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, 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: carnations – 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

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in taxonomy, and groups of populations differing from 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 *Dianthus 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 *Dianthus 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.*, 2022).

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 *Dianthus 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 short, 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* of 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,
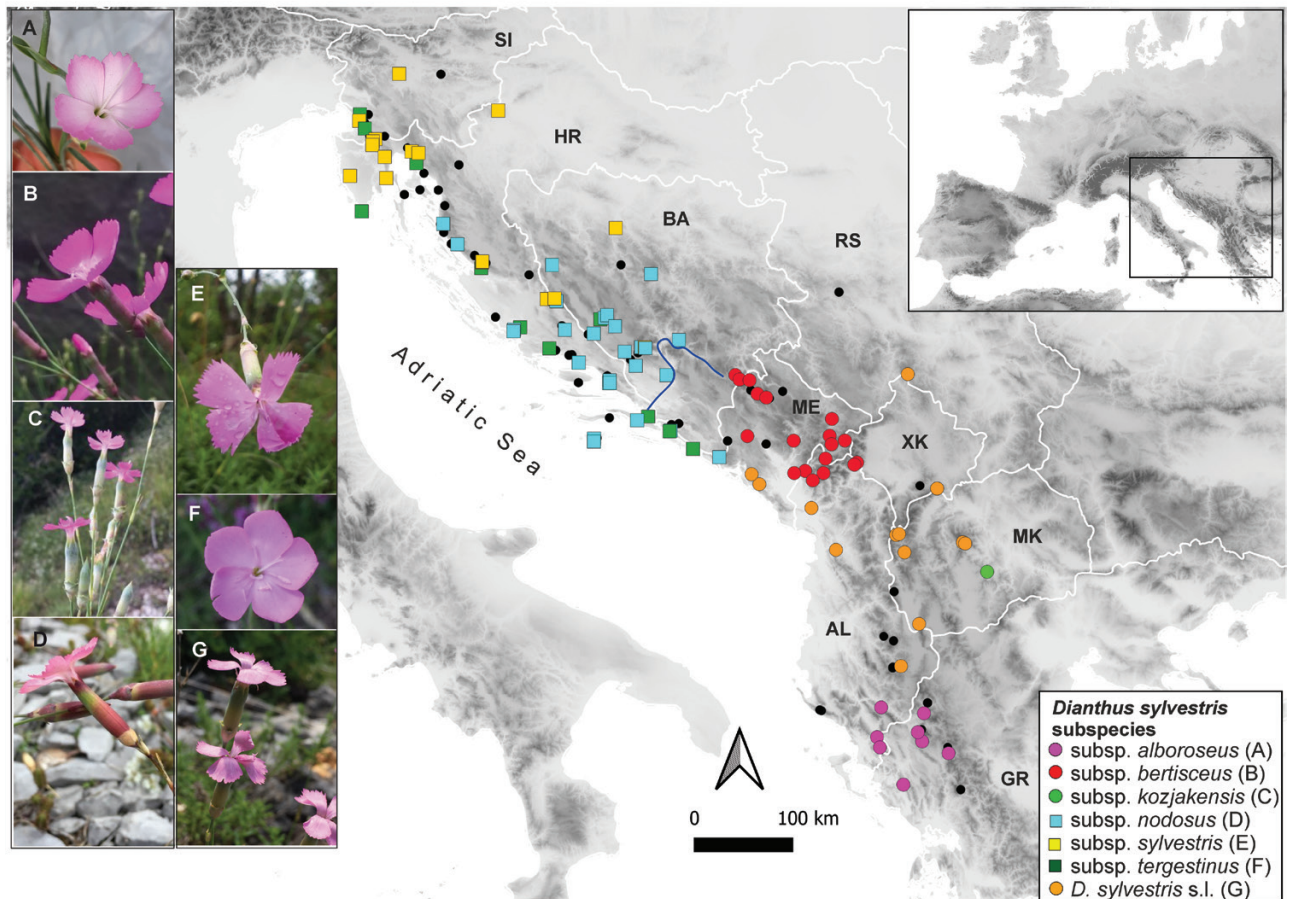
Figure 1. Localities of sampled populations of *Dianthus sylvestris* in the Balkan Peninsula used for characterizing environmental variability (*N* = 183 populations, black dots) and a subset for morphometric analyses (*N* = 97 populations; for details see Table S1, Supporting Information). Squares represent 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. Figure insets A – G represent subspecies of *D. sylvestris* in the Balkan Peninsula: A – *alboroseus*, B – *bertisceus*, C – *kozjakensis*, D – *nodosus*, E – *sylvestris*, F – *tergestinus*, G – *D. sylvestris sensu lato*.

2015), or used as diagnostic characters for subspecies (Hayek, 1924; Rechinger fil., 1935; Micevski, 1990; Meyer, 2011).

