major genetic clustering inferred from Structure at K=2, the two clusters highly corresponded with the two areas of habitat suitability (Fig. 4E), suggesting that the observed genetic structure could be related to species survival in two different glacial refugia.

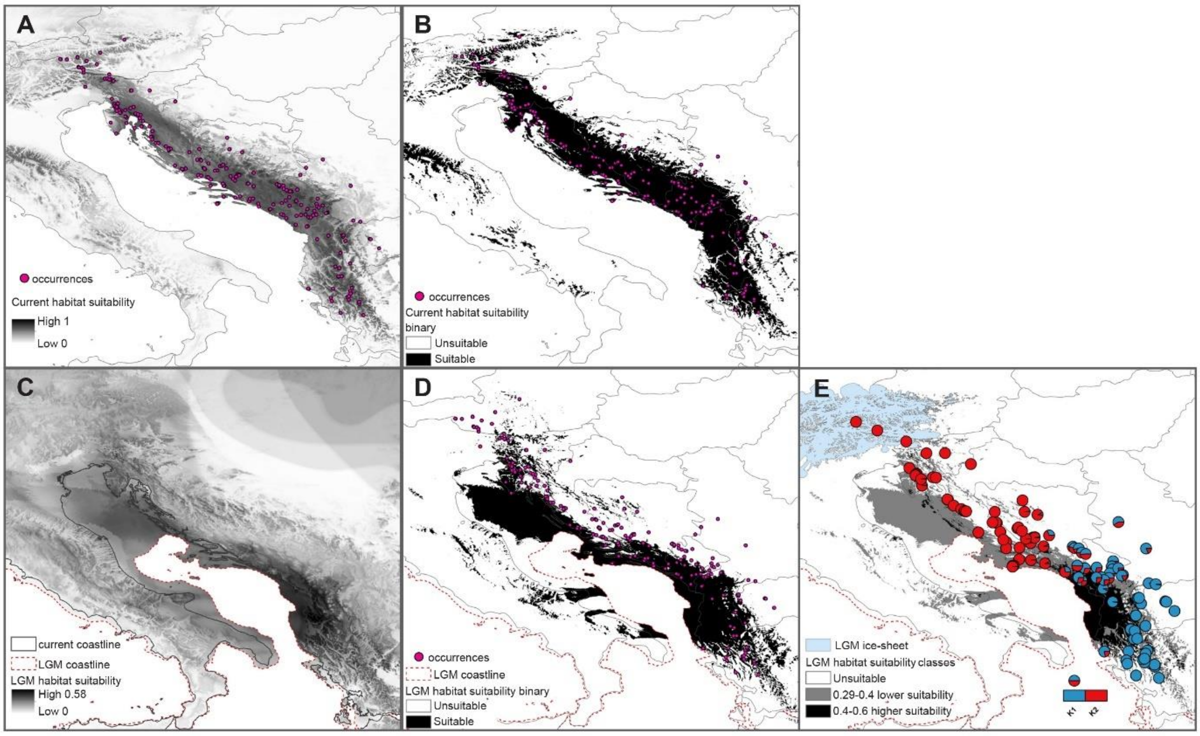

Figure 4. Predicted ensemble habitat suitability maps for *Dianthus sylvestris* s.l. based on 238 occurrences across the Balkan Peninsula for the current (A-B) and the Last Glacial Maximum (LGM) period (C-E). In A and C, continuous habitat suitability maps are shown with darker areas indicating higher suitability. Suitability values range from 0 to 1 for current period and from 0 to maximal suitability value (0.58) for LGM. In B and D, binary habitat suitability maps indicate only suitable or unsuitable areas based on the maximum sensitivity and specificity threshold. In E, habitat suitability classes are overlapped with the Structure output at K=2 based on the Balkan-cline dataset. Extent of the ice-sheet during LGM is also shown on the map.

Discussion

Our phylogenetic analyses based on ML and NJ trees combined with STRUCTURE results strongly supported the presence of three main geographically structured evolutionary lineages within *D. sylvestris,* as recently revealed by Luqman *et al.* (2022). However, our more comprehensive sampling of the Balkan Peninsula revealed the divergence of *D. sylvestris* subsp. *tergestinus* from the rest of the Balkan populations. Furthermore, in-depth population genetic analyses revealed a more complex pattern of genomic variation within the Balkan lineage with hierarchical phylogeographic structure. Population genetic structure of the Balkan lineage suggested that two main genetic groups occur within the Balkan Peninsula, where the NW group seems to be more homogeneous than SE group, which was shown to be more divergent and substructured. Geographical patterns of genetic diversity showed that in general southernmost populations have reduced genetic diversity compared to the northern ones; however, the two main genetic groups seem to harbour similar levels of genetic diversity. Genetic connectivity and migration analyses indicated extensive gene flow among the populations in the NW group, and comparatively much lower gene flow among populations in the SE group, probably promoting their increased divergence. Estimates of directional migration indicated that gene flow mainly occurs from southernmost populations located in Greece, Albania and North Macedonia, approximately up to the area of Montenegro, but not further north, suggesting that SE populations were not the source for the recolonization of the NW. In addition, very limited gene flow was suggested between the populations from the NW and SE groups, indicating barriers to gene flow. Overall, our results suggest that these two groups of populations may represent two separately evolving evolutionary lineages within the Balkan lineage, particularly when combined with potential habitat suitability within the study area during LGM, providing valuable insights into putative refugial areas for *D. sylvestris* within the Balkan Peninsula. We discuss possible phylogeographic scenarios which may have driven the observed genomic variation patterns in *D. sylvestris.*

The three lineages and genetic divergence of Dianthus sylvestris subsp. tergestinus

Using dated phylogeny, the results of Valente *et al.* (2010) suggested that 2.0–1.3 Mya ago an acceleration in *Dianthus* diversification took place, and an association between the climatic aridity in the Pleistocene and the shift in diversification rate of *Dianthus* has been proposed. A trichotomy in the genomic data of the corresponding study area was already revealed by Luqman *et al.* (2022), where *D. sylvestris* is split into three evolutionary lineages originating from distinct glacial refugia: Alpine, Apennine and Balkan lineage. In addition, the former study identified Adriatic Sea as one of the major genetic boundaries between the lineages. However, our STRUCTURE results revealed that at K=3, the populations from the Alps and those from the SE Balkan Peninsula form distinct genetic lineages, whereas the Apennine and the NW Balkan populations share the same genetic pool. This is for example concordant with the phylogeographical pattern revealed in *Silene saxifraga* group (Đurović *et al.* 2017). However, the separation of the three lineages was very well supported in the phylogenetic trees (Fig. 1D, Fig. S2).

Amphi-Adriatic disjunctions are known from a number of studies and have been explained by land connections and disconnections during the Messinian Salinity Crisis (Krijgsman *et al.* 1999) or Quaternary climatic changes (Gridelli 1950). However, phylogeographic connections between Balkan and Apennine populations are well documented and in most cases restricted to central and southern parts of both peninsulas (Đurović *et al.* 2017; Frajman and Schönswetter 2017; Janković *et al.* 2019; Falch *et al.* 2019; Rešetnik *et al.* 2022). Research on *Edraianthus graminifolius* (Surina *et al.* 2014), for example, revealed that the most pronounced genetic discontinuity occurs within the western Balkan Peninsula, rather than across the Adriatic Sea separating the two peninsulas, indicating that, contrary to the expectations, the Adriatic Sea did not represent the main genetic barrier for this high mountain species. Despite of our limited sampling across the Apennine Peninsula, our results do not support the existence of the amphi-Adriatic connections, therefore indicating both the Adriatic Sea and the Balkan topology as barriers to gene flow.

The area between the south-eastern Alps and northern Dinaric mountains was found to be a contact zone between lineages of, e.g., *Knautia drymeia* Heuff. (Rešetnik *et al.* 2016) and the *Heliosperma pusillum* group (Frajman and Oxelman 2007). However, the contact zone between the Alpine and Balkan lineage of *D. sylvestris* is positioned further to the northwest in the Dolomitic Alps (Fig. 1A-B). Nevertheless, all contemporary populations belonging to the Alpine lineage likely evolved independently from the Balkan lineage from an alpine-like LGM refugium located *in-situ* in the Alps from where they expanded westwards (Luqman *et al.* 2022). Consequently, ancestral *D. sylvestris* populations had genotypes closer to those of contemporary populations in current alpine habitats. Our results however imply a different evolutionary history of the Balkan lineage compared to the Alpine one (see below).

Although the STRUCTURE clustering at K=6 was not optimal according to the Evanno statistics, STRUCTURE output together with the phylogenetic trees show a clear separation of *D. sylvestris* subsp. *tergestinus,* from the rest of the Balkan populations despite their co-occurrence in sympatry. This subspecies could be distinguished from the sympatric subsp. *sylvestris* and subsp. *nodosus* by the number of epicalyx scales, but all three subspecies form a morphological gradient in other floral and vegetative traits (Terlević, Temunović, *et al.* 2022). Thus, *D. sylvestris* subsp. *tergestinus* may be an example of a cryptic and evolutionary distinct species (Bickford *et al.* 2007), which deserves an upgraded taxonomic rank. However, its taxonomic designation is out of the scope of this paper.

The Balkan-cline

Majority of our results point to the main pattern of two distinct genetic groups of *D. sylvestris* in the Balkans (NW and SE), suggesting the existence of two evolutionary lineages within the Balkan-cline. This deep genetic split occurring in the vicinity of the Neretva river valley is reflected in the morphological variation of these populations, particularly in the variation of the epicalyx scales shape (Terlević, Temunović, *et al.* 2022). Moreover, the genetic split into two clusters was remarkably congruent with the pattern of habitat suitability during LGM inferred from our SDMs indicating larger area of lower suitability in the NW and smaller area of higher suitability in the SE Adriatic region (Fig. 4C-E). This pattern of habitat suitability could be put in relation with the finding of tetraploid *D. sylvestris* populations in the small area of Istria (Terlević, Bogdanović, *et al.* 2022), which gives additional support for the existence of a distinct refugium in the northern Adriatic region (Rešetnik *et al.* 2020). Finally, results obtained by Treemix and DivMigrate were largely concordant, suggesting almost no migration between the populations of the NW and SE groups, and directional migration was revealed only from the southern-most subgroups (DAPC 1 and 2) up to the area of Montenegro (DAPC group 3), where the migration likely stopped (Fig. 3E). Taken together, along with the similar levels of genetic diversity of the two groups, these results go in favour of the scenario of two distinct glacial refugia (northern and southern) for *D. sylvestris* in the Balkans. The persistence in two different refugia hosting populations with similar levels of genetic diversity was also suggested for e.g., *Viola suavis s.l.* (Mereďa *et al.* 2011) and *Arundo plinii* (Hardion *et al.* 2014).

Similar genetic breaks which coincide with the Neretva valley have been repeatedly observed in the Balkan peninsula, even for taxa of contrasting ecologies (Kučera *et al.* 2010; Đurović *et al.* 2021; Reich *et al.* 2022; Španiel and Rešetnik 2022) and are usually explained with the two main hypothesis. The hypothesis of two ecologically divergent glacial refugia with different climatic conditions along the eastern Adriatic coast during Pleistocene was proposed by Lakušić *et al.* (2013) and Kutnjak *et al.* (2014) where climatic and/or environmental differences between NW and SE Adriatic may have driven the genetic divergence of populations. A contrasting hypothesis by Đurović *et al.* (2021) implies that the Neretva river valley acted as a physical barrier to gene flow in *Cerastium* species, rather than ecological one because suitable climatic conditions for species survival were predicted on both sides of the valley during the LGM. Two distinct genetic groups of *D. sylvestris* within the Balkan lineage and very similar levels of genetic diversity observed for each group indeed indicate the presence of two main glacial refugia, one in the NW and the other in the SE of the east Adriatic coast. Although our predicted habitat suitability pattern for *D. sylvestris* gives more support for the two climatically different refugia during LGM, for the moment, and based on our results, we cannot discard any of the two in our opinion non-exclusive hypotheses described above. It could simply be that the abrupt climatic change from NW to SE of the Adriatic during LGM geographically coincides with the Neretva river valley as proposed by Lakušić *et al.* (2013), and/or with sea level fluctuations during LGM (Sikora *et al.* 2013), whereas the valley itself and the associated lowland habitats unfavourable for some taxa further reinforces the barrier to gene flow. We may assume that populations contracted and expanded to these refugial areas not only during LGM, but over multiple glacial cycles during Pleistocene, as population divergence may have been promoted by isolation on a time scale exceeding a single glaciation (Hewitt 1996). During expansions, probably related to interglacials and the Holocene for the thermophilous low-to-mid-elevation populations, the two lineages may have come into contact in the intermediate area of Montenegro where this event resulted in admixed populations observed here. Similar pattern of admixture in the intermediate area of Montenegro was also proposed for *Gentianella crispata* (Reich *et al.* 2022).

Overall, potential distribution and suitable areas of *D. sylvestris* during LGM were predicted to be very similar to current ones, only with decreased suitability and displaced to lower altitudes. This is possible due to only partially glaciated Balkan mountain tops during LGM, as opposed to Alps where Alpine lineage experienced severe range contraction during LGM and subsequent expansion during the post-glacial period (Milivojević *et al.* 2008; Žebre *et al.* 2019; Luqman *et al.* 2022). Such observed pattern in the Balkan lineage may be particularly true for the cold-adapted, high-elevation populations of *D. sylvestris* which may have followed a different migration pattern compared to their low-elevation counterparts. In such scenario, high-elevation populations would persist during Pleistocene climate oscillations more or less *in situ* shifting their distributions only along the mountain altitudinal clines to track favourable climate (Surina *et al.* 2011; Smyčka *et al.* 2022). This scenario is partly supported with our population graph results showing two main largely disconnected population networks corresponding to the NW and SE group of populations inferred from STRUCTURE, apparently giving additional weight to two distinct refugia within the Balkans. However, unlike other analyses, population graph uncovered several small population groups in the borderline of the Scardo-Pindhic and the Dinaric mountain ranges (2 to 7 populations) that were entirely disconnected from the two main population networks. This suggests, as opposed to admixture of lineages from two distinct refugia, an alternative scenario where these populations were stuck in this intermediate area within several *in situ* microrefugia during the glaciation cycles (not necessarily only since LGM) until today, and that they have diverged independently from the two main Balkan lineages.

In addition, Luqman *et al.* (2022) showed for the Alpine lineage that all contemporary *D. sylvestris* populations, both high-elevation (alpine) and low-elevation (in valleys) evolved from a common alpine-like ancestral adaptive genotype present in the LGM refugia. Moreover, low-elevation populations of the Alpine lineage were characterised by lower diversity, compared to high-elevation populations (Luqman *et al.* 2022). Thus, the genetic diversity of *D. sylvestris* in heterogeneous landscapes seems to be determined by the environmental distance between a population's current habitat and its ancestral alpine-like habitat. If we assume that this is the case for all *Dianthus* lineages (which diverged ca. 200-115 Kya), this suggests that at least part of the Balkan populations which are alpine-like (roughly those above 2000 m altitude corresponding to the Scardo-Pindhic mountain range and Albanian Alps in the SE of the Balkan peninsula) may even had a wider distribution during the glacials than today, and have contracted to the highest mountain areas during unfavourable interglacials where they have remained until today. Consequently, habitat fragmentation during warmer stages (and currently ongoing since the LGM), accompanied by the observed reduced gene flow, may have promoted increased divergence of SE subgroups of populations and decreased genetic diversity in the southernmost areas. Such a case of higher levels of genetic diversity were observed in the northern populations with a decrease toward the southern populations was found in *Haberlea rhodopensis* (Petrova *et al.* 2015), and it was explained as a consequence of severe distribution contractions causing genetic bottlenecks during LGM. The southernmost populations also show many features of rear edge populations, according to Hampe and Petit (2005), and as such, their reduced within-population genetic diversity and increased divergence among populations may be a result of their successful long-term *in situ* persistence in the region that provided suitable conditions for their persistence under both glacials and interglacials. Accordingly, the southernmost Balkan mountains (Albania, Greece) may today, i.e. in the interglacial, represent cryptic southern refugia and/or environmental margin for the high-elevation populations of *D. sylvestris* (Stewart *et al.* 2010).

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Supplementary Figures

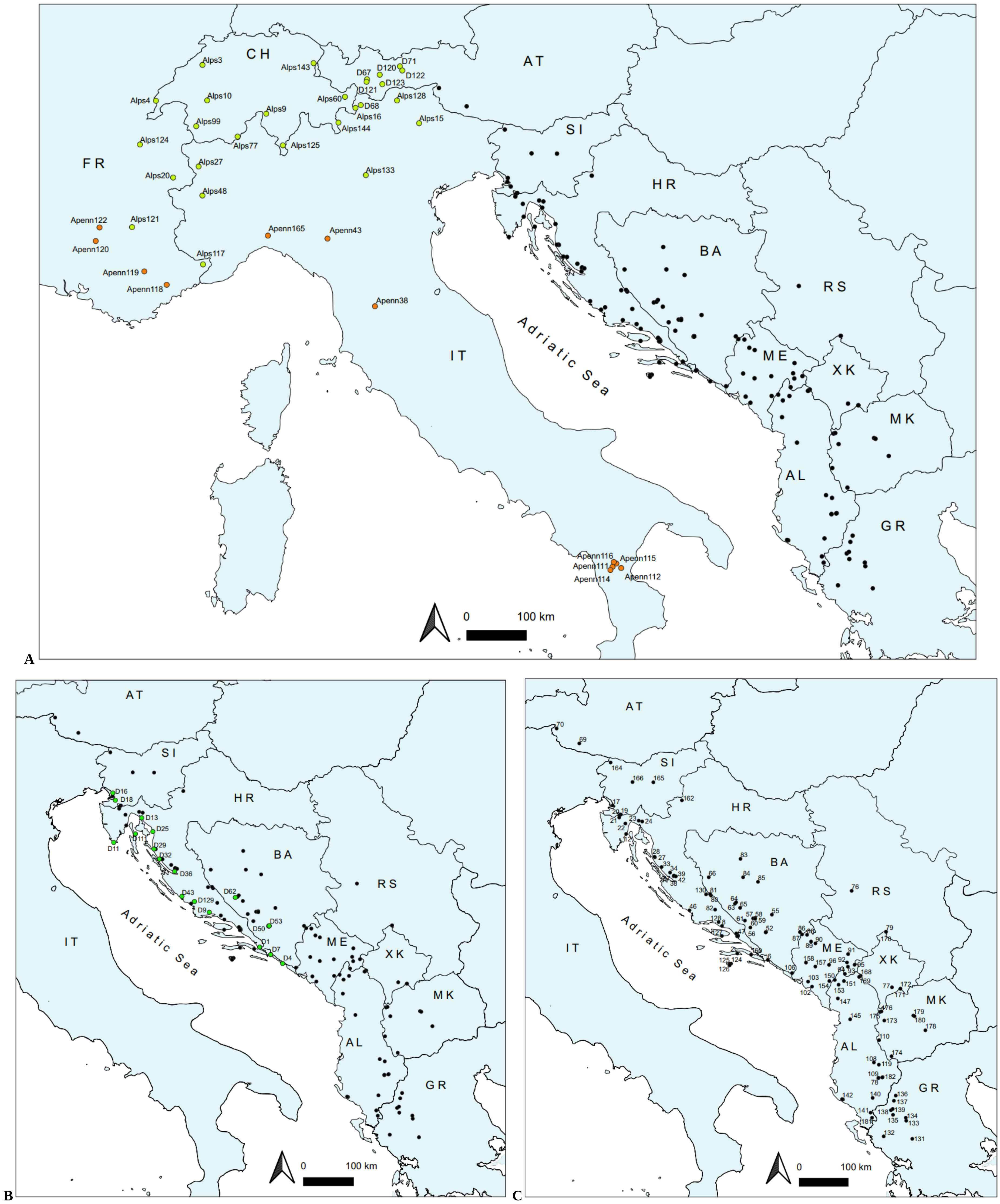

Figure S1. Geographical distribution of 165 sampled populations of *Dianthus sylvestris* s.l. included in the “all-lineage dataset”. Labels corresponding to the A) 33 sequenced populations across the European Alps and Apennines. B) 18 sequenced population of *D. sylvestris* subsp. *tergestinus.* C) 114 sequenced populations of the Balkan cline.

