

Article

Leaf Size and Shape Show Contrasting Relationships with Evolutionary Lineages in *Daphne blagayana* Freyer

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Abstract

Phenotypic variation does not always correspond closely to genetic structure within species, particularly for morphological traits that are also influenced by environmental conditions. We examined whether leaf morphology in *Daphne blagayana* reflects previously identified phylogeographic structures and whether size- and shape-related traits differ in the hierarchical distribution of variation. Leaf morphometric analyses were performed on 3439 leaves collected from 596 shrubs in 21 populations covering the entire distribution range of the species. Ten leaf morphological traits were analysed using nested mixed-effects models, principal component analysis (PCA), MANOVA, Mantel tests and additional conservative mixed-effects models accounting for population-level structure. Leaf size-related traits showed stronger population-level differentiation and clearer geographic structuring, whereas shape-related traits exhibited higher within-shrub variation and weaker correspondence with phylogeographic structure. PCA and MANOVA revealed significant morphological differentiation among the three previously defined evolutionarily significant units (ESUs), although overlap among groups remained considerable. When population-level structure was taken into account, ESU effects were reduced but remained detectable for leaf length and selected shape-related traits. The northwestern ESU was characterized by larger and broader leaves, while differences between the central–southeastern and central ESUs were associated mainly with leaf shape traits. Mantel analysis revealed a weak but significant correlation between geographic and morphological distances among populations, indicating that geographic distance contributes to, but does not fully explain, morphological differentiation. The results show that leaf morphology in *D. blagayana* is associated with the previously identified phylogeographic structure of the species and that size- and shape-related traits differ markedly in their degree of geographic differentiation and hierarchical variation. However, because phylogeographic and geographic structure are spatially confounded in the present dataset, their individual effects on leaf morphology cannot be statistically separated. The study also highlights the importance of hierarchical sampling, appropriate statistical analyses, and cautious interpretation of genotype–phenotype correspondence in observational studies of intraspecific morphological variation.

Keywords: leaf morphology; leaf morphometrics; phylogeographic structure; population differentiation; evolutionarily significant units; intraspecific variation



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1. Introduction

Phenotypic variation does not always correspond closely to genetic structure within species. Common-garden and provenance studies have demonstrated local adaptation in forest tree populations [1], but plant traits can also change in response to environmental conditions [2]. Intraspecific trait variation is increasingly recognised as ecologically and evolutionarily important within and among plant populations and species [3,4]. Leaves are frequently used to study phenotype–genotype relationships, although their morphology is influenced by both environmental and developmental factors. Leaf size and shape affect important functional properties such as heat balance and resource use [5,6], and are themselves shaped by interacting genetic and environmental factors [7]. In long-lived shrubs, leaf traits may integrate inherited differentiation, developmental processes, local microenvironment, and plastic responses to temperature, water availability, and light. Morphological resemblance among populations should therefore not automatically be interpreted as evidence of genetic similarity [2,7]. In this study, we treat leaf phenotype as the outcome of interacting genetic and environmental influences rather than as a direct proxy for lineage identity. Intraspecific variation in leaf traits can be substantial both within and among populations [8].

It remains unclear to what extent leaf morphology reflects phylogeographic structure, and it can vary considerably even among geographically close populations. Previous studies have produced contrasting results. At broad floristic scales, climatic patterns in leaf morphology may largely reflect species turnover across environments rather than within-species plasticity [9]. In contrast, studies comparing historical and contemporary populations suggest that some leaf morphological traits may respond more consistently to climate change than physiological traits [10]. In particular, several authors have emphasized that leaf size and leaf shape are often best considered separately, with their allometric relationship explicitly taken into account, because apparent differences in shape may partly reflect size-related scaling rather than true differences in form [11].

Leaf morphometric data are hierarchically structured because leaves belong to individuals and individuals belong to populations. Variation within species should be analysed across these hierarchical levels rather than represented only by population means [12]. Recent studies suggest that both the amount and hierarchical distribution of within-species variation may carry important ecological information [4]. For multivariate phenotypes, evolutionary units can differ in two ways: they may differ in their average morphology, or in how variable their morphology is within the group [13]. Multivariate analyses may confound differences in group means with differences in within-group variability unless both are analysed separately [14]. Phenotype–genotype studies should examine not only differences in average morphology among lineages, but also how variation is distributed among individuals and populations, and whether some lineages are more variable than others.