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 (van 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 ($r ≥ |0.85|$), 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, 2020). 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 ($P < 0.01$)
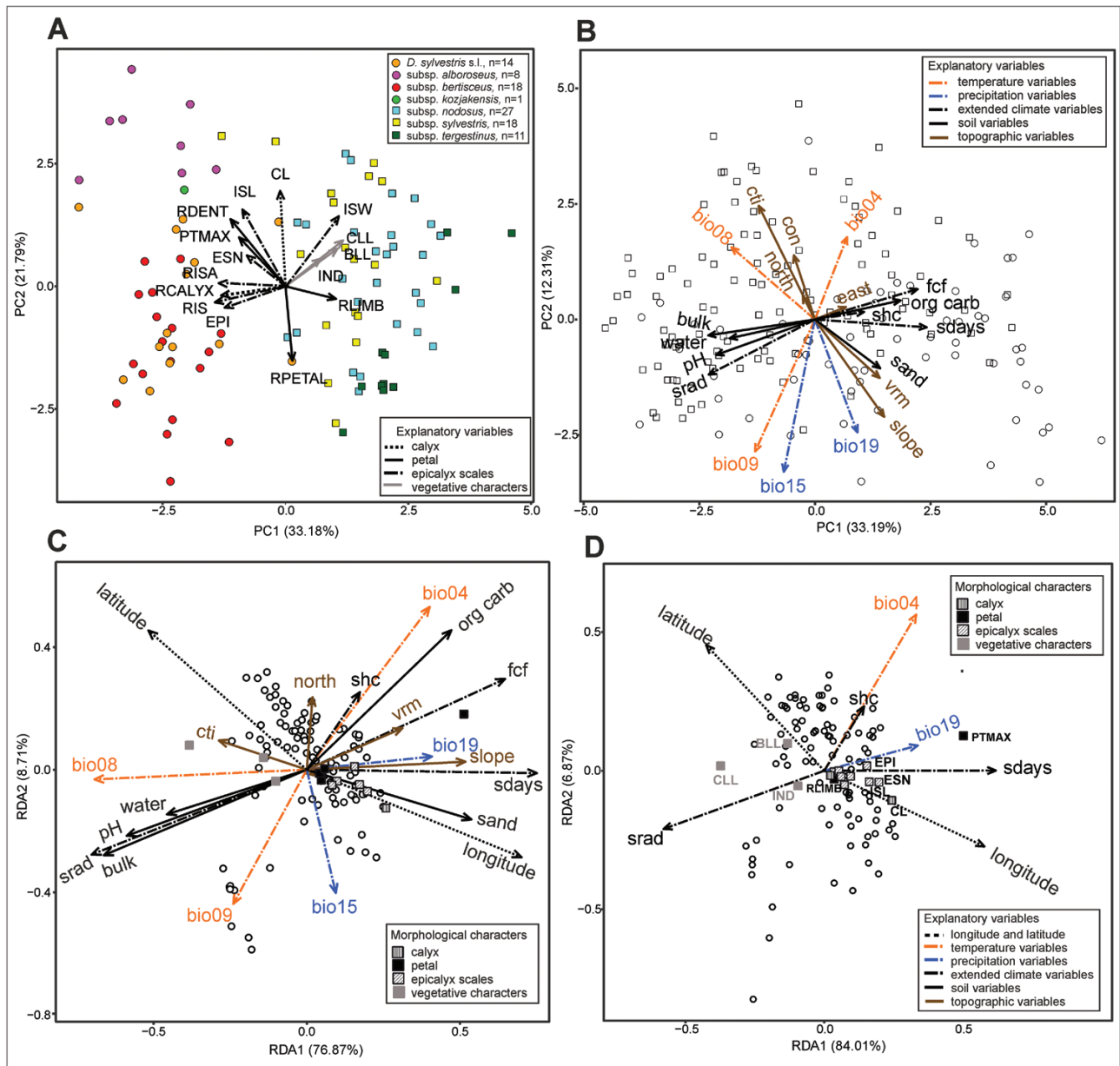
Figure 2. A, Principal component analysis (PCA) based on 15 morphological characters examined on 97 populations of *Dianthus sylvestris,* represented by the first two principal components; taxonomic identification at the intraspecific level, based on regional keys and floras, is projected using differently 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.

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 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 morphospace, 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 Figure S3, 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, 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

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**

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* 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 *Alyssum austrodalmaticum* Trinajstić and *Alyssum 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 (M. 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* subsp. *dalmaticus* (Čelak.) Hayek and *medunensis* (Beck et Szyszył.) Trinajstić 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.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY

The morphological and environmental datasets are available from the corresponding author upon request.

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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. 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 Spearman's 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.

Table S2. Chi-square test between each of the 15 pairs of *Dianthus sylvestris* subspecies, for 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* 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.