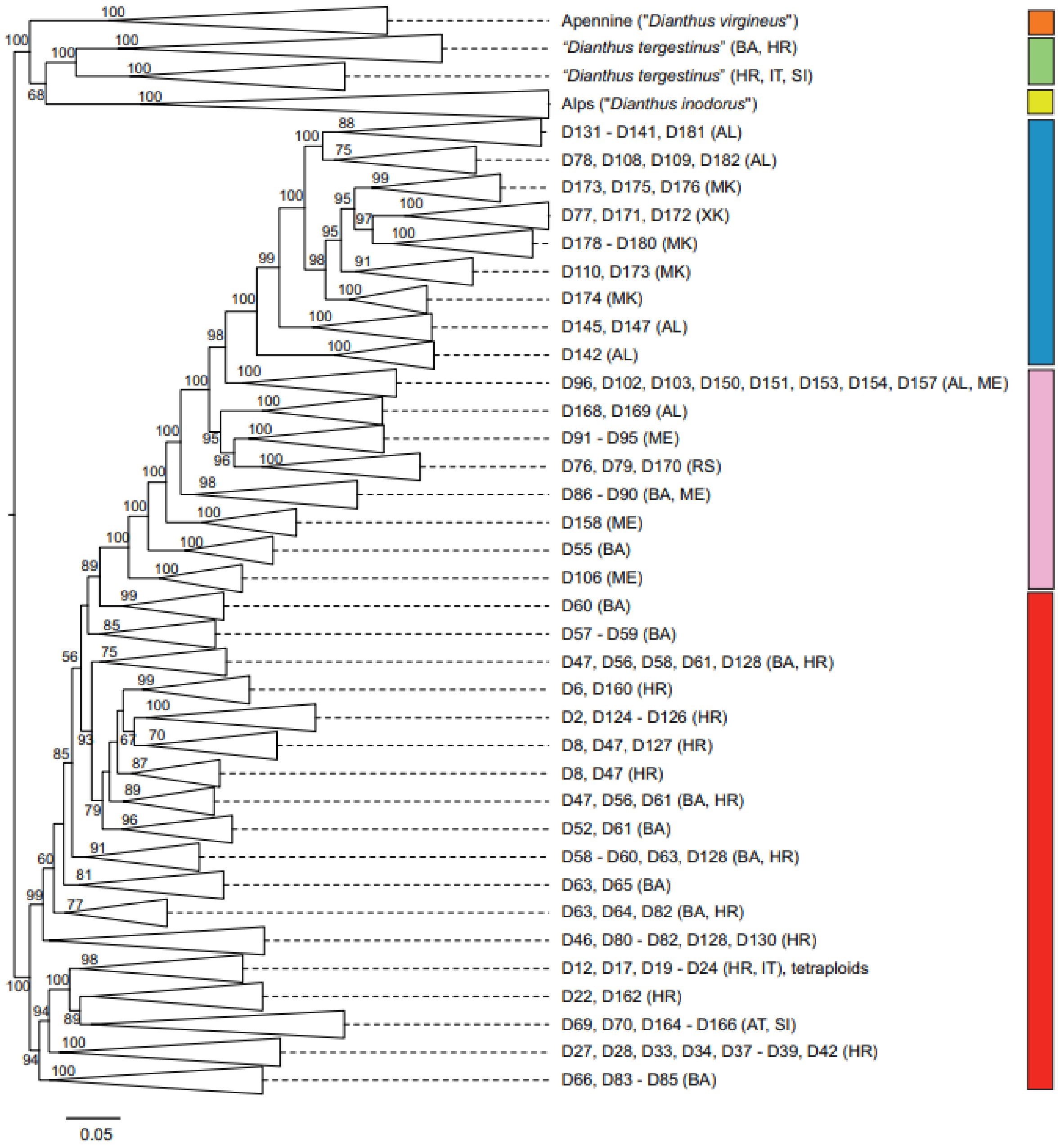

Figure S2. Phylogenetic Maximum Likelihood tree obtained via IQ-TREE based on the "all-lineage dataset" (165 populations, 991 individuals and 5644 SNPs) showing strong support for the three lineages of *Dianthus sylvestris* ("*Dianthus virgineus*" – Apennine lineage, and "*Dianthus inodorus*" – Alpine lineage), as well as for the separation of *D. sylvestris* subsp. *tergestinus* ("*Dianthus tergestinus*") from the rest of the Balkan populations. Colours correspond to genetic groups at K=6 (Fig. 1C). Bootstrap support as inferred for the Maximum Likelihood tree is shown above the branches.

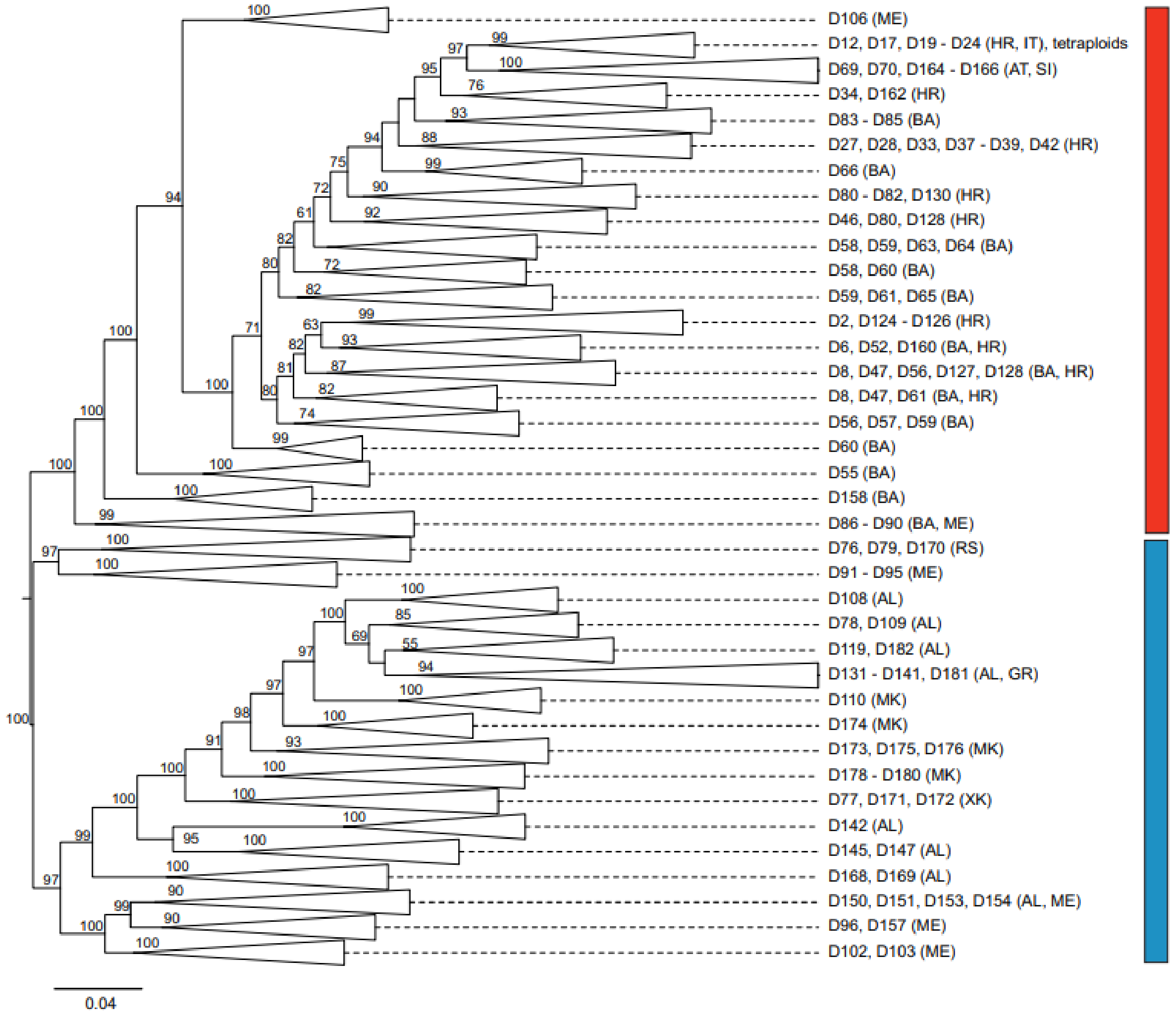

Figure S3. Phylogenetic Maximum Likelihood tree obtained via IQ-TREE based on the "Balkan-cline dataset" (108 populations, 587 Individuals and 6240 SNPs). Colours correspond to genetic groups at K=2 (Fig. 2B). Bootstrap support as inferred for the Maximum Likelihood tree is shown above the branches.

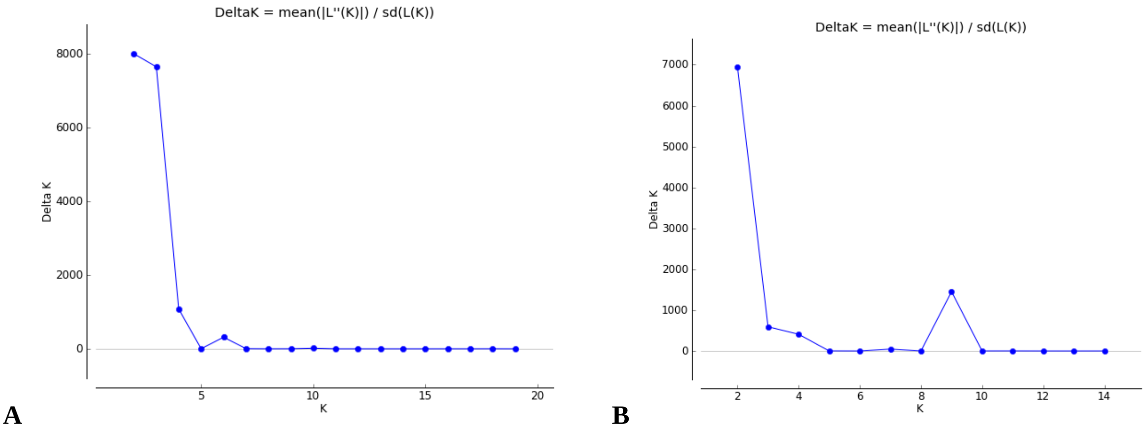

Figure S4. STRUCTUIRE Delta K plot according to Evanno *et al.* (2005) method for A) "all-lineage dataset" and B) "Balkan-cline dataset".

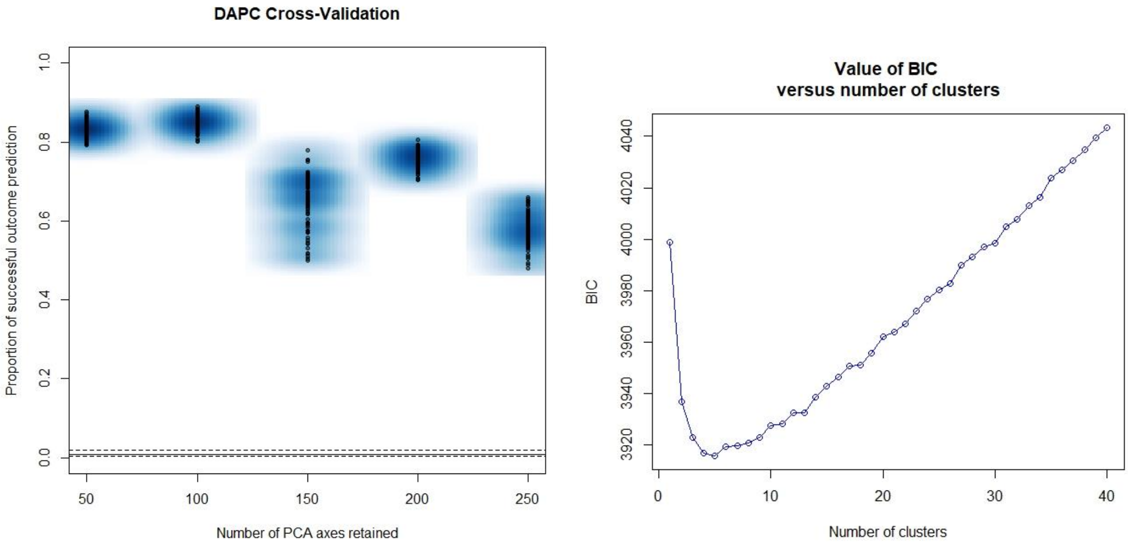

Figure S5. DAPC – BIC plot and Cross validation results.

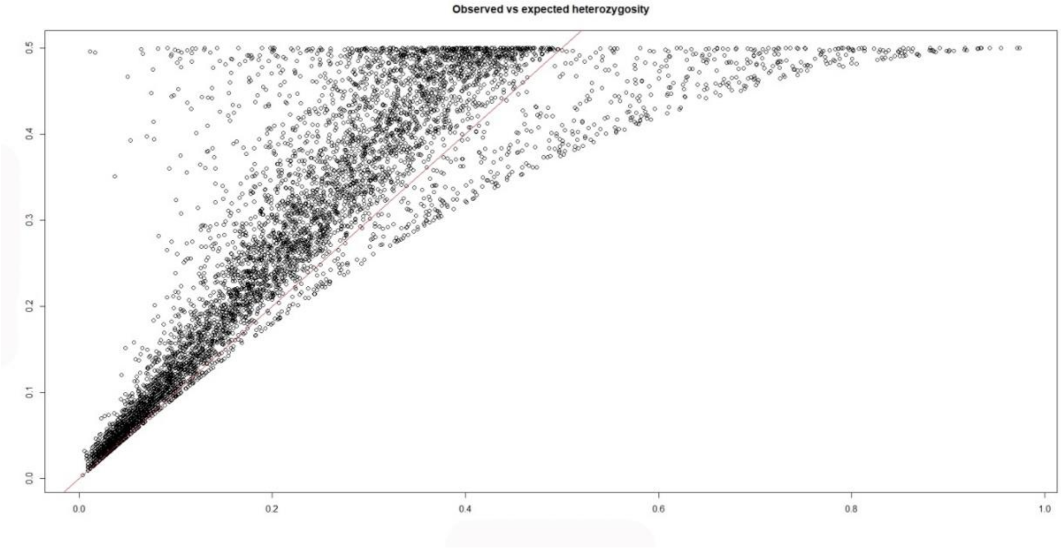

Figure S6. Plot of the mean observed heterozygosity (Ho), which was significantly lower than mean expected heterozygosity (He).

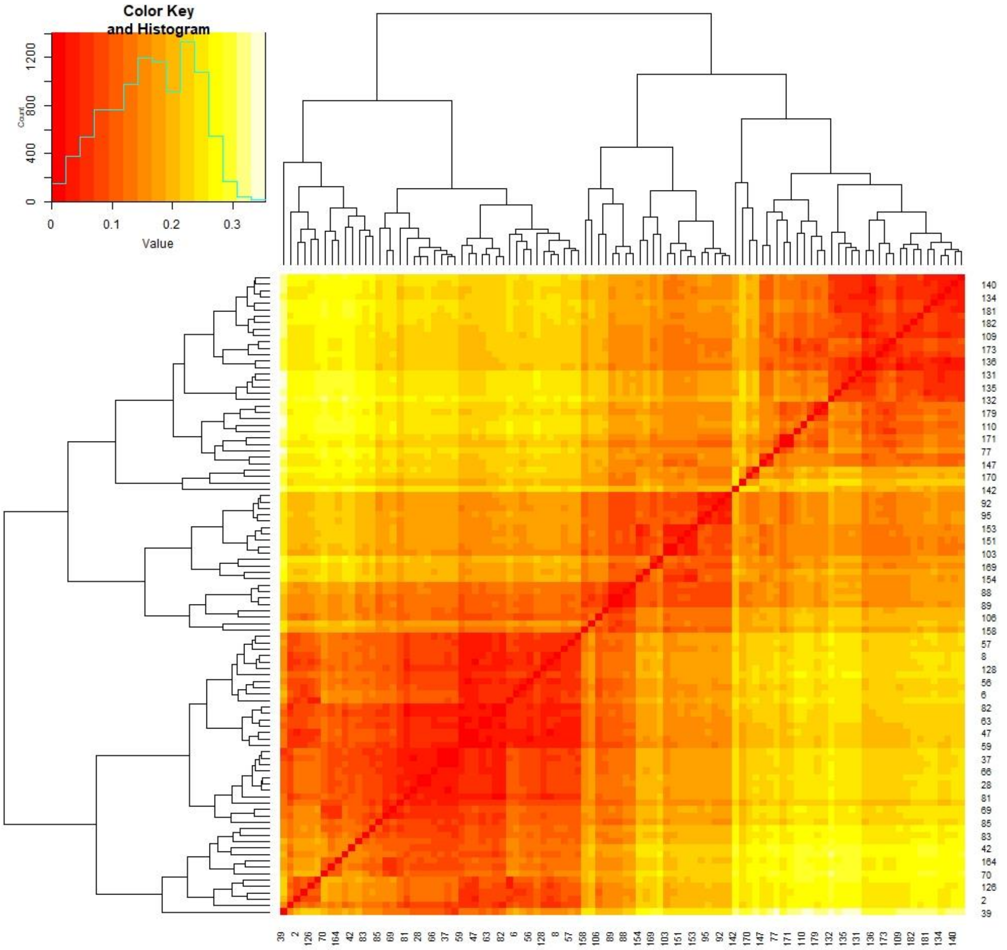

Figure S7. Heatmap of pairwise genetic differentiation (F_{ST} values) between Balkan populations of *D. sylvestris* indicating higher genetic differentiation between populations from the NW and SE groups.

Supplementary Tables

Table S1. Studied populations of the *Dianthus sylvestris* s.l., including the ID label, the number of individuals collected per population (silica No ind.), species (sp) and subspecies (ssp) names, country ISO codes, herbarium voucher ID (herbarium acronyms are according to Index Herbariorum, Thiers 2021). Collectors' names initials: AT (A. Terlević), BF (B. Frajman), DD (D. Dagnino), DG (D. Gargano), DL (D. Lakušić), DS (D. Sincek), DSh (D. Shuka), GT (G. Tomović), HL (H. Luqman), IJ (I. Janković), Ilj (I. Ljubičić), IR (I. Rešetnik), KR (K. Rohweder), LSh (L. Shuka), MD (M. Doboš), MF (M. Falch), MG (M. Grgurev), MGa (M. Gammella), MT (M. Temunović), MZ (M. Zbiljić), NK (N. Kuzmanović), PS (P. Schönswetter), SB (S. Bogdanović), SBu (S. Burg), SC (S. Cambria), SĐ (S. Đurović), SF (S. Fior). Ploidy refers to flow citometry estimations by Terlević, Bogdanović *et al.* 2022.

No	ID	silica No ind.	sp	ssp	Country code	Locality	Habitat	Altitude (m a.s.l.)	Herbarium voucher	Collector	Collection date	Latitude	Longitude	Ploidy	morphometrics dataset	niche analysis dataset	All lineage dataset	Balkan cline dataset	treemix dataset	SDM dataset
1	D1	5	sylvestris	tergestinus	HR	Klek village, mountain road toward Smrdan Grad	rocky grassland	80	ZA-46276	IR, SB	09/05/2018	42.948736	17.564511	2x	+	+	+			+
2	D2	7	sylvestris	nodosus	HR	Korčula island, macadam in shrubland	clearings of the forest edge	250	ZA-46277	IR, SB	11/05/2018	42.940857	17.080551	2x	+	+	+	+	+	+
3	D4	5	sylvestris	tergestinus	HR	Dubrovnik, Srđ hill, summit plateau, Križ	rocky grassland	279	ZA-46279	IR, SB	12/05/2018	42.645206	18.120210	2x	+	+	+			+
4	D6	5	sylvestris	nodosus	HR	Mironja, near the quarry	rocky grassland along the road	332	ZA-46281	IR, SB	13/5/2018, 29/6/2019	42.815694	17.843489	2x	+	+	+	+		+
5	D7	5	sylvestris	tergestinus	HR	between Kuk and Deskovice, near the quarry	rocky grassland along the road	351	ZA-46282	IR, SB	13/05/2018	42.810839	17.830790	2x	+	+	+			+
6	D8	5	sylvestris	nodosus	HR	Omiška Dinara Mt., road near Cetina river	vertical rocks along the road	63	ZA-46283	IR, SB	13/5/2018, 29/6/2019	43.450246	16.698531	2x	+	+	+	+		+
7	D9	5	sylvestris	tergestinus	HR	Kozjak Mt., close to mountain lodge Malačka	rocky grassland	602	ZA-46284	IR, SB	13/05/2018	43.581474	16.325790	2x	+	+	+			+
8	D11	6	sylvestris	tergestinus	HR	Gornji Kamenjak, Gomila peak	rocky grassland	55	ZA-46415	SB, ILj	17/05/2018	44.810971	13.900875	2x	+	+	+			+
9	D12	6	sylvestris	sylvestris	HR	road Zagorje-Šimuni, near Plomin	rocks along the road	240	ZA-46416	SB, ILj	19/5/2018, 13/7/2019	45.123950	14.204677	4x	+	+	+	+		+
10	D13	5	sylvestris	tergestinus	HR	Križišće village, road Hreljin to Kraljevica	rocky grassland along the road	513	ZA-46417	SB, ILj	19/05/2018	45.267597	14.594413	2x	+	+	+			+
11	D16	5	sylvestris	tergestinus	IT	Sežana, border pass	rocky grassland	344	ZA-47416	IR, SB, ILj	13/06/2018	45.701530	13.837293	2x	+	+	+			+
12	D17	4	sylvestris	sylvestris	IT	Trieste, Monte Spacatto	vertical rocks along the road	300	ZA-47417	IR, SB, ILj	13/06/2018	45.642266	13.831103	4x	+	+	+	+		+
13	D18	5	sylvestris	tergestinus	SI	Petrinje village	rocky grassland	422	ZA-47418	IR, SB, ILj	13/06/2018	45.572838	13.906808	2x	+	+	+			+
14	D19	2	sylvestris	sylvestris	HR	Ćićarija, Vodice	rocky grassland	660	ZA-47419	IR, SB, ILj	13/06/2018	45.479168	14.049705		+	+	+	+		+
15	D19	8	sylvestris	sylvestris	HR	Ćićarija, Vodice	rocky grassland	660	ZA-47419	IR, SB, ILj	13/6/2018, 13/7/2019	45.479168	14.049705	4x	+	+	+	+		+
16	D20	5	sylvestris	sylvestris	HR	Ćićarija, Žbevnica, above Brest	rocky grassland along the mountain route	941	ZA-47420	IR, SB, ILj	13/6/2018, 19/7/2020	45.457206	14.016319	4x	+	+	+	+		+