Independently defined evolutionary units provide a useful framework for testing phenotype–genotype relationships. The concept of evolutionarily significant units (ESUs) was originally developed to identify historically independent lineages within species that may represent distinct evolutionary trajectories and therefore deserve separate conservation attention [15]. In practice, ESUs are typically defined using genetic or phylogeographic data independently of morphology, providing an opportunity to test whether phenotypic traits reflect evolutionary structure. More recent interpretations additionally emphasise the importance of adaptive potential and evolutionary diversity under changing environmental conditions [16]. The relationship between phenotypic traits and independently identified evolutionary units is also relevant for conservation and management of forest species. Adaptive responses may depend not only on genetic diversity itself, but also on how that diversity is reflected in phenotypic variation. Because morphological traits can be

assessed more easily than genetic markers in many field situations, they may provide complementary information for identifying patterns of intraspecific variation and for supporting conservation decisions. Understanding how phenotypic variation corresponds to independently defined evolutionary units may therefore contribute to forest genetic conservation by complementing molecular data on evolutionary diversity. This framework is especially relevant in southeastern Europe, where repeated climatic oscillations during the Quaternary strongly shaped the genetic structure and distribution history of many plant and animal taxa [17]. The Balkan Peninsula is considered one of the main European glacial refugial regions, characterised by long-term population persistence, repeated isolation in multiple refugia, and pronounced intraspecific lineage differentiation [18–20].

Daphne blagayana Freyer is a low evergreen, decumbent shrub of the family Thymelaeaceae, typically occurring on calcareous substrates, particularly limestone and dolomite, in open forests and rocky habitats. It is characterized by creeping woody stems, obovate leaves clustered towards the ends of branchlets, and dense terminal clusters of fragrant pale yellow to creamy-white flowers. The species has a fragmented native distribution extending from northeastern Italy and Slovenia through the western and central Balkans to Bulgaria, Greece, and the southern Romanian Carpathians, with its occurrence in Italy, Romania, Bulgaria, North Macedonia and Greece restricted to relatively small areas and small populations [21]. Owing to its restricted and disjunct distribution, it has attracted considerable floristic, ecological, and conservation interest. Leaf morphological characters have also been shown to provide valuable taxonomic information within the genus *Daphne* [22], suggesting that variation in leaf morphology may contribute to understanding evolutionary differentiation within this species. *Daphne blagayana* Freyer is a suitable system for testing these relationships because its evolutionary structure has already been identified independently of the present morphometric dataset. In a range-wide phylogeographic study based primarily on sequence variation in two chloroplast DNA regions, the *trnK-matK* region and the *psbA-trnH* intergenic spacer across 21 populations, [23] identified three major evolutionary lineages corresponding to northwestern, central, and southern clusters, which were interpreted as separate evolutionarily significant units (ESUs). Although these ESUs were defined using plastid DNA markers, they provide an independent evolutionary framework for testing the hypothesis that leaf morphological variation is associated with the previously inferred phylogeographic structure of the species. In that study, leaf morphology was analysed only in a relatively small number of individuals per population and was included mainly as a supplementary component of the broader phylogeographic interpretation. It was not used in the definition of the ESUs. The ESUs were therefore defined independently of the present morphometric analyses and provide a useful basis for testing whether leaf phenotype reflects underlying evolutionary structure. Correspondence between chloroplast-derived ESUs and leaf morphology is plausible if historical isolation has been accompanied by heritable phenotypic differentiation [24]. However, such correspondence need not be complete, because plastid markers capture only the maternal phylogeographic history of the species, whereas leaf morphology may additionally reflect nuclear-genetic effects, plasticity, and local adaptation [15,23]. We therefore expect partial rather than perfect concordance between ESU membership and leaf phenotype.

Morphological differentiation among populations may additionally reflect environmental and climatic gradients across the species' fragmented range. We therefore consider two non-exclusive hypotheses: first, that leaf morphology partly mirrors phylogeographic structure because historically isolated lineages differ phenotypically; and second, that some leaf traits vary in response to climatic or local environmental conditions independently of ESU membership [9,10]. Under the second hypothesis, geographically or climatically similar populations may converge morphologically despite different phylogeographic

affinities. We expected leaf size-related traits (e.g., leaf area, length and width) to be more strongly influenced by environmental conditions because of their high phenotypic plasticity, whereas shape-related traits were expected to be more conservative and thus more likely to reflect genetically based differentiation among evolutionary lineages. These expectations are consistent with previous studies on leaf morphological variation and development [7,25,26].