17	D21	5	sylvestris	sylvestris	HR	Slum, close to village Slum	rocky grassland	553	ZA-47421	IR, SB, ILj	13/6/2018, 19/7/2020	45.423401	14.012134	4x	+	+	+	+		+
18	D22	5	sylvestris	sylvestris	HR	Učka Mt., close to Zrinščak, above Vela Draga	rocky grassland	525	ZA-47422	IR, SB, ILj	13/06/2018	45.316811	14.177338	4x	+	+	+	+		+
19	D23	5	sylvestris	sylvestris	HR	Grobnik, close to Kikovica	rocky grassland	326	ZA-47423	IR, SB, ILj	14/06/2018	45.374679	14.524703		+	+	+	+		+
20	D24	5	sylvestris	sylvestris	HR	Gornje Jelenje, Lazanje	vertical rocks along the road	862	ZA-47424	IR, SB, ILj	14/06/2018	45.361573	14.617238	4x	+	+	+	+		+
21	D25	5	sylvestris	tergestinus	HR	Bunica, close to Senj	littoral rocky coast	8	ZA-47425	IR, SB, ILj	14/06/2018	45.024841	14.884729	2x	+	+	+			+
22	D27	5	sylvestris	nodosus	HR	Plješevica, Gornja Branjevina	rocky grassland	1211	ZA-47427	IR, SB, ILj	15/06/2018	44.707936	14.965827	2x	+	+	+	+	+	+
23	D28	10	sylvestris	nodosus	HR	Butković plan, Balenske brižine	rocky grassland	1020	ZA-47428	IR, SB, ILj	15/06/2018	44.715302	14.956895	2x	+	+	+	+	+	+
24	D29	5	sylvestris	tergestinus	HR	Donji Bileni, above Jablanac	natural shrub	274	ZA-47429	IR, SB, ILj	15/06/2018	44.711960	14.922496	2x	+	+	+			+
25	D32	5	sylvestris	tergestinus	HR	Karlobag, Tatinja draga	vertical rocks along the macadam	12	ZA-47432	IR, SB, ILj	15/06/2018	44.533384	15.065192	2x	+	+	+			+
26	D33	5	sylvestris	nodosus	HR	Baške Oštarije, Ura viewpoint	vertical rocks along the road	905	ZA-47433	IR, SB, ILj	15/06/2018	44.529482	15.143159	2x	+	+	+	+	+	+
27	D34	5	sylvestris	sylvestris	HR	Visočica peak	grassland, along the mountain route	1458	ZA-47434	IR, SB, ILj	16/06/2018	44.429299	15.364303	2x	+	+	+	+		+
28	D36	5	sylvestris	tergestinus	HR	Paklenica, above Starigrad	vertical rocks along the road	397	ZA-47436	IR, SB, ILj	17/06/2018	44.313471	15.453283	2x	+	+	+			+
29	D37	7	sylvestris	sylvestris	HR	Paklenica, Buljma	rocky stones, scrub, edge of forest	1131	ZA-47437	IR, SB, ILj	17/06/2018	44.364749	15.460115	2x	+	+	+	+		+
30	D37	7	sylvestris	sylvestris	HR	Paklenica, Buljma	rocky stones, scrub, edge of forest	1213	ZA-47437	IR, SB, ILj	17/06/2018	44.366496	15.462085	2x	+	+	+	+		+
31	D37	7	sylvestris	sylvestris	HR	Paklenica, Buljma	rocky stones, scrub, edge of forest	1311	ZA-47437	IR, SB, ILj	17/06/2018	44.366662	15.462092	2x	+	+	+	+		+
32	D38	5	sylvestris	sylvestris	HR	Paklenica, Struge toward Buljma	grassland	1368	ZA-47438	IR, SB, ILj	17/06/2018	44.371093	15.465668	2x	+	+	+	+		+
33	D39	2	sylvestris	sylvestris	HR	Paklenica, Struge	rocky grassland	1388	ZA-47439	IR, SB, ILj	17/06/2018	44.375566	15.465719		+	+	+	+		+
34	D42	6	sylvestris	nodosus	HR	Paklenica, Brundo, below Šegestin peak	rocky grassland	1605	ZA-47442	IR, SB, ILj	17/06/2018	44.361124	15.513128	2x	+	+	+	+		+
35	D43	5	sylvestris	tergestinus	HR	Vransko jezero, Banjevački stanovi	maquis and garrigue near macadam	35	ZA-47453	IR, SB, ILj	18/06/2018	43.865234	15.642296	2x	+	+	+			+
36	D46	6	sylvestris	nodosus	HR	Šibenik, Martinska	maquis and garrigue near macadam	18	ZA-47456	IR, SB, ILj	18/06/2018	43.737357	15.871757	2x	+	+	+	+	+	+
37	D47	4	sylvestris	nodosus	HR	Biokovo Mt., above Podgora, Susvid	vertilcal rocks along the road	325	ZA-47457	IR, SB, ILj	18/06/2018	43.259545	17.078427	2x	+	+	+	+		+
38	D47	5	sylvestris	nodosus	HR	Biokovo Mt., road to sv. Jure Peak	rocky grassland	899	ZA-47459	IR, SB, ILj	18/06/2018	43.261003	17.101612	2x	+	+	+	+		+
39	D47	5	sylvestris	nodosus	HR	Biokovo Mt., road to sv. Jure Peak	rocky grassland	1018	ZA-47459	IR, SB, ILj	18/06/2018	43.273612	17.100209	2x	+	+	+	+		+
40	D47	5	sylvestris	nodosus	HR	Biokovo Mt., road to sv. Jure Peak	rocky grassland	1228	ZA-47459	IR, SB, ILj	18/06/2018	43.289186	17.085926	2x	+	+	+	+		+

41	D47	5	sylvestris	nodosus	HR	Biokovo Mt., road to sv. Jure Peak	rocky grassland	1318	ZA-47459	IR, SB, ILj	18/06/2018	43.312484	17.058714	2x	+	+	+	+	+
42	D47	2	sylvestris	nodosus	HR	Biokovo Mt., road to Sv. Jure peak	the edge of the pine forests	535	ZA-47459	IR, SB, ILj	18/06/2018	43.260692	17.090430		+	+	+	+	+
43	D47	2	sylvestris	nodosus	HR	Biokovo Mt., road to Sv. Jure peak	the edge of the pine forests	516	ZA-47459	IR, SB, ILj	18/06/2018	43.262247	17.086312		+	+	+	+	+
44	D50	5	sylvestris	tergestinus	BA	Mostar, road Gornji Vukodol to Donji Vukodol	vertical rocks along the road	250	ZA-47461	IR, SB, ILj	19/06/2018	43.325423	17.792920	2x	+	+	+		+
45	D52	7	sylvestris	nodosus	BA	Mostar, Hum hill	rocky grassland along the road	355	ZA-47463	IR, SB, ILj	19/06/2018	43.325478	17.802829	2x	+	+	+	+	+
46	D53	5	sylvestris	tergestinus	BA	Mostar, Hum hill	rocky grassland along the road	355	ZA-47464	IR, SB, ILj	19/06/2018	43.325875	17.802569	2x	+	+	+		+
47	D52	7	sylvestris	nodosus	BA	Mostar, Hum hill	rocky grassland along the road	369	ZA-47463	IR, SB, ILj	19/06/2018	43.328997	17.802182	2x	+	+	+	+	+
48	D53	5	sylvestris	tergestinus	BA	Mostar, Hum hill	rocky grassland along the road	372	ZA-47464	IR, SB, ILj	19/06/2018	43.328803	17.802206	2x	+	+	+		+
49	D52	7	sylvestris	nodosus	BA	Mostar, Hum hill	rocky grassland along the road	379	ZA-47463	IR, SB, ILj	19/06/2018	43.327339	17.804562	2x	+	+	+	+	+
50	D53	5	sylvestris	tergestinus	BA	Mostar, Hum hill	rocky grassland along the road	389	ZA-47464	IR, SB, ILj	19/06/2018	43.327195	17.804672	2x	+	+	+		+
51	D52	7	sylvestris	nodosus	BA	Mostar, Hum hill	rocky grassland along the road	423	ZA-47463	IR, SB, ILj	19/06/2018	43.328565	17.806905	2x	+	+	+	+	+
52	D53	5	sylvestris	tergestinus	BA	Mostar, Hum hill	rocky grassland along the road	423	ZA-47464	IR, SB, ILj	19/06/2018	43.328315	17.806842	2x	+	+	+		+
53	D55	7	sylvestris	nodosus	BA	Konjic	edge of forest near macadam	360	ZA-47466	IR, SB, ILj	19/06/2018	43.649976	17.967010	2x	+	+	+	+	+
54	D56	5	sylvestris	nodosus	BA	road Posušje to Široki Brijeg, close to village Vranić	vertical rocks along the road	549	ZA-47467	IR, SB, ILj	20/06/2018	43.417465	17.416434	2x	+	+	+	+	+
55	D57	5	sylvestris	nodosus	BA	Blidinje, Škrge, Grgoš	rocky grassland near macadam	1298	ZA-47468	IR, SB, ILj	20/06/2018	43.586683	17.487889	2x	+	+	+	+	+
56	D58	5	sylvestris	sylvestris	BA	Čvrsnica Mt., Široka kosa	rocky grassland near macadam	1928	ZA-47469	IR, SB, ILj	20/06/2018	43.595028	17.540758	2x	+	+	+	+	+
57	D59	6	sylvestris	sylvestris	BA	Čvrsnica Mt., Široka kosa	rocky grassland near macadam	1732	ZA-47470	IR, SB, ILj	20/06/2018	43.586521	17.533781	2x	+	+	+	+	+
58	D60	5	sylvestris	nodosus	BA	Čvrsnica Mt., Čavkarice	rocky grassland near macadam	1556	ZA-47471	IR, SB, ILj	20/06/2018	43.578959	17.540717	2x	+	+	+	+	+
59	D61	5	sylvestris	nodosus	BA	road Posušje to Mesihovina, Trobukva	vertical rocks along the road	904	ZA-47472	IR, SB, ILj	20/06/2018	43.546950	17.279515	2x	+	+	+	+	+
60	D62	5	sylvestris	tergestinus	BA	Livno, above villages Veliki Kabalići and Suhača	edge of forest near macadam	821	ZA-47473	IR, SB, ILj	21/06/2018	43.847624	16.973298	2x	+	+	+		+
61	D63	5	sylvestris	nodosus	BA	Krug plateau, below Cincar Mt.	rocky grassland near macadam	1197	ZA-47474	IR, SB, ILj	21/06/2018	43.859632	17.030663	2x	+	+	+	+	+
62	D64	6	sylvestris	nodosus	BA	Cincar Mt., Begovača	rocky grassland near macadam	1397	ZA-47475	IR, SB, ILj	21/06/2018	43.885435	17.059812	2x	+	+	+	+	+
63	D65	5	sylvestris	nodosus	BA	Between Tomislavgrad and Livno, close to village Galečić	rocky grassland	1096	ZA-47476	IR, SB, ILj	21/06/2018	43.782860	17.160352	2x	+	+	+	+	+
64	D66	6	sylvestris	nodosus	BA	Drvar, slopes of Dejanska glav., Raduklija creek canyon	vertical rocks along the road	716	ZA-47477	IR, SB, ILj	22/06/2018	44.348655	16.359556	2x	+	+	+	+	+
65	D67	5	sylvestris		AT	Prutz, Ladis	dry meadow and Pinus forest	904	ZA-47478	IR	27/06/2018	47.075072	10.653611	2x	+		+		+

66	D68	5	sylvestris		IT	Mals, Tartsch village, Tartscher Bichl hill	dry meadow	1039	ZA-47479	IR	27/06/2018	46.679892	10.558889	2x	+		+			+
67	D69	10	sylvestris		AT	Gailtal Alps, Lienzer Dolomiten, toward Hochstadl peak	rocky grassland	1971	ZA-47480	IR	29/06/2018	46.759219	12.880278	2x	+		+	+	+	+
68	D70	5	sylvestris		AT	Central Eastern Alps, Hohe Tauern, Clarahütte	high mountain meadow	2030	ZA-47481	IR	30/06/2018	47.013058	12.251111	2x	+		+	+	+	+
69	D71	6	sylvestris		AT	Innsbruck, Nordkette Mt., Seegrube	rocky grassland	1906	ZA-47482	IR	08/07/2018	47.304944	11.358611	2x	+		+			+
70	D76	5	sylvestris		RS	Maljen, Tometino polje	serpentinite	640	ZA-48631	SĐ, GT	15/07/2018	44.044240	20.016450	2x		+	+	+		+
71	D77	5	sylvestris		RS	Šar Planina Mts., Ostrvica	alpine rocky grasslands, Dapno-Festucetea, serpentinite	1755	ZA-48632	DL, NK, IJ, MZ	14/07/2018	42.233495	20.915371	2x		+	+	+	+	+
72	D78	5	sylvestris	alboroseus	AL	Maja e Ostrovicës Mts., above the village Marjan	dry rocky grounds, Festuco-Brometea	1386	ZA-48633	DL, NK, IJ, MZ	11/07/2018	40.574101	20.475512	2x		+	+	+	+	+
73	D79	5	sylvestris		RS	NP Kopaonik, SE of Pančićev vrh, Nebeske stolice	ultramafiti	1660	ZA-48634	SĐ, GT	13/07/2018	43.261489	20.839731	2x		+	+	+	+	+
74	D80	8	sylvestris	nodosus	HR	Dinara Mt., above Glavaš village, Pekasova draga	rocky grassland along the mountain route	740	ZA-48607	SB, ILj	30/06/2018	44.014272	16.417037	2x	+	+	+	+		+
75	D81	4	sylvestris	sylvestris	HR	Dinara Mt., Ošljak	rocky grassland along the mountain route	1418	ZA-48606	SB, ILj	30/06/2018	44.038666	16.393428	2x	+	+	+	+	+	+
76	D82	5	sylvestris	nodosus	HR	Svilaja Mt., road Zelovo to Gornje Ogorje	rocky grassland near macadam	934	ZA-48608	SB, ILj	01/07/2018	43.751194	16.523219	2x	+	+	+	+		+
77	D83	5	sylvestris	sylvestris	BA	Banja Luka, Vrbas river canyon, Tijesno	vertical rocks along the road	207	ZA-48609	SB, ILj	10/07/2018	44.685469	17.179697	2x	+	+	+	+	+	+
78	D84	4	sylvestris	sylvestris	BA	Jajce, Pliva river bridge	under the bridge, along the dam	380	ZA-48610	SB, ILj	10/07/2018	44.346803	17.242528	2x	+	+	+	+	+	+
79	D85	5	sylvestris	nodosus	BA	Vlašić Mt., above Babići village	vertical rocks along the road	1100	ZA-48611	SB, ILj	10/07/2018	44.258524	17.624191	2x	+	+	+	+		+
80	D86	6	sylvestris	bertisceus	BA	Sutjeska river canyon, close to Tjentište	rocky grassland	638	ZA-48662	SB, ILj	11/07/2018	43.315479	18.668211	2x	+	+	+	+		+
81	D87	5	sylvestris	bertisceus	BA	Maglić Mt.	calcareous and calcshist screes	1580	ZA-48663	SB, ILj	12/07/2018	43.273085	18.719551	2x	+	+	+	+		+
82	D88	5	sylvestris	bertisceus	ME	Piva river canyon, road Šćepan to Plužine	river canyon, vertical rocks along the road	735	ZA-48664	SB, ILj	12/07/2018	43.263261	18.845226	2x	+	+	+	+	+	+
83	D89	5	sylvestris	bertisceus	ME	Pivska planina, close to Lokvice village	vertical rocks along the road	1680	ZA-48665	SB, ILj	13/07/2018	43.137130	18.940890	2x	+	+	+	+	+	+
84	D90	5	sylvestris	bertisceus	ME	Durmitor Mt., Sedlo mountain pass	planinske rudine	2002	ZA-48666	SB, ILj	13/07/2018	43.100445	19.050844	2x	+	+	+	+	+	+

85	D91	5	sylvestris	bertisceus	ME	Lim river canyon, Tivranska klisura	river canyon, vertical rocks along the road	711	ZA-48612	SB, ILj	13/07/2018	42.882032	19.861470	2x	+	+	+	+		+
86	D92	6	sylvestris	bertisceus	ME	Lim river canyon, close to Andrijevica	vertical rocks along the road	746	ZA-48667	SB, ILj	13/07/2018	42.728919	19.824579	2x	+	+	+	+	+	+
87	D93	6	sylvestris	bertisceus	ME	Zeletin, Goleš, Plana	planinske rudine	2011	ZA-48668	SB, ILj	14/07/2018	42.649709	19.844121	2x	+	+	+	+	+	+
88	D94	5	sylvestris	bertisceus	ME	Popadija peak	rocky grassland on silicates	1989	ZA-48669	SB, ILj	14/07/2018	42.520644	19.761424	2x	+	+	+	+		+
89	D95	5	sylvestris	bertisceus	ME	Planinica peak	planinske rudine	1948	ZA-48670	SB, ILj	15/07/2018	42.679973	20.011363	2x	+	+	+	+	+	+
90	D96	5	sylvestris	bertisceus	ME	Morača river canyon, N of Podgorica	river canyon, vertical rocks along the road	278	ZA-48613	SB, ILj	15/07/2018	42.696833	19.374310	2x	+	+	+	+		+
91	D102	5	sylvestris		ME	Budva to Cetinje road, close to Obzovica village	rocky grassland	931	ZA-48619	SB, ILj	16/07/2018	42.305811	18.931887	2x	+	+	+	+	+	+
92	D103	5	sylvestris		ME	Lovčen Mt., Jezerski vrh	planinske rudine	1681	ZA-48620	SB, ILj	16/07/2018	42.400299	18.838067	2x	+	+	+	+		+
93	D106	5	sylvestris	nodosus	BA	Sitnica border pass	rocky stones, scrub, edge of forest	915	ZA-48623	SB, ILj	17/7/2018, 29/6/2019	42.564323	18.443123	2x	+	+	+	+	+	+
94	D108	5	sylvestris		AL	gorge between Kukur and Roves villages	calcareous cliffs	969	ZA-48625	BF, PS	12/07/2018	40.866944	20.384444	2x		+	+	+		+
95	D109	5	sylvestris		AL	Maja e Ostrovicës Mts., above the village Marjan	alpine meadow	1406	ZA-48626	BF, PS	13/07/2018	40.579722	20.477222	2x		+	+	+		+
96	D110	4	sylvestris		MK	Struga, Jablanica Mts, Labuniški Bačila	stabilised limestone screes	1759	ZA-48627	BF, PS	15/07/2018	41.272778	20.535000	2x		+	+	+		+
97	D111	5	sylvestris	tergestinus	HR	Island of Cres, port of Merag	rocky grassland along the road, forest edge	40	ZA-48628	SB, SC	21/10/2018	44.977214	14.445000	2x	+	+	+			+
98	D119	4	sylvestris		AL	Valamara Mts., ridge above the Shkumbin river	grasslands in Pinus heldreichii subalpine zone, ophiolites	1670	ZA-54922	MF	16/07/2018	40.821667	20.501667	2x	+	+	+	+	+	+
99	D120	5	sylvestris		AT	Ötztal	rocky slopes	1180	ZA-54923	MF	25/07/2018	47.157310	10.925250							+
100	D121	5	sylvestris		AT	Oberes Gericht	pineforest, dry and sunny, quite steep	1160	ZA-54924	MF	16/07/2018	47.036690	10.646640	2x			+			+
101	D122	5	sylvestris		AT	Innsbruck S	open area above a cliff, pineforest	880	ZA-54925	MF	03/09/2018	47.244250	11.418530	2x			+			+
102	D123	5	sylvestris		AT	Ötztal	pineforest, dry and sunny, quite steep	1266	ZA-54926	MF	25/07/2018	47.018720	10.997420				+			+
103	D124	7	sylvestris	nodosus	HR	Island of Lastovo, Podanje	rocky grassland along the road	48	ZA-54927	IR, SB	04/06/2019	42.755188	16.923785	2x	+	+	+	+		+
104	D125	5	sylvestris	nodosus	HR	Island of Lastovo, Bozonji dol	rocky grassland along the road	126	ZA-54928	IR, SB	04/06/2019	42.749941	16.887758		+	+	+	+	+	+
105	D126	5	sylvestris	nodosus	HR	Island of Lastovo, Struga Peninsula	rocky grassland above the sea	46	ZA-54929	IR, SB	04/06/2019	42.723823	16.885311		+	+	+	+		+
106	D127	7	sylvestris	nodosus	HR	Island of Brač, Smokovje	vertical rocks along the road	150	ZA-54930	ILj, AT, MG	04/06/2019	43.266081	16.693588	2x	+	+	+	+		+

107	D128	5	sylvestris	nodosus	HR	Mosor Mt., above Gornje Sitno	rocky grassland along the mountain road	810	ZA-54931	SB, ILj, IR, AT, MG	05/06/2019	43.522366	16.611583	2x	+	+	+	+		+
108	D129	5	sylvestris	tergestinus	HR	Šibenik to Drniš road, close to Gradina village	rocky grassland along the road	186	ZA-54932	SB, ILj, IR, AT, MG	05/06/2019	43.773985	15.956080	2x	+	+	+			+
109	D130	5	sylvestris	sylvestris	HR	Knin, Podinarje, Krčić creek	rocky grassland along the road	345	ZA-54933	SB, ILj	14/06/2019	44.033978	16.292465	2x	+	+	+	+		+
110	D131	5	sylvestris	alboroseus	GR	Pindus Mts., SE of Theodoriana village	silicates	1023	ZA-54934	SB, ILj, IR, AT	21/06/2019	39.426471	21.215909	2x	+	+	+	+		+
111	D132	5	sylvestris	alboroseus	GR	Gorilla Mt., Eleftherochori village	vertical rocks along the road	664	ZA-54935	SB, ILj, IR, AT	21/06/2019	39.497535	20.540515	2x	+	+	+	+		+
112	D133	5	sylvestris	alboroseus	GR	Lygeres, close to Chrysovitsa, Siolades village	silicates along the road	744	ZA-54936	SB, ILj, IR, AT	22/06/2019	39.766384	21.093540	2x	+	+	+	+		+
113	D134	7	sylvestris	alboroseus	GR	Lygeres, Aoös lake	silicates along the road	1398	ZA-54937	SB, ILj, IR, AT	22/06/2019	39.820819	21.087621	2x	+	+	+	+	+	+
114	D135	5	sylvestris	alboroseus	GR	Kapesovo village	silicates	1135	ZA-54938	SB, ILj, IR, AT	22/06/2019	39.886151	20.786382	2x	+	+	+	+	+	+
115	D136	5	sylvestris	alboroseus	GR	Gramos Mt., Theotokos village	silicates, edge of Quercus trojana forest	765	ZA-54939	SB, ILj, IR, AT	22/06/2019	40.237462	20.873948			+	+	+		+
116	D137	5	sylvestris	alboroseus	GR	Smolikas Mt., Aoös river	silicates along the road	631	ZA-54940	SB, ILj, IR, AT	22/06/2019	40.144235	20.823990	2x	+	+	+	+		+
117	D138	6	sylvestris	alboroseus	GR	Astraka peak, above Mikro Papingo village	carbonates	1296	ZA-54941	SB, ILj, IR, AT	23/06/2019	39.972951	20.740756	2x	+	+	+	+		+
118	D139	5	sylvestris	alboroseus	GR	Pindus Mts, Tymfi Mt., Drakolimni lake	rocky limestone above sea	2093	ZA-54942	SB, ILj, IR, AT	23/06/2019	39.994937	20.785990	2x		+	+	+	+	+
119	D140	6	sylvestris	alboroseus	AL	Nemërçka Mts., Maja e Dhembelit Mt.	carbonates	1523	ZA-54943	SB, ILj, IR, AT	24/06/2019	40.215069	20.316456	2x	+	+	+	+		+
120	D141	6	sylvestris	alboroseus	AL	Gjirokastër, above Drinos river valley	carbonate clifs along the road	400	ZA-54944	SB, ILj, IR, AT	25/06/2019	39.945037	20.251086	2x	+	+	+	+		+
121	D142	3	sylvestris	alboroseus	AL	Ceraunian Mts., Llogara National Park, Maja Qores peak	alpine meadow	1600	ZA-54945	SB, ILj, IR, AT	25/06/2019	40.207207	19.599520	2x		+	+	+		+
122	D142	5	sylvestris	alboroseus	AL	Ceraunian Mts., Llogara National Park	carbonate clifs along the road	950	ZA-54946	SB, ILj, IR, AT	25/06/2019	40.214363	19.579591	2x	+	+	+	+		+
123	D145	5	sylvestris		AL	Mat river canyon, Madhesh village	silicates along the road	145	ZA-54947	SB, ILj, IR, AT	26/06/2019	41.679535	19.847393	2x	+	+	+	+		+
124	D147	8	sylvestris		AL	Shkodër, close to Bardhaj village	silicates	129	ZA-54953	SB, ILj, IR, AT	27/06/2019	42.072188	19.564231	2x	+	+	+	+		+
125	D150	6	sylvestris	bertisceus	AL	Leqet e Hotit, Rrapsh serpentine	vertical rocks along the road, carbonate	773	ZA-54956	SB, ILj, IR, AT	27/06/2019	42.415086	19.503708	2x	+	+	+	+		+
126	D151	7	sylvestris	bertisceus	AL	Bjeshkët e Nemuna, Thethi, Maja Shtegut	rocky carbonate clifs and pastures	1735	ZA-54957	SB, ILj, IR, AT	28/06/2019	42.388436	19.730210	2x	+	+	+	+		+
127	D153	5	sylvestris	bertisceus	AL	Bjeshkët e Nemuna, Thethi	carbonate rocky clifs above road	665	ZA-54959	SB, ILj, IR, AT	28/06/2019	42.324863	19.593453	2x	+	+	+	+	+	+

128	D154	5	sylvestris	bertisceus	ME	Podgorica, Cijevna river canyon	river canyon Cijevna, carbonate rocks along the road	139	ZA-54960	SB, ILj, IR, AT	28/06/2019	42.398352	19.367112	2x	+	+	+	+	+	+
129	D157	1	sylvestris	bertisceus	ME	Ostroška Greda Mt., Ostrog	rocky carbonate clifs along the road	600	ZA-54963	SB, ILj, IR, AT	28/06/2019	42.673681	19.030135		+	+	+	+		+
130	D158	5	sylvestris	bertisceus	ME	Nikšić region, Slano jezero	rocky pastures near Quercus pubescens forest	900	ZA-54964	SB, ILj, IR, AT	29/06/2019	42.751109	18.799159	2x	+	+	+	+	+	+
131	D160	5	sylvestris	nodosus	HR	Pelješac, Popova luka village	rocky stones along the road	160	ZA-54966	SB, ILj, IR, AT	29/06/2019	42.915273	17.426740	2x	+	+	+	+	+	+
132	D162	5	sylvestris	sylvestris	HR	Samoborsko gorje, Oštrc peak	the edge of the forests, grassland	740	ZA-54968	AT, MG	05/07/2019	45.763765	15.648604	2x	+	+	+	+		+
133	D164	6	sylvestris		SI	Julian Alps, Triglav NP, close to Vršič pass	calcareous and calcshist screes	1954	ZA-54970	AT, ILj	09/07/2019	46.431321	13.736592	2x	+		+	+	+	+
134	D165	5	sylvestris	sylvestris	SI	Litija, Sava village	rocky stones, scrub, edge of forest	295	ZA-54971	AT, ILj	09/07/2019	46.088952	14.887297	2x	+	+	+	+	+	+
135	D166	5	sylvestris	sylvestris	SI	Polhov Gradec, Polhograjska Grmada hill	near mountain road, carbonates	900	ZA-54972	AT, ILj	10/07/2019	46.084072	14.334739	2x	+	+	+	+	+	+
136	D168	5	sylvestris	bertisceus	AL	Lumi i Tropojës	limestone	1115	ZA-54974	DSh	23/07/2019	42.473717	20.151667		+	+	+	+		+
137	D169	5	sylvestris	bertisceus	AL	Shkëlzeni Mountain	limestone	2253	ZA-54975, ZA-54976	DSh	24/07/2019	42.457408	20.122222	2x	+	+	+	+		+
138	D169	5	sylvestris	bertisceus	AL	Shkëlzeni Mountain	limestone	1963	ZA-54975, ZA-54976	DSh	24/07/2019	42.453953	20.111389	2x	+	+	+	+		+
139	D170	6	sylvestris		RS	Kopaonik Mt., Nebeske stolice	alpine grassland	1787	ZA-54977	SB, MD, MG, IR	10/07/2019	43.261512	20.836531	2x	+	+	+	+	+	+
140	D171	5	sylvestris		MK	Šar planina, Ljuboten peak	alpine grassland	2255	ZA-54978	SB, MD, MG, IR	11/07/2019	42.199482	21.123851	2x	+	+	+	+	+	+
141	D172	5	sylvestris		MK	Šar planina, Ljuboten peak	alpine grassland	2255	ZA-54979	SB, MD, MG, IR	11/07/2019	42.199482	21.123851	2x	+	+	+	+	+	+
142	D173	6	sylvestris		MK	Bistra Mt.	rocky grassland along the road	1687	ZA-54980	SB, MD, MG, IR	12/07/2019	41.629296	20.684806	2x	+	+	+	+		+
143	D174	5	sylvestris		MK	Galičica Mt.	rocks along the road	1510	ZA-54981	SB, MD, MG, IR	12/07/2019	40.965656	20.818794	2x	+	+	+	+		+
144	D175	6	sylvestris		MK	Korab Mt., Nistrovski Korab ridge	rocky places witih alpine grassland	2112	ZA-54982	SB, MD, MG, IR	13/07/2019	41.791897	20.592906	2x	+	+	+	+		+
145	D176	5	sylvestris		MK	Korab Mt., Radika river canyon	rocky blocks along the road in the canyon	1379	ZA-54983	SB, MD, MG, IR	13/07/2019	41.799585	20.628008	2x	+	+	+	+		+
146	D178	6	sylvestris	kozjakensis	MK	Kozjak Mt, above Krstec village	rocky grassland in Fagus sylvatica forest	1292	ZA-54985	SB, IR	14/07/2019	41.409144	21.678418	2x	+	+	+	+	+	+
147	D179	5	sylvestris		MK	Jakupica Mt., Solunska Glava	calcareous rocky alpine grassland	2274	ZA-54986	SB, MG, IR	17/07/2019	41.692786	21.403810	2x	+	+	+	+	+	+
148	D180	5	sylvestris		MK	Jakupica Mt., Solunska Glava	along the road, forest edge	1235	ZA-54987	SB, MG, IR	17/07/2019	41.680442	21.432421	2x	+	+	+	+	+	+
149	D181	5	sylvestris	alboroseus	AL	Leshnica e Sipërme	limestone	700	ZA-54988	DSh	07/06/2019	39.849242	20.281111		+	+	+	+		+

150	D182	5	sylvestris		AL	Skorushë, Ura e Hasanit	serpentine	1430	ZA-54989, ZA-54990	LSh	27/07/2019	40.588833	20.579722	2x	+	+	+	+	+	+
151	D182	5	sylvestris		AL	Skorushë, Ura e Hasanit	serpentine	1170	ZA-54989, ZA-54990	LSh	27/07/2019	40.583694	20.570556	2x	+	+	+	+	+	+
152	Alps124	21	sylvestris		FR			206		HL	10/06/2017	45.743560	5.887870				+			
153	Apenn165	4	sylvestris		IT			530		DD	31/01/2018	44.600550	8.809254				+			
154	Alps121	20	sylvestris		FR			1803		HL	07/06/2017	44.499080	5.950400				+			
155	Apenn112	30	sylvestris		IT			604		DG	21/07/2015	39.829403	16.303786				+			
156	Alps3	21	sylvestris		CH			673		HL	28/07/2016	47.047807	7.012920				+			
157	Apenn38	19	sylvestris		IT			280		MGa	2011	43.666700	11.160000				+			
158	Alps4	22	sylvestris		CH			1456		HL	29/07/2016	46.424684	6.104079				+			
159	Alps117	20	sylvestris		FR			1453		HL	04/06/2017	44.070350	7.516830				+			
160	Alps15	23	sylvestris		IT			2220		HL	04/08/2016	46.466060	11.859290				+			
161	Alps133	23	sylvestris		IT			1051		HL	17/07/2017	45.632300	10.780350				+			
162	Alps77	25	sylvestris		CH			2340		SF	04/08/2016	46.036944	7.952500				+			
163	Apenn119	4	sylvestris		FR			1705		HL	06/06/2017	43.863010	6.321030				+			
164	Apenn43	21	sylvestris		IT			590-600		MGa	08/04/2011	44.633300	10.066700				+			
165	Alps125	20	sylvestris		CH			828		HL	26/06/2017	45.975350	8.944370				+			
166	Apenn111	30	sylvestris		IT			585		DG	22/07/2015	39.847182	16.133714				+			
167	Apenn114	30	cfr.		IT			1824		DG	04/08/2015	39.798429	16.092498				+			
168	Apenn115	31	cfr.		IT			2134		DG	13/08/2015	39.897224	16.210160				+			
169	Apenn116	30	cfr.		IT			2181		DG	06/08/2015	39.919789	16.158453				+			
170	Alps60	23	sylvestris		CH			2080-2210		KR	25/06/2016, 30/06/2016	46.785515	10.198479				+			
171	Alps16	18	sylvestris		CH			1300		HL	03/08/2016	46.632160	10.446993				+			
172	Alps27	14	sylvestris		IT			2130		HL	28/09/2016	45.525360	7.191370				+			
173	Alps144	7	sylvestris		CH			1874		HL	28/09/2017	46.390290	10.102070				+			
174	Alps20	23	sylvestris		FR			1986		HL	19/08/2016	45.314010	6.680270				+			
175	Alps10	16	sylvestris		CH			1790		HL	24/07/2016	46.527342	7.208130				+			
176	Apenn122	17	sylvestris		FR			428		HL	07/06/2017	44.428779	5.277236				+			
177	Apenn118	20	sylvestris		FR			900		HL	05/06/2017	43.703900	6.815060				+			
178	Alps9	10	sylvestris		CH			1420		HL	01/07/2016	46.424250	8.518020				+			
179	Alps143	20	sylvestris		CH			1512		HL	27/09/2017	47.250760	9.445180				+			
180	Apenn113	31	sylvestris		IT			909		DG	30/07/2015	39.806289	16.139899				+			
181	Alps99	20	sylvestris		CH			900		SBu	17/08/2016	46.121950	7.039067				+			
182	Alps48	7	sylvestris		IT			880		MGa	2011	45.098000	7.343000				+			
183	Apenn120	20	sylvestris		FR			569		HL	06/06/2017	44.219720	5.237900				+			
184	Alps128	17	sylvestris		IT			1774		HL	14/07/2017	46.789070	11.345210				+			
185	SDM1		sylvestris	sylvestris	SI	Sežana municipality resting place	vertilcal rocks along the road	351		IR, SB, ILj	13/06/2018	45.702937	13.946861			+				+
186	SDM2		sylvestris	tergestinus	IT	Trieste, Pesek village	rocky grassland	491		IR, SB, ILj	13/06/2018	45.627242	13.900654			+				+
187	SDM3		sylvestris	tergestinus	SI	Kozina village	rocky grassland	472		IR, SB, ILj	13/06/2018	45.601953	13.930488			+				+
188	SDM4		sylvestris	tergestinus	SI	Koper municipality	rocky grassland	411		IR, SB, ILj	13/06/2018	45.560501	13.891776			+				+
189	SDM5		sylvestris	sylvestris	HR	Ćićarija, close to Jelovice village	the edge of the forests	639		IR, SB, ILj	13/06/2018	45.506661	13.974236			+				+
190	SDM6		sylvestris	sylvestris	HR	between the villages Slum and Nugla	rocky grassland	567		IR, SB, ILj	13/06/2018	45.424990	14.007932			+				+
191	SDM7		sylvestris	sylvestris	HR	Grobnik	rocky grassland	330		IR, SB, ILj	14/06/2018	45.379421	14.544188			+				+

192	SDM8	sylvestris	tergestinus	HR	Crikvenica	vertilcal rocks along the road	25	IR, SB, ILj	14/06/2018	45.176634	14.693055	+	+
193	SDM9	sylvestris	sylvestris	HR	Jadranska magistrala, close to Prizna	vertilcal rocks along the road	30	IR, SB, ILj	15/06/2018	44.636572	14.965935	+	+
194	SDM10	sylvestris	nodosus	HR	Visočica	grassland, along the mountain route	1501	IR, SB, ILj	16/06/2018	44.429109	15.366957	+	+
195	SDM11	sylvestris	tergestinus	HR	Paklenica, close to Veliki Vaganac	rocky grassland	513	IR, SB, ILj	17/06/2018	44.338871	15.444109	+	+
196	SDM12	sylvestris	tergestinus	HR	Šibenik, Guberina, next to the old bridge	maquis and garig near macadam	30	IR, SB, ILj	18/06/2018	43.763085	15.845444	+	+
197	SDM13	sylvestris	nodosus	HR	Biokovo Mt, SE of Mali Troglav	the edge of the forests	1440	IR, SB, ILj	18/06/2018	43.327629	17.057692	+	+
198	SDM14	sylvestris	nodosus	BA	Posušje	vertilcal rocks along the road	551	IR, SB, ILj	20/06/2018	43.473272	17.353415	+	+
199	SDM15	sylvestris	nodosus	BA	Blidinje to Posušje road, close to Rakitno village	vertilcal rocks along the road	734	IR, SB, ILj	20/06/2018	43.536338	17.447936	+	+
200	SDM16	sylvestris	bertisceus	BA	Sutjeska river canyon, close to Tjentište	rocky grassland	620	SB, ILj	11/07/2018	43.304243	18.649676	+	+
201	SDM17	sylvestris	bertisceus	ME	Maglić Mt., Trnovačko jezero	rocky grassland	1526	SB, ILj	12/07/2018	43.251793	18.721639	+	+
202	SDM18	sylvestris	bertisceus	BA	Maglić Mt.	calcareous and calcshist screes	1647	SB, ILj	12/07/2018	43.285038	18.719106	+	+
203	SDM19	sylvestris	bertisceus	ME	Pivsko jezero, serpentines on the road to Žabljak	river canyon, vertical rocks along the road	809	SB, ILj	13/07/2018	43.172169	18.858826	+	+
204	SDM20	sylvestris	bertisceus	ME	Durmitor Mt, road Žabljak Trsa Plužine	vertical rocks along the road	1692	SB, ILj	13/07/2018	43.092165	19.089705	+	+
205	SDM21	sylvestris	bertisceus	ME	Tara river canyon, close to Vrela village	vertical rocks along the road	1346	SB, ILj	13/07/2018	43.152615	19.257936	+	+
206	SDM22	sylvestris	nodosus	HR	Velebit Mt., road Sveti Juraj to Krasno	the edge of the forests, rocky grassland	803	SB, ILj	06/09/2018	44.883093	14.971827	+	+
207	SDM23	sylvestris	sylvestris	HR	top ridge between the peaks Svilaja and Vršina	rocky grassland	1411	MT	07/01/2018	43.787163	16.490082	+	+
208	SDM24	sylvestris	sylvestris	HR	peak Svilaja	rocky grassland	1462	MT	07/01/2018	43.789100	16.485000	+	+
209	SDM25	sylvestris	sylvestris	HR	Svilaja Mt. Umac	rocky grassland near macadam	1090	MT	07/01/2018	43.778099	16.511263	+	+
210	SDM26	sylvestris	tergestinus	HR	Kozjak Mt., above Kaštel Gomilica	rocky grassland	251	ILj, AT, MG	04/06/2019	43.563016	16.409640	+	+
211	SDM27	sylvestris	tergestinus	HR	Žrnovnica canyon, between Sridivica hill and Gradac	rocky grassland along the road	100	SB, ILj, IR, AT, MG	05/06/2019	43.517604	16.575629	+	+
212	SDM28	sylvestris	tergestinus	HR	Lička Plješivica Mt., Poštak peak	rocky grassland along the road	743	SB, ILj, IR, AT, MG	06/06/2019	44.256939	16.065089	+	+
213	SDM29	sylvestris	nodosus	BA	Trebinje, Klobuk border pass	edge of forest	937	SB, ILj, IR, AT	29/06/2019	42.711594	18.551172	+	+
214	SDM30	sylvestris	nodosus	BA	Ravno, Rupni Do, Gradac	edge of forest	500	SB, ILj, IR, AT	29/06/2019	42.880243	17.947038	+	+

215	SDM31	sylvestris	nodosus	HR	Čepikuće border pass, Oblik hill slopes		400	SB, ILj, IR, AT	29/06/2019	42.872725	17.854560	+	+
216	SDM32	sylvestris	sylvestris	SI	Polhov Gradec, Polhograjska Grmada hill	near mountain road, carbonates	758	AT, ILj	10/07/2019	46.085547	14.346393	+	+
217	SDM33	sylvestris	sylvestris	HR	Klek, Ogulin	Klek peak	1029	DS	02/08/2020	45.260345	15.145431	+	+
218	SDM34	sylvestris	sylvestris	HR	Klek, Ogulin	helicopter takeoff near Klek peak	1104	DS	02/08/2020	45.260182	15.144568	+	+
219	Balkan DB 1			RS				SĐ		43.800511	19.519803		+
220	Balkan DB 3			RS				SĐ		43.586220	19.347322		+
221	Balkan DB 4			MK				SĐ		41.756200	20.618400		+
222	Balkan DB 5			BA				SĐ		43.925400	16.628080		+
223	Balkan DB 6			BA				SĐ		44.344230	17.253380		+
224	Balkan DB 7			AL				SĐ		42.054450	19.537890		+
225	Balkan DB 8			AL				SĐ		41.786988	20.180390		+
226	GBIF DSYL BALKANS 1052816295			ME				GBIF		42.825130	18.917570		+
227	GBIF DSYL BALKANS 1144595356			AT				GBIF		47.592780	14.140280		+
228	GBIF DSYL BALKANS 1264995318			IT				GBIF		46.438330	13.568610		+
229	GBIF DSYL BALKANS 1264995334			AT				GBIF		47.075560	12.751110		+
230	GBIF DSYL BALKANS 152921058			SI				GBIF		45.599720	13.912060		+
231	GBIF DSYL BALKANS 164145531			AT				GBIF		46.861820	13.325860		+
232	GBIF DSYL BALKANS 165105849			SI				GBIF		45.599740	14.411990		+
233	GBIF DSYL BALKANS 214525133			AT				GBIF		46.783330	12.783330		+
234	GBIF DSYL BALKANS 296454179			AT				GBIF		47.007440	12.439940		+
235	GBIF DSYL BALKANS 874692609			IT				GBIF		46.194720	12.980830		+
236	GBIF DSYL BALKANS 874757553			IT				GBIF		46.194720	12.980830		+
237	GBIF DSYL BALKANS 911950894			AT				GBIF		47.209980	13.425830		+
238	GBIF DSYL BALKANS 9268476			AT				GBIF		46.706100	12.925600		+
239	GBIF DSYL BALKANS 9268477			AT				GBIF		46.634700	12.935000		+