The aim of this study was to examine whether leaf morphology in *D. blagayana* reflects evolutionary structure and whether size- and shape-related traits differ in their degree of phylogeographic differentiation. We also examined how morphological variation is distributed across hierarchical levels, from leaves and individuals to populations and ESUs.

2. Materials and Methods

2.1. Sampling

Leaf material was collected from 21 natural populations covering the entire distribution range of *Daphne blagayana* (Table 1; Figure 1). In each population, we aimed to sample approximately 20 adult shrubs, or more when population size and field conditions allowed. In small or spatially restricted populations, fewer shrubs were sampled. The sample therefore ranged from 13–35 adult shrubs per population, resulting in a total of 596 sampled shrubs. To minimise the possibility of sampling ramets of the same genet, only shrubs located at least 10 m apart were sampled. Clonal independence was inferred from spatial separation in the field but was not genetically verified. Sampling was not strictly proportional to population size, and exact population sizes were not quantitatively estimated.

Table 1. Sampled populations and their assignment to evolutionarily significant units (ESUs).

No.	Acronym	Pop. Name	ESU *	Coordinates	Country
1	ITA	Inglagna Valley	1	46.220 N, 12.647 E	Italy
2	TG	Trebuša Valley	1	46.061 N, 13.844 E	Slovenia
3	ID	Idrija	1	46.052 N, 14.040 E	Slovenia
4	SA	Samija	1	46.016 N, 14.210 E	Slovenia
5	JE	Jetrbenk	1	46.097 N, 14.392 E	Slovenia
6	GRA	Gračnica Valley	1	46.127 N, 15.219 E	Slovenia
7	KP	Kozje	1	46.088 N, 15.534 E	Slovenia
8	MI	Mirtoviški potok	1	45.506 N, 14.794 E	Slovenia
9	SM	Smolin	2	44.463 N, 17.890 E	Bosnia and Herzegovina
10	MA	Maoča	2	45.136 N, 18.629 E	Bosnia and Herzegovina
11	KO	Konjuh	2	44.336 N, 18.514 E	Bosnia and Herzegovina
12	KR	Kreševo	2	43.868 N, 18.053 E	Bosnia and Herzegovina
13	ST	Stolac	3	43.779 N, 19.389 E	Bosnia and Herzegovina
14	CE	Čelebići	3	43.433 N, 18.987 E	Bosnia and Herzegovina
15	ME	Meštrovac	3	43.401 N, 19.016 E	Bosnia and Herzegovina
16	CG	Berane	3	42.506 N, 19.521 E	Montenegro
17	MK	Kožuf	3	41.150 N, 21.938 E	North Macedonia
18	GRC	Katara Pass	2	39.792 N, 21.205 E	Greece
19	BG	Stara planina	2	42.789 N, 24.559 E	Bulgaria
20	BV	Buila Vanturaita	2	45.225 N, 24.075 E	Romania
21	PM	Piatra Mare	2	45.557 N, 25.654 E	Romania

* Populations were assigned to evolutionarily significant units (ESUs) according to the phylogeographic structure identified by [23].