240	Luqman Dinaric Fortress	HR	HL	44.016560	16.415360	+
241	Luqman Dolina Glinscice	IT	HL	45.619583	13.873778	+
242	Luqman Dubrava	HR	HL	43.508210	16.623780	+
243	Luqman Durmitor	ME	HL	43.098944	19.051111	+
244	Luqman Grossglockner	AT	HL	47.065730	12.756470	+
245	Luqman Kapetanovo Jezero	ME	HL	42.816440	19.230430	+
246	Luqman Komen	SI	HL	45.841944	13.772181	+
247	Luqman Konitsa	MK	HL	40.070000	20.837800	+
248	Luqman Kotor	ME	HL	42.422230	18.785500	+
249	Luqman Laserzsee	AT	HL	46.766720	12.800410	+
250	Luqman Mangart	SI	HL	46.443540	13.639610	+
251	Luqman Mostar	BA	HL	43.247770	17.966450	+
252	Luqman Movraz	SI	HL	45.482972	13.913364	+
253	Luqman Mramorje	ME	HL	42.925167	19.023417	+
254	Luqman Mt Stitan	ME	HL	42.573817	19.559967	+
255	Luqman Orjen	BA	HL	42.572461	18.458978	+
256	Luqman Parun	AL	HL	42.322222	19.656944	+
257	Luqman Rotspitze	AT	HL	47.018970	12.240970	+
258	Luqman Sneznik	SI	HL	45.565373	14.318294	+
259	Luqman Soca High	SI	HL	46.412170	13.693990	+
260	Luqman Soca Low	SI	HL	46.404810	13.705320	+
261	Luqman Ucka	HR	HL	45.284200	14.202050	+
262	Luqman Valbone	AL	HL	42.410100	19.817400	+
263	Luqman Vela Draga	HR	HL	45.316517	14.169567	+
264	Luqman Visitor	ME	HL	42.614806	19.893222	+
265	Luqman Vlaka	BA	HL	42.736230	18.101060	+
266	VHA 10	IT	VirtualHerbariaAustria	46.194722	12.980833	+
267	VHA 12	AT	VirtualHerbariaAustria	46.958333	13.021944	+
268	VHA 13	ME	VirtualHerbariaAustria	42.112778	19.211667	+
269	VHA 15	AT	VirtualHerbariaAustria	46.710556	12.933056	+
270	VHA 5	ME	VirtualHerbariaAustria	42.375000	19.238056	+
271	VHA 7	AT	VirtualHerbariaAustria	47.069444	12.752500	+

Table S2. Genome-wide population genetic diversity summary for 100 diploid *Dianthus sylvestris* populations from the Balkan lineage. Sample size (n), Allelic richness (Ar) observed (Ho) and expected heterozygosity (He).

POP_ID	x	y	n	Ar	Ho	He
102	18.93189	42.30581	5	1.227	0.229	0.227
103	18.83807	42.4003	5	1.226	0.229	0.226
106	18.44312	42.56432	5	1.225	0.229	0.224
108	20.38444	40.86694	5	1.215	0.224	0.214
109	20.47722	40.57972	4	1.213	0.214	0.214
110	20.535	41.27278	4	1.203	0.212	0.203
119	20.50167	40.82167	4	1.211	0.212	0.211
124	16.92379	42.75519	7	1.206	0.208	0.206
125	16.88776	42.74994	5	1.219	0.217	0.220
126	16.88531	42.72382	5	1.211	0.222	0.210
127	16.69359	43.26608	7	1.224	0.221	0.224
128	16.61158	43.52237	5	1.229	0.227	0.229
130	16.29247	44.03398	5	1.208	0.220	0.207
131	21.21591	39.42647	5	1.206	0.214	0.204
132	20.54052	39.49754	5	1.197	0.208	0.195
133	21.09354	39.76638	5	1.203	0.208	0.203
134	21.08762	39.82082	7	1.216	0.218	0.216
135	20.78638	39.88615	5	1.204	0.207	0.204
136	20.87395	40.23746	5	1.219	0.222	0.219
137	20.82399	40.14424	5	1.215	0.218	0.215
138	20.74076	39.97295	6	1.210	0.213	0.210
139	20.78599	39.99494	5	1.210	0.221	0.209
140	20.31646	40.21507	6	1.216	0.228	0.215
141	20.25109	39.94504	6	1.200	0.189	0.202
142	19.59952	40.20721	8	1.192	0.197	0.192
145	19.84739	41.67954	5	1.210	0.224	0.208
147	19.56423	42.07219	8	1.209	0.209	0.208
150	19.50371	42.41509	6	1.222	0.225	0.222
151	19.73021	42.38844	7	1.222	0.223	0.222
153	19.59345	42.32486	5	1.223	0.220	0.224
154	19.36711	42.39835	5	1.215	0.221	0.214
158	18.79916	42.75111	4	1.216	0.218	0.216
160	17.42674	42.91527	5	1.206	0.212	0.206
162	15.6486	45.76377	5	1.212	0.212	0.212
164	13.73659	46.43132	6	1.209	0.220	0.208
165	14.8873	46.08895	5	1.211	0.215	0.211
166	14.33474	46.08407	5	1.226	0.225	0.226
168	20.15167	42.47372	5	1.207	0.213	0.206
169	20.11681	42.45568	4	1.226	0.265	0.220
170	20.83653	43.26151	6	1.201	0.211	0.200
171	21.12385	42.19948	5	1.206	0.209	0.206
172	21.12385	42.19948	5	1.211	0.213	0.211
173	20.68481	41.6293	6	1.213	0.210	0.214
174	20.81879	40.96566	5	1.218	0.222	0.217
175	20.59291	41.7919	6	1.214	0.216	0.214
176	20.62801	41.79959	5	1.203	0.200	0.203
178	21.67842	41.40914	6	1.200	0.216	0.198
179	21.40381	41.69279	5	1.210	0.220	0.209
180	21.43242	41.68044	5	1.206	0.207	0.206
181	20.28111	39.84924	5	1.212	0.210	0.212
182	20.57514	40.58626	5	1.222	0.252	0.218
2	17.08055	42.94086	7	1.211	0.218	0.210
23	14.5247	45.37468	5	1.277	0.359	0.266
27	14.96583	44.70794	5	1.231	0.232	0.232
28	14.9569	44.7153	10	1.230	0.230	0.230

33	15.14316	44.52948	5	1.232	0.237	0.232
34	15.3643	44.4293	5	1.234	0.231	0.233
37	15.46143	44.36597	7	1.231	0.233	0.231
38	15.46567	44.37109	5	1.232	0.231	0.232
39	15.46572	44.37557	2	1.186	0.223	0.168
42	15.51313	44.36112	6	1.209	0.230	0.207
46	15.87176	43.73736	6	1.220	0.226	0.220
47	17.08662	43.28407	11	1.232	0.232	0.232
52	17.80412	43.32759	7	1.225	0.224	0.224
55	17.96701	43.64998	6	1.222	0.224	0.221
56	17.41643	43.41747	5	1.225	0.216	0.225
57	17.48789	43.58668	5	1.223	0.232	0.223
58	17.54076	43.59503	5	1.220	0.232	0.218
59	17.53378	43.58652	6	1.237	0.233	0.237
60	17.54072	43.57896	5	1.225	0.233	0.224
61	17.27952	43.54695	5	1.238	0.235	0.239
6	17.84349	42.81569	5	1.222	0.223	0.221
63	17.03066	43.85963	5	1.230	0.230	0.230
64	17.05981	43.88544	6	1.238	0.238	0.238
65	17.16035	43.78286	5	1.224	0.219	0.225
66	16.35956	44.34866	6	1.232	0.234	0.232
69	12.88028	46.75922	9	1.221	0.219	0.222
70	12.25111	47.01306	5	1.201	0.198	0.202
76	20.01645	44.04424	5	1.198	0.209	0.197
77	20.91537	42.2335	5	1.199	0.209	0.198
78	20.47551	40.5741	5	1.220	0.219	0.221
79	20.83973	43.26149	5	1.197	0.207	0.197
80	16.69853	43.45025	8	1.235	0.235	0.234
8	16.41704	44.01427	5	1.220	0.215	0.220
81	16.39343	44.03867	4	1.228	0.231	0.228
82	16.52322	43.75119	5	1.236	0.238	0.236
83	17.1797	44.68547	5	1.215	0.215	0.215
84	17.24253	44.3468	4	1.206	0.209	0.205
85	17.62419	44.25852	5	1.226	0.236	0.224
86	18.66821	43.31548	6	1.210	0.230	0.209
87	18.71955	43.27309	5	1.228	0.225	0.228
88	18.84523	43.26326	5	1.228	0.230	0.227
89	18.94089	43.13713	5	1.234	0.230	0.235
90	19.05084	43.10045	5	1.229	0.232	0.228
91	19.86147	42.88203	5	1.219	0.219	0.218
92	19.82458	42.72892	6	1.222	0.225	0.222
93	19.84412	42.64971	6	1.219	0.226	0.219
94	19.76142	42.52064	5	1.220	0.222	0.220
95	20.01136	42.67997	5	1.216	0.217	0.216
96	19.37431	42.69683	5	1.207	0.220	0.206

Table S3. Geographical patterns of genetic diversity - relationship between latitude/longitude and allelic richness across all loci (Ar), observed heterozygosity (Ho) and expected heterozygosity (He).

	Ar		**Ho**		**He**	
	R^2	***p***	**R^2**	***p***	**R^2**	***p***
x (longitute)	0.168	**<0.0001**	0.096	**0.002**	0.148	**<0.0001**
y (latitude)	0.131	**<0.001**	0.083	0.074	0.115	**0.001**

Appendix I

Environmental variability of *Dianthus sylvestris* Wulfen (Caryophyllaceae) on the Balkan Peninsula

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During the review process of the manuscript “Morphological and environmental variability of *Dianthus sylvestris* Wulfen (Caryophyllaceae) on the Balkan Peninsula”, criticism on performing niche analysis on the weakly supported subspecies were raised several times. In line with the reviewer’s and editor’s comments we decided to completely remove the niche analysis from the manuscript. Since the second research objective of this thesis was to “develop models of habitat suitability for the present and the past and analyse the ecological niches of *D. sylvestris* subspecies with the aim of investigating the relationship between their morphological, genetic and environmental divergence”, this Appendix I is attached here along with publications to meet this objective. Furthermore, and according to the concept of integrative taxonomy, we think that by incorporating niche analysis in addition to morphometrics and population genomics, one can obtain more robust evidence which would elucidate the recognition of intraspecific entities.

INTRODUCTION

In addition to morphometrics performed to capture morphological diversity and the investigation of the relationship between morphological variability and environment and geography (**Publication IV**), we performed environmental niche analyses to capture the environmental diversity between the currently accepted subspecies of *D. sylvestris* s.l. Furthermore, we compared their flowering periods to test the shift in flowering time of *D. sylvestris* subsp. *tergestinus*.

The development of niche analysis methods allowed for a quantification of species' niche and assessment of how niches vary between taxa (Broennimann *et al.* 2012). Niche analyses have been increasingly used for characterising the environmental niche of species or subspecies (Aguirre-Gutiérrez *et al.* 2015; Jaime *et al.* 2015; Janišová *et al.* 2018; Moreno-Contreras *et al.* 2020), and in integrative approaches for resolving taxonomically challenging groups (Mota-Vargas and Rojas-Soto 2016; Otero *et al.* 2019; Lin *et al.* 2021). Species' niche may be estimated by relating data on its occurrences with environmental data (climatic, edaphic and topographic) in the form of GIS layers. Based on the degree of relatedness between species, niches may be conserved (more similar than expected), constrained (divergent within a limited subset of available niches) or divergent (less similar than expected; Pyron *et al.*, 2015). Niche divergence may not always support taxon boundaries at species level, but it may be a consequence of local adaptation and reflect phenotypic diversity at intraspecific level (Dagnino *et al.* 2017). Accordingly, when distributed along an environmental gradient, such as the case of *D. sylvestris*, ecologically marginal populations may show some morphological differentiation (Alcántara-Ayala *et al.* 2020; Buitrago Aristizábal *et al.* 2020) which may lead to potentially erroneous inference on taxon boundaries. Nevertheless, as part of an integrative approach in dealing with taxonomical issues, niche quantification provides an additional line of evidence and thus may help in reaching a decision on delimitation of taxon boundaries (Dagnino *et al.* 2017; Otero *et al.* 2019).

By incorporating environmental variation, *D. sylvestris* represents a promising model to explore the population differentiation in terms of morphological variability and niche divergence/conservatism. Hence, the aim was to compare the environmental niches and test for niche conservatism/divergence between *D. sylvestris* subspecies. Therefore, a comprehensive environmental dataset was created based on populations from across the geographical range of these subspecies on the Balkan Peninsula.

MATERIAL AND METHODS

ENVIRONMENTAL NICHE COMPARISON BETWEEN THE SUBSPECIES

We initially obtained 182 occurrences of *D. sylvestris* for environmental niche analysis, which included 97 populations used for the morphometric and RDA analyses (**Publication IV**). To reduce the sampling bias in environmental niche analysis, occurrences with pair-distances < 1 km were excluded using the “thin” function in the “spThin” R package version 0.2.0 (Aiello-Lammens *et al.* 2015). After thinning, a total of 162 populations of *D. sylvestris* were used for the environmental niche analysis (Fig. 1).

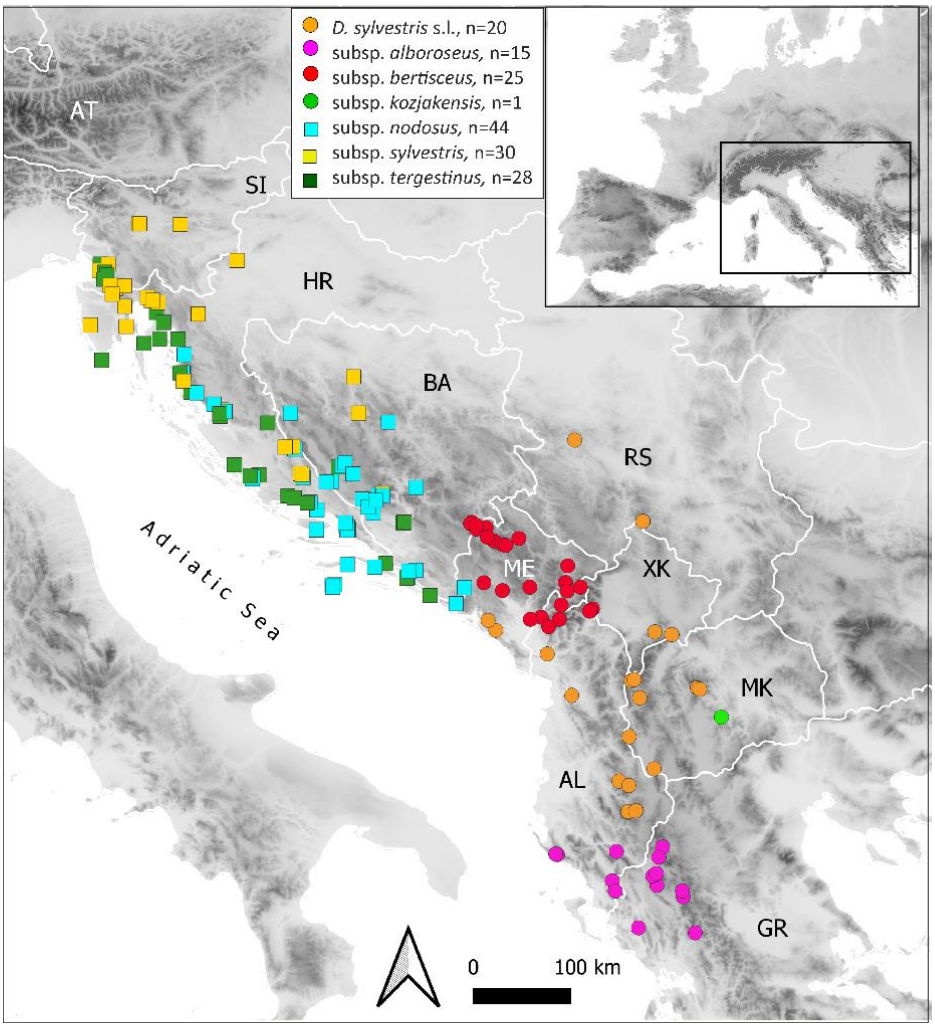

Figure 1. Localities of sampled populations of *Dianthus sylvestris* on the Balkan Peninsula used for environmental niche analyses (n = 182 populations; for details see Table S1 in **Publication IV**).

To characterise the environmental niche for each subspecies, we used environmental data from three databases: climate data from the Chelsa database (Karger *et al.* 2017, 2020), soil properties from the SoilGrids (Hengl *et al.* 2017) and topographic variables from the EarthEnv database (Amatulli *et al.* 2018). An initial set of 43 environmental (predictor) variables were evaluated for collinearity with the "vifstep" function of the "usdm" R package, and therefore the number of variables was reduced to 20 (Table 1). The "vifstep" function calculates Variance Inflation Factor (VIF) for all variables, excludes one with the highest VIF (greater than the threshold set at 10), and repeats the procedure until no variables with VIF greater than threshold remain. First, we tested and visualised the niche differences in univariate environmental space between the subspecies using Kruskal Wallis test, Dunn's post-hoc tests and boxplots in R. Then, niche comparison and niche overlap in multivariate environmental space between the subspecies was performed using PCA-env approach (Broennimann *et al.* 2012) as implemented in the "ecospat" R package version 3.0 (Di Cola *et al.* 2017). The obtained environmental space was divided into a 1000 × 1000 grid. We used Schoener's D metric as a summary statistic for niche overlap, which ranges from 0 (no overlap) to 1 (complete overlap).

The extent of niche differences was further evaluated using tests for niche equivalency and niche similarity for all pairs of subspecies with 1000 random permutations to evaluate the significance (p = 0.05; Warren, Glor, & Turelli, 2008; Broennimann *et al.*, 2012). Niche equivalency test determines if the two observed niches are identical, where a statistically significant result indicates that the two niches are not identical. Niche similarity test determines whether the niches are more similar than would be expected at random, given the niches available across the geographic range of the species (Warren *et al.* 2008). We tested for niche conservatism, i.e. whether the niches are more similar than expected at random (alternative = "greater"), as well as for niche divergence, i.e. whether the niches are less similar than expected at random (alternative = "lower"; Broennimann *et al.*, 2012; Di Cola *et al.*, 2017). Niche similarity test includes specification of a background area, such that niches are similar relative to that area (Warren *et al.* 2008). Background area is an area in geographical space that has been accessible to the species through dispersal over relevant time periods (Barve *et al.* 2011). We applied an approach of estimating the available background according to Barve *et al.* (2011) in which we considered historical environmental changes on the landscape of interest. Thus, we depicted the background area by extracting environmental data from 10000 random points across the environment available to all of *D. sylvestris* subspecies given their putative geographic range.

PHENOLOGY

Data on flowering periods were accessed from herbarium records of the collected populations. Most national floras and identification keys define flowering periods using a range of months, for example June-August. Thus, each collected population was assigned to the months when the plants were flowering. We used “wilcox.test” R function for performing the non-parametric Mann-Whitney U Test, to see if there is statistically significant difference in flowering period between each of the taxa pairs. The response variable is ordinal, i.e., months labelled as numbers from 4 to 8 (April to August).

RESULTS

ENVIRONMENTAL NICHE VARIABILITY BETWEEN THE SUBSPECIES

In the environmental PCA (Fig. 2A) PC1 explained 26.09% of the variance and mainly reflected soil organic carbon content, soil bulk density, soil pH and terrain slope in the study area. PC2 explained 16.75% of the variance corresponding to the mean daily air temperatures of the driest (bio09) and wettest (bio08) quarter, mean monthly precipitation of the coldest quarter (bio19) and temperature seasonality (bio04). Environmental niche space for each of the *D. sylvestris* subspecies is visualised in Fig. S1. Environmental niche overlap quantified with the *D* metric (Fig. 2B) varied between the subspecies, ranging from no or very limited overlap (0-0.2) to moderate overlap (0.4-0.6) following the classification provided by Rödder & Engler (2011). *Dianthus sylvestris* subsp. *bertisceus* and subsp. *tergestinus* showed the lowest overlap of 0.01, whereas subsp. *alboroseus* and subsp. *nodosus* showed the highest overlap of 0.62 (Fig. 2B).

The niche equivalency test showed that the niches were not equivalent in the pairwise comparison for seven pairs (Fig. 2B, red asterisk), but also not divergent according to the niche similarity test. Furthermore, four pairs had equivalent and conserved niches (Fig. 2B, black asterisk), and four pairs had equivalent but not conserved niches (Fig. 2B, without an asterisk). Not a single pair showed a statistically significant result when testing for niche divergence in a similarity test.

Kruskal Wallis test (Table 1) showed significant differences in 16 out of 20 environmental variables, as depicted also in the boxplots ($p < 0.01$; Fig. S2). Climate variables had the greatest statistical significance in delimiting between the subspecies, followed by soil variables.

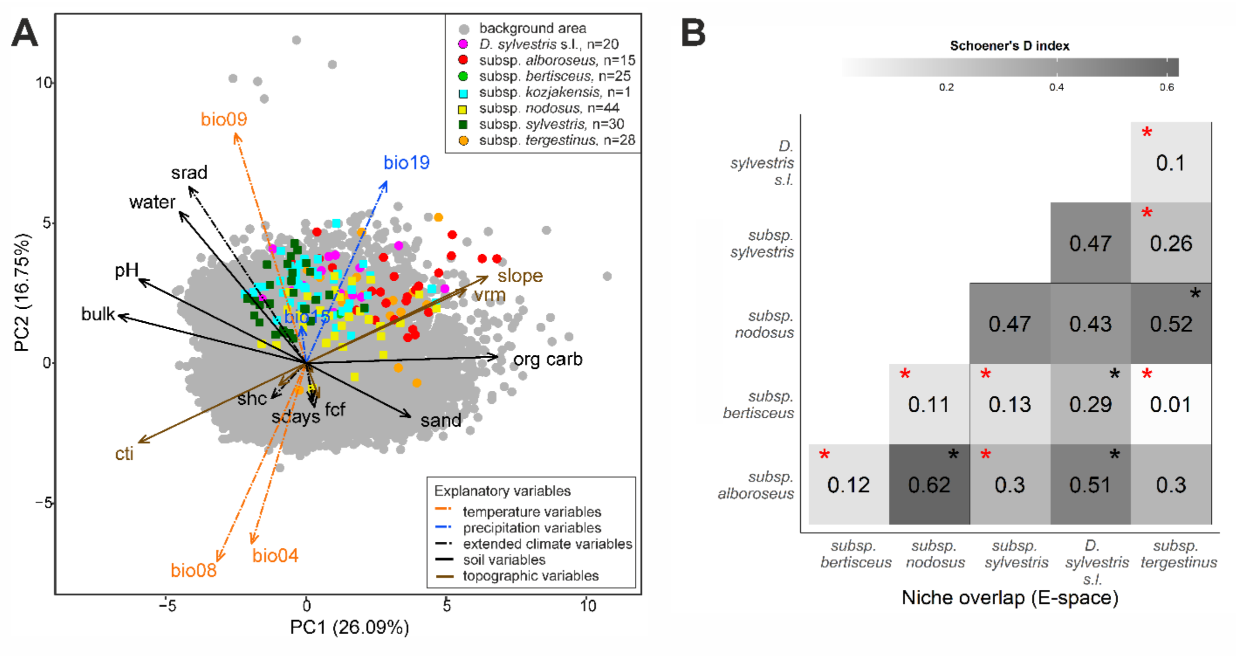

Figure 2. A, Environmental niche space along the first two axes of the environmental PCA based on 20 environmental variables. Coloured points: populations, grey points: available background. **B**, Summarised pair-comparison between the observed values of niche overlap (*Schoener's* D index) in the environmental niche space (E-space) and the simulated overlap from the 1000 pseudo-replicated data sets. Significant values for the similarity test (alternative = "greater") are depicted as black *, whereas significant values for the equivalency test are depicted as red *, $p < 0.05$.

PHENOLOGY

Mann-Whitney U test was performed for each pair of taxa and on two datasets, one including all sampled populations spanning from 8 to 2274 m altitude, and one including a subset of populations from 8 to 1023 m altitude. The results showed significant difference in the flowering period of *D. sylvestris* subsp. *tergestinus* in both datasets, and of *D. sylvestris* subsp. *bertisceus* only in the "all populations" dataset ($p < 0.01$, Table 2, Fig. 3).

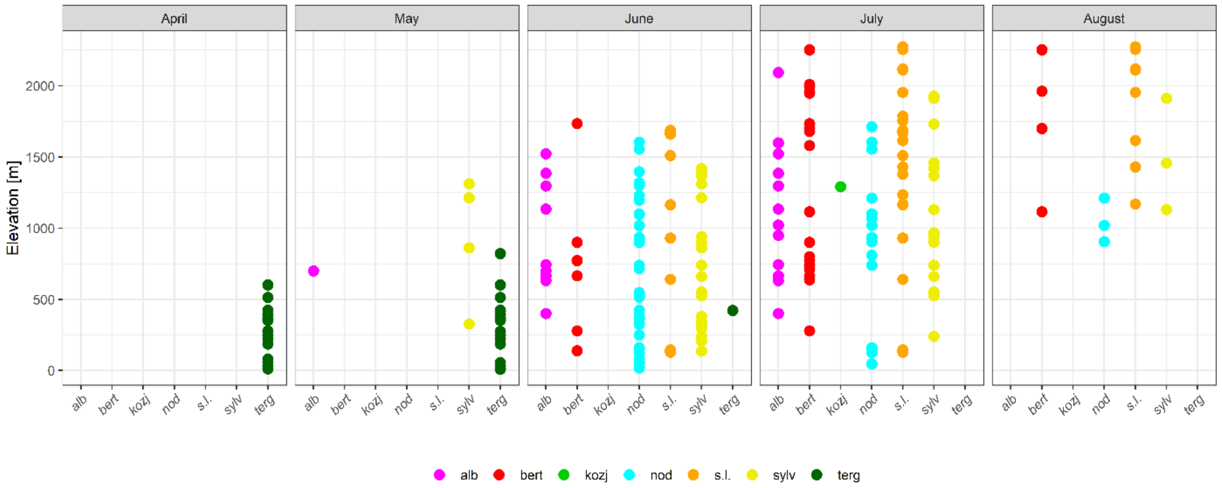

Figure 3. Scatterplot of populations according to their altitude and flowering period.

DISCUSSION

NOT EQUIVALENT, BUT STILL SIMILAR ENVIRONMENTAL NICHES

While Bacchetta *et al.* (2010) gave notes on ecology for species/populations belonging to *D. sylvestris* in central and southern Italy, Sicily and Sardinia, there is no such data describing ecological preferences of subspecies/populations on the Balkan Peninsula. To fill this knowledge gap, we characterised and compared their environmental niches. A lack of taxonomic confidence for the subspecies may lead to uncertainties regarding ecological meaning (Mota-Vargas and Rojas-Soto 2016), and consequently the taxonomic ambiguity could be reflected in the modelled niche (Smith *et al.* 2019). Therefore, our criteria for assigning populations to subspecies were based on morphological information, geographic distribution and current taxonomy of *D. sylvestris.*

The ecological niche differentiation between subsp. *bertisceus* and subsp. *tergestinus* corresponded well to their morphological differentiation. Non-equivalent environmental niches and almost no overlap observed between the two subspecies (*D* = 0.01, Fig. 2B, Fig. S1) could be explained by their geographical distance and different altitudinal affinities. Significant climatic niche differentiation may help to clarify species boundaries between highly morphologically similar species (Lin *et al.* 2021). Our study showed it to be true for the non-equivalent niches of subsp. *sylvestris* and subsp. *tergestinus,* which share parts of their geographic distributions and partially overlap in morphological space. Aside from occupying the narrowest environmental niche of all subspecies, *D. sylvestris* subsp. *tergestinus* was tested regarding the “temporal niche”, since we observed during fieldwork that this subspecies flowered first in April–May (Fig. 3). Earlier flowering time of this subspecies may be an adaptation to habitats with higher temperatures and lower soil moisture in order to complete reproduction prior to the onset of unfavourable growing conditions, or it may be constrained by the temporal niche of its pollinator (Levin 2006). Therefore, temporal isolation could be an important barrier to gene flow between subsp. *tergestinus* and the two sympatric subsp. *nodosus* and subsp. *sylvestris.* On the other hand, the significant difference revealed for *D. sylvestris* subsp. *bertisceus* (Table 2) can be explained by the wide altitudinal range this subspecies inhabits (Fig. 3), and the fact that at higher altitude the flowering season starts later.

Non-equivalent niches of subsp. *bertisceus* and subsp. *alboroseus* may be an example of how morphological extremes along environmental gradients can mistakenly be described as subspecies (Caković *et al.* 2015; Buitrago Aristizábal *et al.* 2020) and form an artificial taxonomy. In some cases, the equivalency test may be prone to rejecting the null hypothesis of niche equivalency when such is not the case, i.e. it may be prone to over-represent niche differentiation (Peterson 2011). For example, in the ecological niche comparisons of the Mexican white pines by Aguirre-Gutiérrez *et al.* (2015), some pairs of species had non-equivalent niches according to the equivalency test, while more similar niches than expected at random according to the similarity test. The investigated populations with geographic distribution and morphology between subsp. *bertisceus* and subsp. *alboroseus*, were placed in *D. sylvestris* s.l. Although niche overlap between subsp. *bertisceus* and *D. sylvestris* s.l. is low (0.2-0.4), similarity test indicated a certain degree of similarity within the environmental space, which may suggest an ongoing differentiation and a certain degree of retention of ancestral environmental characteristics of these two groups over time (Peterson *et al.* 1999). Moreover, the niche is multidimensional, and in any group some aspects may be conserved while others diversify (Wiens *et al.* 2010).

Although the niche similarity test suggested niche conservatism, subsp. *alboroseus* and subsp. *nodosus* were differentiated by quantitative morphometrics (Fig. 2A) and had moderate niche overlap (D = 0.62, Fig. 2B), which could suggest that their morphological distinctiveness is not determined by environmental factors. Phenotypic variability can result from environmental plasticity or genetic differentiation (Halbritter *et al.* 2018; Hardion *et al.* 2020). For example, Hardion *et al.* (2020) in their revision of *D. superbus* subspecies provided a mixed case with some subspecies supported by phylogenetic markers, and others representing environmental plasticity, while Gammella (2016) suggested that the difference in flowering time found between *D. sylvestris* populations growing on limestone vs. serpentine soils have genetic basis. In general, our findings indicate that the niche similarity is more common, since there was not a single case of niche divergence among the subspecies.

CONCLUSION

Here, 162 populations of *D. sylvestris* were evaluated regarding the variability of 20 environmental variables. By quantitatively assessing the niche overlap, equivalency and similarity of *D. sylvestris* subspecies, niche similarity was more common than divergence. Environment, rather than geography, plays a potentially more important role in shaping the morphological variability of this taxon. Additionally, the earlier flowering time of *D. sylvestris* subsp. *tergestinus* (April–May) is an indication of its divergence from the sympatric *D. sylvestris* subsp. *sylvestris* and subsp. *nodosus* which flower through June and July.

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The authors are grateful to Israel Moreno-Contreras for providing the R script for Fig. 2B.

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TABLES AND SUPPLEMENTARY FIGURES

Table 1. Environmental variables used for environmental niche comparisons and RDA analysis, with their abbreviated and full names/definitions, units and types. Significant environmental niche differences along 20 variables between the *Dianthus sylvestris* subspecies as revealed by Kruskal Wallis test ($p < 0.01$) are shown in bold.

Abbreviation	**Full name**	**Unit**	**Variable type**	**Kruskal Wallis rank sum statistic**	***P*-value**
bio04	temperature seasonality	°C/10	climate	43.270955	< **0.01**
bio08	mean daily mean air temperatures of the wettest quarter	°C/10	climate	60.168164	< **0.01**
bio09	mean daily mean air temperatures of the driest quarter	°C/10	climate	45.394996	< **0.01**
bio15	precipitation seasonality	kg m^{-2}	climate	70.492235	< **0.01**
bio19	mean monthly precipitation amount of the coldest quarter	kg m^{-2}	climate	42.245848	< **0.01**
fcf	frost change frequency	No days	extended climate	55.416456	< **0.01**
sdays	number of snow days	No days	extended climate	53.952482	< **0.01**
shc	Selyaninov's Hydrothermic Coefficient	(kg m^{-2}/10)/°C	extended climate	37.439972	< **0.01**
srad	surface solar radiation downwards	kJ m^{-2}	extended climate	44.888935	< **0.01**
dtm_slope	slope	°	topographic	43.184477	< **0.01**
dtm_east	eastness	index	topographic	12.865335	0.024674
dtm_north	northness	index	topographic	11.065501	0.050097
soil_org_carb	soil organic carbon content	5 x g kg^{-1}	soil properties	34.50189	< **0.01**
soil_bulk	soil bulk density	10 x kg m^{-3}	soil properties	50.816375	< **0.01**
soil_sand	sand content	%	soil properties	58.20695	< **0.01**
soil_ph_h2o	soil pH x 10 in H2O	pH	soil properties	39.250477	< **0.01**
soil_water	soil water content at 33kPa		soil properties	29.883328	< **0.01**
dtm_con	convergence	index	topographic	4.8926719	0.429119
dtm_cti	compound topographic index	index	topographic	8.3510321	0.137921
dtm_vrm	vector ruggedness measure	index	topographic	32.938803	< **0.01**

Table 2. Results from the Mann-Whitney U Test. Differences in flowering periods between subspecies pairs of *Dianthus sylvestris* were tested on two datasets: 1) dataset including all sampled populations spanning from 8 to 2274 m altitude, 2) subset of populations from 8 to 1023 m altitude to account for the altitudinally driven later start of flowering. Statistically significant differences at $p < 0.01$ are indicated in bold.

	Balkan pops **8-2274 m alt**		Balkan pops **8-1023 m alt**			Balkan pops **8-2274 m alt**	Balkan pops **8-1023 m alt**
Subspecies pairs	*Wilcoxon test statistic*	*p*-value	*Wilcoxon test statistic*	*p*-value	Subspecies	No of populations	No of populations
alb - s.l.	583	0.01941	50	0.8961	*alboroseus*	13	7
alb - bert	219	0.017	69.5	0.4064	*bertisceus*	21	10
alb - nod	685.5	0.3806	257	0.6784	*nodosus*	42	30
alb - sylv	512	0.5582	177.5	0.4589	*sylvestris*	26	16
alb - terg	777.5	**2.224e-10**	419.5	**3.004e-07**	*tergestinus*	20	20
bert - s.l.	606	0.9406	48	0.5482	*sensu lato*	23	4
bert - nod	1203	**0.00025**	353	0.09894			
bert - sylv	890	**0.002684**	241	0.06104			
bert - terg	1077	**8.652e-13**	501.5	**1.607e-08**			
nod - s.l.	1579	**0.0001731**	180	0.5247			
nod - sylv	1190.5	0.9177	557	0.5746			
nod - terg	1998.5	**2.2e-16**	1427	**2.273e-14**			
sylv - s.l.	1176	**0.001868**	124	0.367			
sylv - terg	1498	**2.134e-13**	908.5	**6.874e-11**			
terg - s.l.	1435	**2.219e-14**	286	**4.014e-06**			

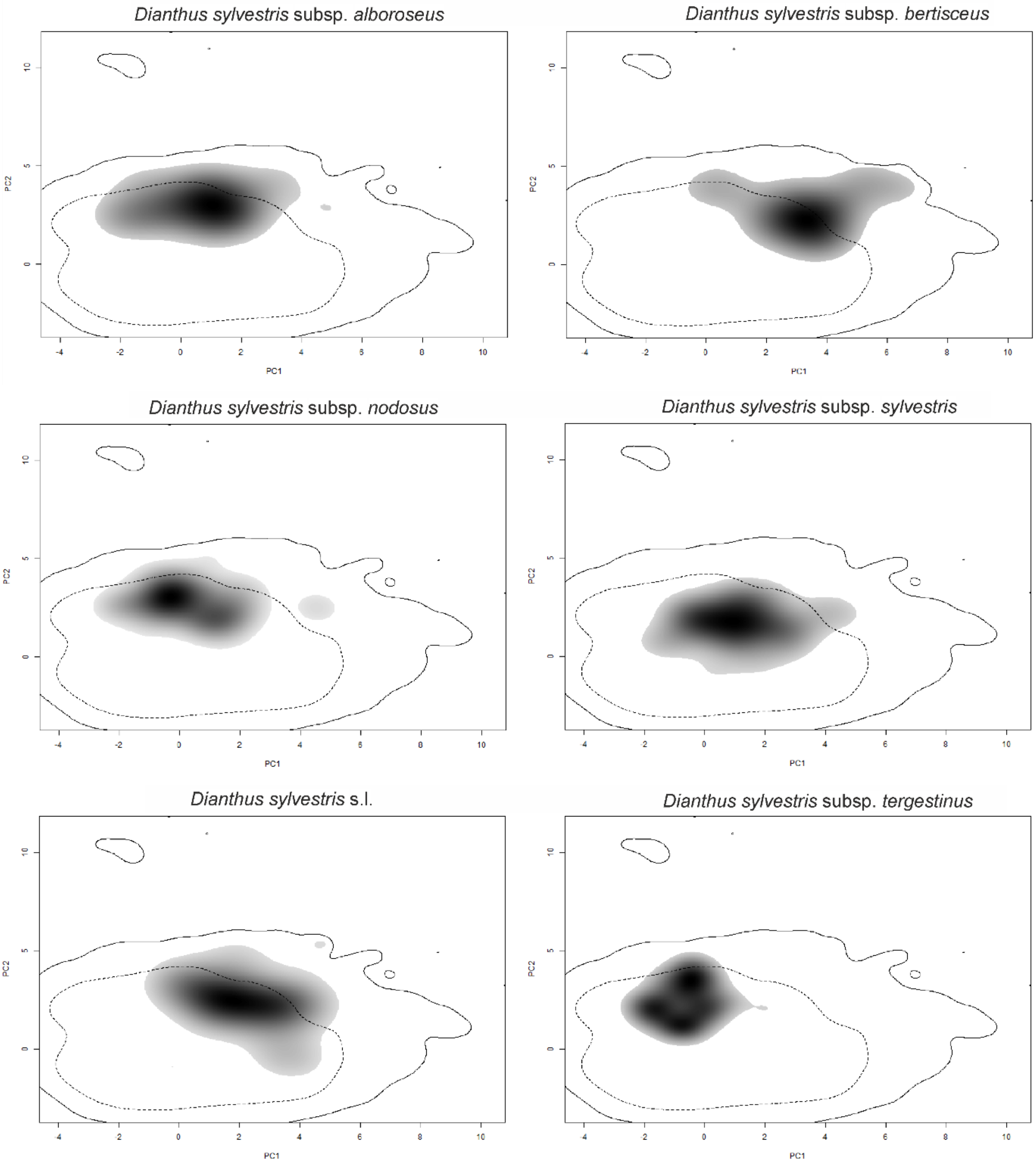

Figure S1. Occurrence density of *Dianthus sylvestris* subspecies along environmental space depicted by the first two axes of an environmental principal component analysis (see also Figure 2). Solid grey line indicates 100% of the available environmental niche space, and dashed line indicates 50% of the available environmental space. The available environmental niche space was obtained by extracting environmental data from 10000 random points across the environment available to all of *D. sylvestris* subspecies given their putative geographic range.

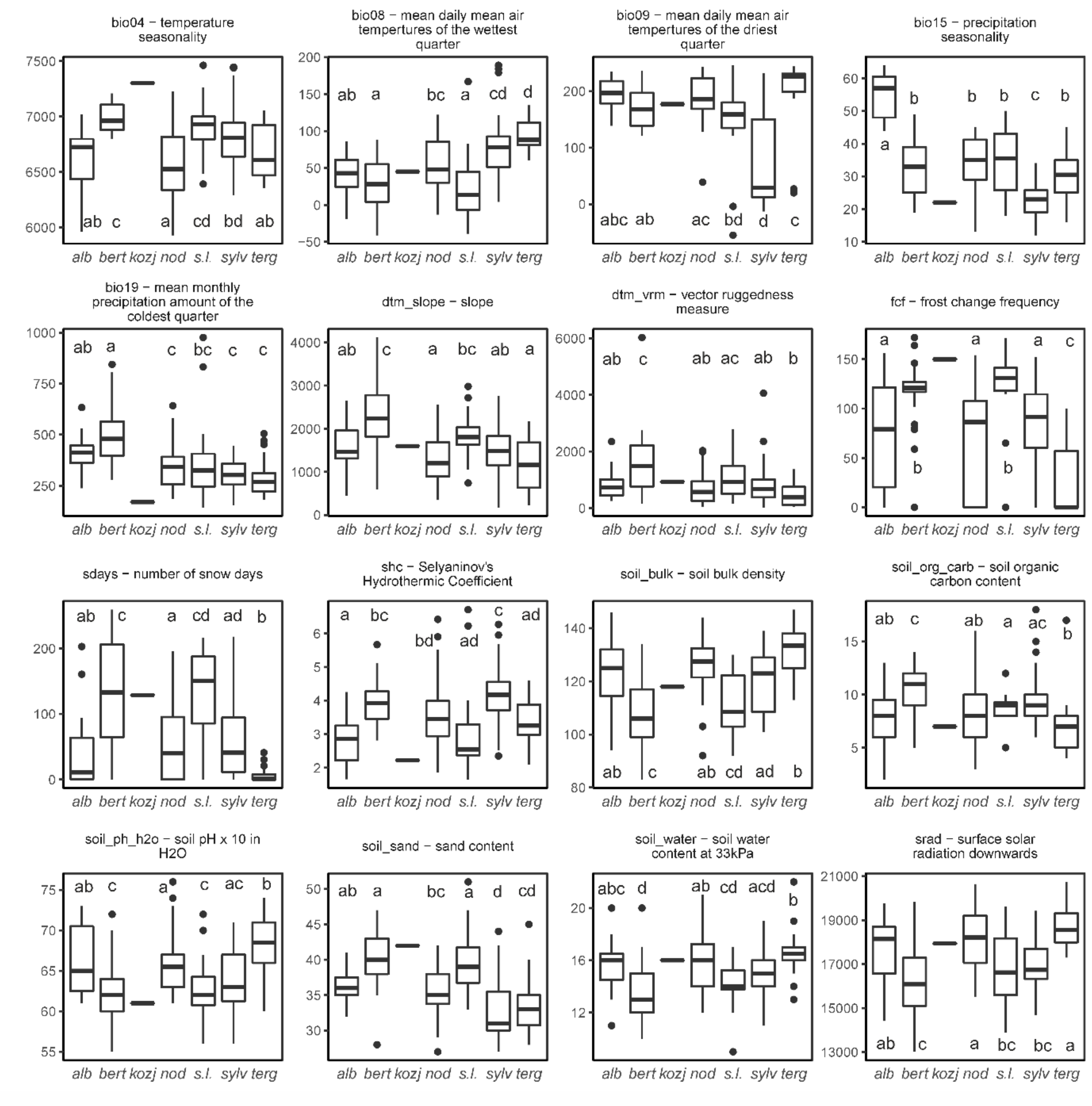

Figure S2. Boxplots showing environmental niche differences along the 16 environmental variables, revealed by Kruskal Wallis test as significantly different between *Dianthus sylvestris* subspecies (Table 1). Abbreviated labels: *alb* - subsp. *alboroseus*, *bert* - subsp. *bertisceus*, *kozj* - subsp. *kozjakensis*, *nod* - subsp. *nodosus*, *s.l.* - *D. sylvestris sensu lato*, *sylv* - subsp. *sylvestris*, *terg* - subsp. *tergestinus*. Means not significantly different at $p < 0.01$, according to Dunn's post-hoc test, are indicated by the same letter.

Appendix II

Taxonomic treatment of *Dianthus sylvestris* Wulfen (Caryophyllaceae) on the Balkan Peninsula

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Our proposed taxonomic treatment is based on results obtained from an extensive population sampling across the Balkan Peninsula: morphometric analysis (461 individuals from 97 populations, **Publication IV**), phylogenomic analysis and analyses of genetic structure and divergence (587 individuals from 108 populations, including 6240 SNPs, **Publication V**), and environmental niche analysis and phenology (162 populations, **Appendix I**). STRUCTURE results together with the Neighbour joining tree (992 individuals from 165 populations the – "All-lineage-dataset", **Publication V**) showed clear divergence of *D. sylvestris* subsp. *tergestinus,* whereas the rest of the Balkan populations were split into two groups. Considering its genetic divergence, the shift in flowering time (**Appendix I**) and its morphological characteristics, this taxon is circumscribed here at the species level, *Dianthus tergestinus.* The RAD-seq SNP data including only the "Balkan-cline-dataset" (587 individuals from 108 populations), revealed two genetic groups, the south-eastern and the north-western group, which were also distinguished according to the results of morphometric analysis. These two distinct entities are circumscribed here at the subspecies level. An identification key for *D. sylvestris* taxa on the Balkan Peninsula is also provided.

Dianthus sylvestris Wulfen **subsp. *sylvestris*** in Collectanea 1: 237. 1786.

Type: "in montibus illis prope Ponewitsch Baronis Wolkensberg in Carniolia, tum in M. Utocsek prope Pillichgraz; in iis. Vallis Rablensis; denique & in iis Vallis Canalensis &c."; lectotype designated by Domina *et al.* (2021)

Synonyms: see Fassou *et al.* (2022)

Distribution: Bosnia and Herzegovina, Croatia, Italy and Slovenia.

Habitat and ecology: Sub-Mediterranean to alpine carbonate or dolomite rocky places, grasslands, rocky crevices in canyons, forest fringes, from 20 up to 2000 m alt., mesophilous to alpine.

Dianthus sylvestris* subsp. *bertisceus Rech. f. in Repert. Spec. Nov. Regni Veg. 38: 150. 1935.
Type: Fuß des Peklen (319); Schlucht bei Pec, Felsritzen (98); Schlucht der Susica (444); Lumbardska Planina, ca. 1800 m (578); Djaravica, Kalk (1232); Greben, ca. 1100 m (1540).

By examining herbarium specimens, six syntypes were found in G (G00418895!, G00418896!, G00418897!, G00418898!, G00418899!, G00418900!) and three syntypes in K belonging to the same collection (K000725353!, K000725354!, K000725355!). A lectotype was chosen and the rest were designated as isolectotypes.
Lectotype designated here: KOSOVO: BERTISCUS (Alpes boreales albanicae): In fauce fluvii Paćska Bistrica prope oppidum Peć (Ipek). In fissuris rupium. alt. 600–750 m s.m., substr. calc., 3.VII.1933., Rechinger fil. et Scheffer No. 98 (G00418898!, Fig. 1).

Isolectotypes designated here: KOSOVO: BERTISCUS (Alpes boreales albanicae): In fauce fluvii Sušica prope oppidum Peć (Ipek). In saxosis calc. alt. 750–950 m s.m., VII.1933., Rechinger fil. et Scheffer No. 444 (G00418895!); In monte Djaravica (Derviš Kom) prope Dečani. alt. 2000–2400 m s.m., substr. calc., 18.VII.1933., Rechinger fil. et Scheffer No. 1232 (G00418896!); In monte Lumbardska Planina prope oppidum Peć (Ipek), ca. 800 m s.m., substr. calc., 8.–9.VII.1933., Rechinger fil. et Scheffer No. 578 (G00418899!); In collibus circa oppidum Peć (Ipek) versus montem Peklen, alt. 500–700 m s.m., 5.VII.1933., Rechinger fil. et Scheffer No. 319 (G00418900!). MONTENEGRO: In monte Greben prope Gusinje, alt. ca. 1100 m s.m., substr. calc., 25.VII.1933., Rechinger fil. et Scheffer No. 1540 (G00418897!).

Synonyms:
= *Dianthus sylvestris* subsp. *alboroseus* F.K.Meyer, in Haussknechtia, Beih. 15: 53. 2011.
= *Dianthus sylvestris* subsp. *kozjakensis* Micevski in Prilozi Oddel. Biol. Med. Nauki Makedonska Akad. Nauk. Umet. 8: 43. 1990.
≡ *Dianthus bertisceus* (Rech.f.) E.Mayer and Trpin in Biol. Vestn. 13: 57. 1965
≡ *Dianthus bertisceus* (Rech.f.) Trinajstić in Suppl. Fl. Anal. Jug. 6: 7. 1979
Distribution: Albania, Bosnia and Herzegovina, Greece, Kosovo, Montenegro, North Macedonia and Serbia.

Habitat and ecology: Sub-Mediterranean to alpine carbonate and silicate rocky crevices, fissures, alpine grasslands, from 200 up to 2300 m alt., mesophilous to alpine.

Figure 1. Lectotype of the name *Dianthus sylvestris* subsp. *bertisceus* Rech. f. (G00418898!)

Dianthus tergestinus (Rchb.) A.Kern., Sched. Fl. Exs. Austro-Hung.: no. 545. 1882

Basionym: Dianthus virgineus var. *tergestinus* (Rchb., Icon. Fl. Germ. Helv. 6: 47, t. 5049b. 1844

Type: none [but Trieste, Italy, can be easily inferred from the epithet “*tergestinus*” that means “from Trieste”]; lectotype designated by Domina et al. (2021).

Synonyms: see Domina et al. (2021) and Fassou et al. (2022)

Habitat and ecology: Eu-Mediterranean to sub-Mediterranean dry rocky grasslands and garrigues, carbonate rocks from sea level up to 600(–800) m alt., thermophilous.

Distribution: Triestine karst (Italy), Slovenian karts, along the north-eastern Adriatic coast (Albania, Bosnia and Herzegovina, Croatia and Montenegro).

Identification key to *Dianthus sylvestris* taxa from the Balkan Peninsula

1. Spring flowering plants (April to May); petal limb entire or slightly eroded; epicalyx scales 2(4), appressed, with mucro (0.3–)0.5–0.8(–0.9) mm long …..………………... ***D. tergestinus***

1. Summer flowering plants (late May, June to August); petal limb denticulate, rarely entire; epicalyx scales 4(6), appressed to divaricated, with mucro (0.4–)0.5–1.6(–3) mm long........... **2**

2. Calyx teeth (2.8–)3.6–5.2(–8.3) mm long, incised ¼ to 1/3 calyx; inner scales ovate to subrounded, (3.8–)4.6–5.6(–7) mm wide, with mucro (0.7–)1.1–1.6(–3) mm long, divaricated ………………………………………………………………… ***D. sylvestris* subsp. *bertisceus***

2. Calyx teeth (2.5–)3–3.9(–4.5) mm long, incised less than ¼; inner scales transversally elliptical, (3.8–)6.4–7.4(–8.7) mm wide, with mucro (0.4–)0.5–0.8(–1.1) mm long, appressed ……………………………………………………………...***D. sylvestris* subsp. *sylvestris***

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DISCUSSION

The results of studies featured in this thesis present an integrative approach to elucidate the patterns and processes that were paramount in shaping the current diversity of *Dianthus sylvestris* populations on the Balkan Peninsula. All the aims and questions within this thesis were addressed and answered through four published scientific publications (**I – IV**), one scientific paper in preparation (**Publication V**), and two unpublished results (**Appendix I – II**) enclosed.

Phylogeographic patterns of Dianthus sylvestris on the Balkans

Deciphering the evolutionary relationships among certain groups has been persistently difficult due to poor resolution within phylogenies as a result of rapid diversification. Such case was reported for genus *Dianthus* which was inferred to be the prime example of fast non-adaptive allopatric diversification (Valente *et al.* 2010). *Dianthus sylvestris* is one of its lineages that rapidly evolved in the central Mediterranean. Recent study by Luqman *et al.* (2022) revealed the existence of three geographically separated lineages within *D. sylvestris*, the Alpine, Apennine and Balkan lineages, that diverged ca. 200.000-115.000 years ago. Each of the three evolutionary lineages occupied distinct Pleistocene glacial refugia and each experienced independent evolutionary histories (Luqman *et al.* 2022).

With the additional comprehensive sampling through the Balkan Peninsula, in **Publication V** we confirm the genetic divergence of *D. sylvestris* populations collected across the Balkan and Apennine Peninsulas and the Alps into three lineages. In **Publication III** further evidence for independent evolutionary divergence of Alpine and Balkan lineages was observed in abruptly different relative genome sizes (RGS). The populations genetically belonging to the Alpine lineage exhibited larger RGS, whereas populations from the eastern part of the Southern Alps, genetically affiliated to the Balkan lineage, had smaller RGS that corresponds to the RGS of other Balkan populations.

The differentiation within the Balkan lineage is characterised through two main events (**Publication V**). First, the genetic structure clearly demonstrates the divergence of *D. sylvestris* subsp. *tergestinus* from the rest of the Balkan populations. Second, the remaining Balkan populations (hereafter termed Balkan cline) exhibit further hierarchical phylogeographic structure, however with more complex underlying patterns of genetic variation.

The divergence of *D. sylvestris* subsp. *tergestinus* populations was strongly supported in the Maximum Likelihood (ML) analysis and inferred from STRUCTURE analysis at K=6 (**Publication V**). Interestingly, although the *D. sylvestris* subsp. *tergestinus* populations are occurring in sympatry with *D. sylvestris* subsp. *sylvestris* and *D. sylvestris* subsp. *nodosus* along the eastern Adriatic coast, only slight admixture was observed. The possible reason for the lack of gene flow may be sought in observed noteworthy shift in flowering time of *D. sylvestris* subsp. *tergestinus,* reported in **Appendix I**. *Dianthus sylvestris* subsp. *tergestinus* flowers first in April and May, whereas *D. sylvestris* subsp. *sylvestris* and subsp. *nodosus* flower through June and July. The distinction of *D. sylvestris* subsp. *tergestinus* is further seen in morphological features such as the petal limb shape, number of epicalyx scales and the mucro length (**Appendix II**).

The differentiation of the Balkan cline was likely influenced through complex interplay between spatial and environmental drivers that have influenced the migration through Pleistocene oscillations. One of the potential evolutionary processes that could have driven the diversification of *D. sylvestris* on the Balkans is allopatric differentiation promoted primarily by heterogeneous Balkan topography (**Publication V**). A sharp genetic discontinuity in the area of Montenegro and a split into two main groups of populations, the north-western (hereafter NW Group) and the south-eastern group (hereafter SE Group), suggest two main evolutionary lineages within the Balkan cline. Moreover, the detailed exploration of morphological characters (**Publication IV**) reveals a gradual change that follows the spatial cline, clustering the populations into two groups, the north-western and the south-eastern. The congruent genetic and morphological split is positioned in the area between rivers Neretva and Sutjeska in Bosnia and Herzegovina. This area is a known geographical barrier that has been documented in various Balkan taxa (Španiel and Rešetnik 2022), e.g., in *Campanula pyramidalis - C. austroadriatica* (Lakušić *et al.* 2013), *Cerastium dinaricum* (Kutnjak *et al.* 2014) and *Gentianella crispata* (Reich *et al.* 2021), whereas in the *Heliosperma pusillum* (Frajman and Oxelman 2007) the genetic break matches the Sutjeska and Drina valleys.

Contemporary patterns of genetic variation in plants often reflect evolutionary processes linked to past climate events. Molecular clock-based estimations highlight the importance of Pleistocene climatic fluctuations across taxonomic groups (Španiel and Rešetnik 2022). For the Balkan cline, suitable habitats during the LGM were identified along the whole eastern-Adriatic coast, with higher suitability predicted in the south of the Balkans compared to the north (**Publication V**). However, the rate of migration between the two main groups was shown to be very limited, i.e., no migration from the NW Group to the SE Group was observed (**Publication V**). Moreover, there were no connectivity patterns identified among two main groups (**Publication V**). Together with the pronounced genetic separation into two groups, the presented data indicate the presence of two main glacial refugia, comparable to examples seen in *Viola suavis* s.l. (Mereďa *et al.* 2011), *Edraianthus graminifolius* (Surina *et al.* 2014) and

Gentianella crispata (Reich *et al.* 2021). Within each of these refugia, populations could have expanded and contracted over multiple cycles of climatic oscillations, resulting in distinct patterns.

The refugial area of the NW Group is postulated to have been in the NW part of the east Adriatic coast with possible extension in the nowadays northern Adriatic Sea (**Publication V**). Northern Dinaric glacial refugia were suggested for a number of trees and understory herbs and for some it presented a source for their postglacial colonisation of more northerly regions (see Gömöry *et al.* 2020; Španiel and Rešetnik 2022). The finding of *D. sylvestris* tetraploid populations ($2n = 4x = 60$) in the small area of Istria (**Publication III**) gives further evidence for the existence of northern Dinaric refugium where the species persisted during glacial stages. Up to this discovery, it was assumed that only diploids are present on the Balkan Peninsula. The only tetraploids within all three *D. sylvestris* lineages were known from the geographically distant Maritime Alps in France (Carolin 1957; Moore 1982), thus our results are adding evidence of a multiple independent origin of polyploids (Bardy *et al.* 2010). As the tetraploids evolved only within the NW Group, we may speculate that the more pronounced climatic oscillations in the northern Balkan Peninsula, compared to the more stable conditions in the south (Tzedakis 2004), were probably a driving force for polyploidization. Possibly, the postglacial recolonization of this area by previously isolated populations resulted in their secondary contact and hybridization which led to the formation of polyploids. Such scenario of postglacial expansion with secondary contacts that resulted in the formation of polyploids was inferred for example in *Alyssum austrodalmaticum* (Zozomová-Lihová *et al.* 2020). Since the tetraploids in Istria do not differ morphologically from their geographically close diploids (**Publication III**), and show high genetic similarity with them (**Publication V**), an autopolyploid origin has been suggested (Levin 2002), such as in *Euphorbia spinosa* (Stevanoski *et al.* 2020) and *Veronica chamaedrys* (Bardy *et al.* 2010).

The southernmost part of the Adriatic coast and adjacent eastern area was indicated as a highly suitable LGM refugial region of the SE Group (**Publication V**). This area was also found as suitable for e.g., *Edraianthus graminifolius* (Surina *et al.* 2014), *E. tenuifolius* (Glasnović *et al.* 2018) and *Salvia officinalis* (Rešetnik, Baričevič, *et al.* 2016). The populations of the SE Group exhibited more noticeable diversification and lower gene flow supported also through lower rates of directional migration. Additionally, the southernmost populations have reduced genetic diversity. Interestingly, the southernmost populations exhibited highest RGS among the Balkan taxa, and the more pronounced RGS variation in this geographic region compared to the NW Group was discovered (**Publication III**). Similar more pronounced genetic differentiation of southern compared to northern populations has been observed in several plant groups (e.g., *Silene saxifraga,* Đurović *et al.* 2017; *Euphorbia myrsinites,* Falch *et al.* 2019) and is in this case further detected in higher variability of morphological characters (**Publication IV**). The most interesting are the populations growing in the area of Montenegro. At the STRUCTURE K=2 these populations show highly admixed pattern comprising large fractions of two main gene pools (**Publication V**). Such specific structure could be a consequence of the admixture of divergent lineages through secondary contact after postglacial colonisation, as seen in *Veronica chamaedrys* (Bardy *et al.* 2010)*, Campanula fenestrellata* (Rešetnik *et al.* 2020) and *Gentianella crispata* (Reich *et al.* 2021). However, we consider this scenario of between-lineage admixture to be a false inference (Luqman *et al.* 2022). Instead, more likely these populations persisted in situ and experienced small-scale differentiation that is visible in the isolation by distance pattern which reveals the existence of small groups consisted of 2 to 7 populations across the borderline area of the Dinaric and Scardo-Pindhic mountain ranges (**Publication V**). Moreover, in **Publication III** pronounced genome size variation at small geographic distances on the same area is revealed, likely indicating at allopatric diversification.

Environmental variability of Dianthus sylvestris subspecies on the Balkan Peninsula

The great variation in RGS (**Publication III**), morphology (**Publication IV**) and genetic diversity (**Publication V**) among the Balkan populations of *D. sylvestris* is demonstrated to have a limited correlation with its traditional taxonomy. The observed pattern of geographical, rather than taxonomical segregation of phylogeographical structure has been previously inferred for other species groups with high taxonomic diversity on the Balkan Peninsula, such as *Heliosperma pusillum* (Frajman and Oxelman 2007) and *Silene saxifraga* (Đurović *et al.* 2017). These groups have a similar diversification pattern, where regional morphological diversification likely resulted from adaptation to different ecological pressures.

In the wide-range species, such as *D. sylvestris* that occupy broad elevational gradients, an array of environmental variables can enforce spatial changes connected to ecological adaptations and environmental niche shifts. For example, the tightly-linked interplay of migration and adaptation was established between high- and low-elevational populations of *D. sylvestris* in the Alpine lineage (Luqman *et al.* 2022). Within the continuous distributions, the division in populations that locally adapt to different environmental niches may be reflected in their morphological variability. In **Publication IV** we investigated the relationships between morphological variability of the Balkan populations and environmental conditions, i.e., whether morphological characters are constrained by a set of environmental variables. As the distribution of *D. sylvestris* encompasses a wide elevational range across the Balkan Peninsula, it is shown that the selection operating on vegetative characters is environment-mediated, thus these characters are not taxonomically indicative. When looking at reproductive morphological characters, the shape of epicalyx scales and calyx teeth incision show an association with geography, i.e., a gradual change in northwest – southeast direction. However, the overall morphological variability of this taxon is primarily shaped by environment, rather than geography. Regardless of the elevation, the common feature of *D. sylvestris* habitats is xerothermity. Water deficit is the type of stress to which *D. sylvestris* is adapted, while some other environmental stressors cause differences in plant size, habit, phenology, etc., among populations (**Publication IV**).

Furthermore, the comparison of the environmental niches and test for niche conservatism/divergence between *D. sylvestris* subspecies was done (**Appendix I**). The narrowest environmental niche was found in *D. sylvestris* subsp. *tergestinus*, in addition to the observed non-equivalent niche with the sympatric *D. sylvestris* subsp. *sylvestris*. The existence of climatic niche differentiation may represent an additional line of evidence needed to clarify species boundaries between highly morphologically similar species (Lin *et al.* 2021). A very limited niche overlap between *D. sylvestris* subsp. *tergestinus* and *D. sylvestris* subsp. *bertisceus* likely represents adaptation to two environmental extremes of the available niche of *D. sylvestris* (constrained niches, Pyron *et al.* 2015). When looking at the geographical distributions of these two subspecies, the difference in their niches reflect the transition from the coastal Mediterranean environment to the alpine environment. It may also represent an example of a process through time where a species tracks favourable ecological niche, slowly diverging from the ancestral niche (Ackerly 2003), as evidenced for high- and low-elevational populations of *D. sylvestris* in the Alpine lineage (Luqman *et al.* 2022).

Apart from niche divergence, populations may exhibit niche conservatism as well, which is possible due to the tendency for populations to retain ancestral ecological characteristics over time (phylogenetic niche conservatism, Peterson *et al.* 1999; Pyron *et al.* 2015). We observed niche conservatism between *D. sylvestris* subsp. *nodosus* and *D. sylvestris* subsp. *alboroseus*, two geographically distant and morphologically distinct subspecies belonging to separate genetic groups. At this point we may hypothesize that the type of environment where *D. sylvestris* subsp. *nodosus* and *D. sylvestris* subsp. *alboroseus* grow is particularly suitable for *D. sylvestris* in general, and thus selection for that niche may have persisted for a relatively long period of evolutionary time, leading to niche conservatism (Pyron *et al.* 2015). Overall, niche analyses suggested niche similarity to be more common, since there was not a single significant case of niche divergence.

Although we have not investigated the pollinators, we can speculate that the observed cline in floral characters (**Publication IV**), whose direction follows that of the genetic diversity cline, is pollinator-mediated. Pollinators are key dispersal agents, and as such, they may drive the distribution of neutral genetic variation of populations within plant species (Burgin and Hopkins 2022). In **Publication V** it was found that some sampled populations are more genetically similar than predicted by geographic distance (i.e., IBD), namely the populations from the Central Dinaric Mountains up to the Carnic Alps in south Austria. One of the possible explanations for this phenomenon is that pollinator movement between spatially distant populations maintains population connectivity (Skogen *et al.* 2019). Indeed, long-tongued Lepidopteran pollinators which are able to reach the nectar at the bottom of the calyx tube (Bloch 2009), can cover greater flight distances than other insects, and therefore are able to sustain a higher effective population size (Schmitt 1980). This assumption that pollinators affect the distribution of genetic variation (beyond IBD) may be quantitatively tested using pollinator movement patterns as an explanatory variable for the plant genetic structure (Burgin and Hopkins 2022). However, the interaction between *D. sylvestris* and its pollinator, and whether its population structure is pollinator driven, remains unexplored.

Taxonomic implications

Intuitive recognition of intraspecific entities may be sometimes successful in yielding a classification. However, fragmentating diversity into smaller and smaller units throughout the history of systematic botany, botanists have often created complex taxonomic situations at specific and especially intraspecific levels. A group of closely related taxa displaying continuity in morphological variability and a wide ecological preference, is hard to classify. *Dianthus sylvestris* represents an example where botanists over centuries forced this continuity into discrete units (intraspecific taxa), creating an impractical taxonomical clutter. The species is still inconsistently treated across its distribution area and the formation of a stable taxonomy for *D. sylvestris* presents a challenging task. For example, the observed tendency of botanists investigating Tuscan populations in Italy has been to assign the plants from higher altitudes to *D. sylvestris*, and the more thermophilous ones to *D. longicaulis* Ten. (Astuti *et al.* 2020). Afterwards, Domina *et al.* (2021) applied the name *D. virgineus* L. to the Apennine populations so far named *D. longicaulis*, being the oldest available name referring to plants that fall within the morphological variability of the *D. sylvestris* species complex (Bacchetta *et al.* 2010).

Moreover, the three main genetic lineages, as revealed both in Luqman *et al.* (2022) and in **Publication V**, can be distinguished morphologically (Peruzzi and Franzoni, unpublished) and suggest the possibility of their treatment as separate species. The name *Dianthus sylvestris* s.str. would then apply to populations from the Balkan genetic lineage, *D. virgineus* to those from the central Mediterranean genetic lineage, and finally, the name *D. inodorus* (L.) Gaertn. would apply to populations from the Alpine genetic lineage.

Our herbarium and literature investigation of the names applied to plants on the Balkan Peninsula revealed the inconsistent nomenclatural treatment of populations from the same locality by numerous collectors and botanists (**Publication I**). This inconsistency also led to the recognition of more than one putative subspecies co-occurring in the same locality, e.g., subsp. *sylvestris* and subsp. *nodosus,* where the subspecies rank lacks a clear geographical and/or ecological structure. Therefore, the classical subspecies concept stating that subspecies are geographically isolated populations belonging to the same species (Wilson and Brown 1953) is difficult to apply in such case. **Publication II** contributes to strengthening the applications of names lacking type material, so that the assignment of natural populations to subspecies could be facilitated by comparing with the designated types.

Publications III-V and Appendix I contribute to the morphological, ecological and genetic characterisation of Balkan populations, in order to offer a sensible taxonomy. Morphological delimitation and the revealed pattern of genetic divergence correlated mostly with the taxonomic classification of *D. sylvestris* subsp. *tergestinus*. **Publication IV** highlights the morphological distinctiveness of only two groups of Balkan populations, where the split between them corresponds to the split between allopatric NW populations and SE populations. The most evident morphological difference between the two groups is the variation in shape of epicalyx scales, from transversally elliptical in NW populations to ovate in SE populations. The likely persistence of the species in at least two glacial refugia within the Balkans (**Publication V**) may have triggered this morphological divergence of epicalyx scales shape.

Here, the gathered evidence from morphological, environmental and molecular patterns for each of the traditionally recognised subspecies of *D. sylvestris* on the Balkan Peninsula is summarised, and a revised taxonomic treatment is proposed.

The typical subspecies, *D. sylvestris* subsp. *sylvestris* is reported for the entire Balkan area: Slovenia, Croatia, Montenegro, Albania, North Macedonia and Greece (Rohlena 1942; Trinajstić 1979; Micevski 1990; Tutin and Walters 1993; Martinčič 2007; Dimopoulos *et al.* 2013; Stešević and Caković 2013; Raab-Straube and Raus 2013; Vangjeli 2015; Barina *et al.* 2018; Nikolić 2020). However, the description of morphological variability from the Wulfen's protologue (1786) and the illustration in Jacquin (1781–1787, Tav. 82) do not morphologically correspond to any of the plants from south-eastern Balkan countries. Instead, evidence suggest that the typical *D. sylvestris* populations are those belonging to the NW morphological group (**Publication IV**) and genetic NW Group (**Publication V**).

Dianthus sylvestris subsp. *nodosus* is the only name for which the original material is unknown, since the original herbarium collection of Tausch was not found within the extensive herbaria survey in the course of the Thesis. It was described as *D. nodosus* by Tausch (1828) from the Croatian Dalmatia, and it has been recorded in the flora of Croatia, Bosnia and Herzegovina, Montenegro and Albania (Rohlena 1942; Trinajstić 1979; Stešević and Caković 2013; Vangjeli 2015; Barina *et al.* 2018; Nikolić 2020). Hayek (1924) placed *D. sylvestris* subsp. *nodosus* to a subspecific level and mentions that the stem in a typical variety of subsp. *nodosus* is puberulous, and he cites Visiani's *D. caryophyllus* L. var. *pubescens* Vis. (Visiani 1852) from the island of Hvar under its synonyms. Since Beck-Mannagetta (1909) and Hayek (1924) reported this trait, it appears in floras and identification keys as diagnostic for this taxon (e.g., stems usually puberulent below, in Tutin and Walters 1993), although there is no mention of indumentum description in the protologue. Moreover, in **Publication IV**, we showed the intrapopulation variability of indumentum coverage and consequently its questionable taxonomic importance. Adding the results of population genetic analyses which also do not support the recognition of this taxon (**Publication V**), we suggest here that there is no basis for distinction of *D. sylvestris* subsp. *nodosus* from *D. sylvestris* subsp. *sylvestris* and, therefore, it does not merit formal taxonomic recognition. Thus, we suggest treating *D. sylvestris* subsp. *nodosus* as a synonym of *D. sylvestris* subsp. *sylvestris*.

Dianthus sylvestris subsp. *tergestinus* was described as *D. virgineus* var. *tergestinus* by Reichenbach (1844), and this basionym was lectotypified in **Publication II**. It has been recorded for the flora of Slovenia, Croatia, Bosnia and Herzegovina, Montenegro and Albania (Trinajstić 1979; Tutin and Walters 1993; Martinčič 2007; Stešević and Caković 2013; Vangjeli 2015; Barina *et al.* 2018; Nikolić 2020). According to the illustration by Reichenbach (Tab. CCLXVI, 5049. β, 1844), the taxon has one pair of epicalyx scales, and this trait is reported in floras and keys as diagnostic. Along with pale pink colour of petals and entire petal limbs, these characters distinguish it from other subspecies of *D. sylvestris* as confirmed in **Publication IV**. Moreover, we found flowering period to be an indicative character discriminating this subspecies (**Appendix I**) and its distinction was validated with genomic data (**Publication V**). **Publications IV and V** together show that this subspecies occurs only along the eastern Adriatic coast with its southernmost locality on Srđ hill near Dubrovnik in

Croatia. However, according to the floras of Montenegro and Albania, the distribution of *D. sylvestris* subsp. *tergestinus* also includes these countries. Possibly we have not recorded it there because our fieldwork across these countries was conducted in late June and July when the plants are in late drying fruiting stage and thus inconspicuous. Considering together all the gathered evidence so far, we regard *D. sylvestris* subsp. *tergestinus* at the specific level as *D. tergestinus* (Rchb.) Kern. (**Appendix II**).

Specimens from the Albanian Alps (Prokletije Mt., Mons Bertiscus) were named by Rechinger fil. (1935) as *D. sylvestris* subsp. *bertisceus*, and N. Fumeaux (G) designated them in 2018 as syntypes. This subspecies has been recorded for the flora of Montenegro, North Macedonia, Serbia and Albania (Rohlena 1942; Trinajstić 1979; Micevski 1990; Tutin and Walters 1993; Stešević and Caković 2013; Vangjeli 2015; Barina *et al.* 2018). Rechinger fil. (1935) noted the morphological similarity between the one-flowered small plants from Prokletije Mts. and alpine *D. sylvestris* from the European Alps. In **Publication III** we reported significantly different genome size estimates between these two groups, and in **Publication V** we report that these two groups of populations belong to two different genetic groups. Moreover, populations of *D. sylvestris* subsp. *bertisceus* display the most complex genetic structure within the Balkan cline. The farther from the type localities, the less typical its morphological diagnostic characters are, and plants gradually display morphologies more typical to geographically adjacent *D. sylvestris* subsp. *kozjakensis* and/or *D. sylvestris* subsp. *alboroseus* (**Publication IV**). Thus, defining the geographical and morphological extents for this subspecies is ambiguous.

Dianthus sylvestris subsp. *kozjakensis* was described from Mt. Kozjak in North Macedonia (Micevski 1990) and it is the only known locality so far. *Dianthus sylvestris* subsp. *alboroseus* is a pale pink to white flowering form from south Albania, described by Meyer (2011). Since it is a relatively new subspecies, it has been reported only for the flora of Albania by Vangjeli (2015). According to Meyer (2011), petal denticulation is generally much more pronounced than in other subspecies, and petal limbs are shorter as well. Its often-divaricated epicalyx scales are similar to those of *D. sylvestris* subsp. *bertisceus.* The holotypes of *D. sylvestris* subsp. *alboroseus* and *D. sylvestris* subsp. *kozjakensis* are very much alike and the results of morphometric and genetic analyses group these two subspecies together (**Publication IV and V**). All the three aforementioned subspecies approximate in some morphological characteristics one another, mainly in the epicalyx scales shape, and thus we suggest treating them as a single subspecies, namely *D. sylvestris* subsp. *bertisceus* (**Appendix II**). Since *D. sylvestris* subsp. *bertisceus* was described prior to both *D. sylvestris* subsp. *alboroseus* and subsp. *kozjakensis*, and these three names are available for the populations from northern Greece, Albania, Montenegro and North Macedonia, the older validly published name is accepted and used (Turland *et al.* 2018).

CONCLUSIONS

The ***First hypothesis*** could not be rejected since we demonstrate the association between genetic variability and the existence of microrefugia on the Balkan Peninsula in **Publications III-V**. The ***Second hypothesis*** could not be rejected when considering *D. sylvestris* subsp. *tergestinus*, as well as the two main Balkan genetic groups, the NW and the SE, for they can be identified according to their morphological diagnostic characters.

The most important conclusions that have arisen from this doctoral thesis are:

1. Remarkable genetic and morphological variation in studied populations of *Dianthus sylvestris* on the Balkan Peninsula showed that only the thermophilous and earlier flowering *D. sylvestris* subsp. *tergestinus* forms a genetic group separated from the rest of the Balkan cline.
2. Both morphological and genetic diversity of populations of the Balkan cline follow a geographic structure in the northwest – southeast direction. This gradient showed one major discontinuity around the Neretva River valley, thus revealing two main groups of Balkan populations, the NW and the SE group, morphologically distinct mainly in the shape of epicalyx scales, calyx teeth incision and petal denticulation.
3. Patterns of genetic variation together with species distribution models suggested that *D. sylvestris* on the Balkan Peninsula persisted during the Pleistocene glaciations in two separate glacial refugia located along the eastern-Adriatic coast. The NW group was more homogeneous with higher gene flow and connectivity, whereas the SE group was more divergent with less gene flow and lower migration rates. Moreover, very limited gene flow was suggested between the populations from the NW and SE groups, indicating barriers to gene flow.
4. Tetraploid populations were found within the NW group and they are distributed only regionally in Istria. Within tetraploids, genome downsizing was observed and they significantly differ from their diploid counterparts in an array of morphological and environmental characteristics. A deep RGS divergence between Alpine and Balkan lineages was observed with Alpine lineage exhibiting larger RGS. Moreover, the increase of RGS towards the southernmost distribution margins of the Balkan lineages was observed.
5. Based on our integrative approach considering multiple lines of evidence, populations traditionally referred to *D. sylvestris* subsp. *nodosus* and one population of *D. sylvestris* subsp. *kozjakensis* are not recognised as distinct intraspecific entities. Furthermore, *D.*

sylvestris subsp. *bertisceus* and *D. sylvestris* subsp. *alboroseus* approximate in some flower traits one another, and thus their taxonomic treatment as one morphologically variable subspecies is suggested. Finally, a new taxonomic treatment of *D. sylvestris* on the Balkan Peninsula is proposed: *D. sylvestris* is a genetically, morphologically and ecologically variable species with two subspecies, *D. sylvestris* subsp. *sylvestris* including the NW Balkan populations, and *D. sylvestris* subsp. *bertisceus* including the SE Balkan populations, whereas *D. tergestinus* is regarded as a separate species.

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CURRICULUM VITAE

Ana Terlević was born in Pula, Croatia, 12 August 1993. She finished elementary and high school in Pula and continued education at the University of Zagreb, Faculty of Science, Department of Biology. She finished undergraduate study of biology in 2015, and graduate study of experimental biology, module botany, in 2018. Prior to starting her doctorate, Ana spent three months at the Jagiellonian University in Krakow in the frame of Erasmus+ traineeship, working on habitat suitability modelling of invasive plant species. Her employment as research assistant and her PhD programme at the University of Zagreb, Faculty of Science, Department of Biology, started in October 2018 and November 2018, respectively. During her PhD, she was working in the frame of the Croatian Science Foundation project "Phylogeography and evolution of three ecologically divergent groups of amphi-Adriatic plants" (Amphi-Adri-Plant). Ana published 3 short communications and 5 scientific articles, she has 13 conference proceedings with 9 active participations and received the best oral presentation award at the 8th Balkan Botanical Congress. Additionally, she participated in educational work as a teaching assistant on two undergraduate courses, Systematic Botany and Statistics. She has also received training in the Research Group Evolutionary Systematic lab, Department of Botany at the University of Innsbruck, Austria. Together with colleagues from Switzerland, Italy, and Germany working on *Dianthus*, Ana initiated a proposal of herbarium collections digitalization including several natural history museums in European cities, and the proposal received funds within the SYNTHESYS+ Virtual Access project. Ana held two popular scientific lectures for the Croatian Botanical Society and one presentation of her investigations at the PhD Café meeting of PhD students at the University of Zagreb, in the organization of the Croatian Science Foundation.

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ORIGINAL SCIENTIFIC PAPERS:

Terlević A, Temunović M, Bogdanović S, Grgurev M, Ljubičić I, Rešetnik I. 2022. Morphological and environmental variability of *Dianthus sylvestris* Wulfen (Caryophyllaceae) on the Balkan Peninsula. Botanical Journal of the Linnean Society. *In press*. doi: 10.1093/botlinnean/boac058.

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Terlević A, Koopman J, Więcław H, Rešetnik I, Bogdanović S (2021): *Carex phyllostachys* (Cyperaceae), a new species in Croatia. Acta botanica Croatica, 80, 106–111. doi:10.37427/botcro-2021-002.

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Tokić P, **Terlević A**, Ljubičić I, Rešetnik I, Bogdanović, S. (2022) Morphological variability of the *Dianthus ciliatus* complex (Caryophyllaceae) in the Adriatic region. Book of abstract, 7th Croatian Botanical Symposium.

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Terlević A, Bogdanović S, Rešetnik I. (2021) Morphological variability in *Dianthus sylvestris* Wulfen s.l. on the Balkan Peninsula. Book of Abstracts, 5th PhD Student Symposium.

Terlević A, Rešetnik I. (2020) Genome size variation in *Dianthus sylvestris* group (Caryophyllaceae) across the Balkan Peninsula. Book of Abstracts, 4th PhD Student Symposium.

Terlević A, Rešetnik I. (2019) Inventory of the historical *Dianthus sylvestris* herbarium material from ZA and ZAHO. Book of abstracts, 6th Croatian Botanical Symposium.

Terlević A, Jelaska, S. (2019) Chainsaw and axe still has the highest impact on temperate forest flora in Medvednica Mt., Croatia, over a 20-year period. Book of abstracts, 6th Croatian Botanical Symposium

Budisavljević A, Mihelić P, Papković D, Špadina B, **Terlević A**, Šegota, V. (2016) Vascular flora inventarisation and mapping in Dotrščina memorial park. Book of abstracts, 5th Croatian Botanical Symposium.

SCIENTIFIC PROJECTS:

(2018 – 2022) Research assistant on UIP-2017-05-2882 (AmphiAdriPlant - Phylogeography and evolution of three ecologically divergent groups of amphi-Adriatic plants)

(2020 – 2022) Collaborator on Croatia-Austria Bilateral project (Genome size evolution and polyploidisation in amphi-Adriatic *Aurinia* (Brassicaceae) and the *Cerastium tomentosum* and *Dianthus sylvestris* species groups (Caryophyllaceae))

WORKSHOPS:

(2022) Introduction to Bayesian Inference in Practice, Transmitting Science, online

(2022) Programming in Python, University Computing Centre SRCE, University of Zagreb

(2021) Introduction to Python programming, University Computing Centre SRCE, University of Zagreb

(2021) Environmental variables: how to obtain and process them with R, Transmitting Science, online

(2021) How to use Isabella Computer Cluster, University Computing Centre SRCE, University of Zagreb

(2021) Introduction to Bioinformatics for Biosystematics, ForBio – Research School in Biosystematics, The Arctic University Museum of Norway

(2020) Tidy data in R, University Computing Centre SRCE, University of Zagreb

(2020) Introduction to QGIS, Exatto d.o.o.

(2020) Basic programming in R, University Computing Centre SRCE, University of Zagreb

(2019) Introduction to R syntax and its application in basic statistical and graphical data analysis, University Computing Centre SRCE, University of Zagreb

(2019) 4th Int'l Summer School on Data Science, Faculty of Electrical Engineering, Mechanical Engineering and Naval Architecture, University of Split

(2013-2018) Popular scientific manifestation ″Biology Night″ at University of Zagreb, Faculty of Science, Biology Department

SCIENTIFIC TRAINING:

(2021) Scientific research in the Evolutionary Systematics laboratory, University of Innsbruck, Institute of Botany

(2020) Visit of the Plant Ecological Genetics research group at ETH Zürich, Institute of Integrative Biology, Department of Environmental Systems Science, ETH Zürich

(2019) Scientific research in the Evolutionary Systematics laboratory, University of Innsbruck, Institute of Botany

ORGANIZING EXPERIENCE:

(2022) Member of technical support at 7th Croatian botanical symposium, Zagreb, Croatia

(2019) Member of technical support at 6th Croatian botanical symposium, Zagreb, Croatia

(2017) Research and educational camp “Insula Tilagus” in organisation of Biology student association (BIUS)

(2016) Member of technical support at 2nd Croatian symposium on invasive species, Zagreb, Croatia

(2016) Research and educational camp “Mura – Drava” in organisation of Biology student association (BIUS)

(2015-2016) Head of Ornithology section in Biology student association (BIUS)

(2015) Research and educational camp “Papuk” in organisation of Biology student association (BIUS)

(2013) Research and educational camp “Apsyrtides” in organisation of Biology student association (BIUS)