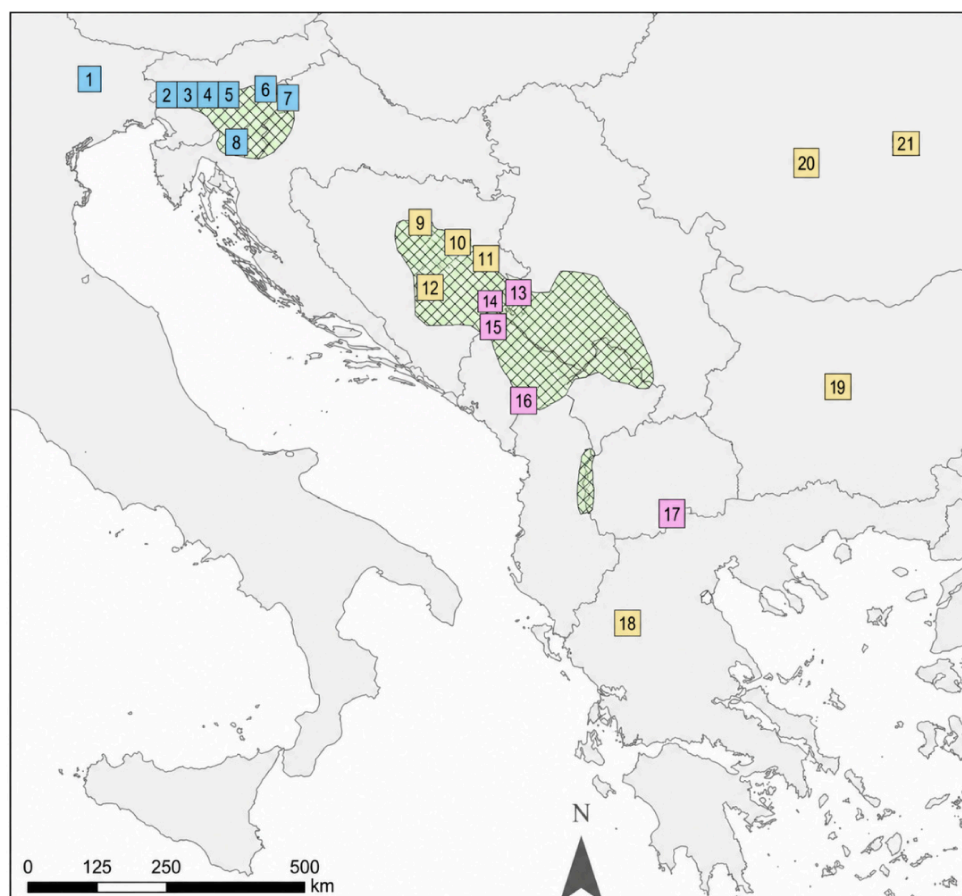


Figure 1. Locations of the 21 sampled populations of *Daphne blagayana* and their assignment to the three evolutionarily significant units (ESUs) defined by [23]: northwestern ESU (blue), central-southeastern ESU (yellow), and central ESU (purple). The green hatched areas indicate the main continuous parts of the species' distribution range, whereas small, isolated local occurrences, including some sampled populations, cannot be shown at this scale. Population numbers correspond to the localities listed in Table 1.

As *D. blagayana* is a small, low-growing shrub and individual shrubs often bear only a limited number of suitable leaves, entire shoots were collected from each sample shrub and transported to the laboratory. All fully developed and undamaged leaves were collected, while undeveloped and damaged leaves were excluded. The number of suitable leaves consequently varied among shrubs and ranged from 3 to 9. In total, 3439 leaves were pressed and dried as herbarium specimens before being digitized in colour using an Epson Expression 1680 flatbed scanner.

2.2. Morphometric Analysis

Leaf morphology was analysed using digital image analysis as described in previous morphometric studies of *Daphne blagayana* [23] and other woody plant taxa [27]. Scanned leaves were analysed using the image-processing software WinFOLIA Pro 2014 (Regent Instruments Inc., Quebec, QC, Canada) at a scanning resolution of 0.03 mm. The leaves of *D. blagayana* are simple, entire, smooth-margined and lack lobes or teeth. Therefore, classical linear and outline-based morphometric traits were used rather than landmark-based approaches, which require discrete homologous points that are more readily defined in leaves with distinct marginal features or pronounced lobing [28]. The selected traits (Figure 2; Table 2) describe the main components of leaf variation: overall size, proportions, position of maximum width, and the shape of the basal and distal parts of the blade. Leaf

area (A), length (L), and maximum width (W) were used as standard size descriptors, while the width/length ratio (W/L) and form coefficient (coef) described general leaf shape and elongation. The form coefficient was calculated as $4\pi A/P^2$, where A is leaf area and P is leaf perimeter, and corresponds to a circularity index used in WinFOLIA Pro 2014. The distance to maximum width (dW) described the position of the widest part of the leaf. W85 and W95 quantified distal narrowing and apex-region shape, whereas A10 and A25 described variation in the basal to lower-middle part of the leaf blade.

Table 2. List of morphological characters examined.

Abbr.	Morphological Trait
A	leaf area
L	leaf length
W	leaf width
W/L	leaf width/length ratio
coef	$4\pi A/P^2$ (P = leaf perimeter)
dW	distance from the leaf's base to the point of maximal width
W85	width of leaf on 85% of leaf's length from leaf's base up
W95	width of leaf on 95% of leaf's length from leaf's base up
A10	angle at 10% of leaf's length from leaf's base up
A25	angle at 25% of leaf's length from leaf's base up

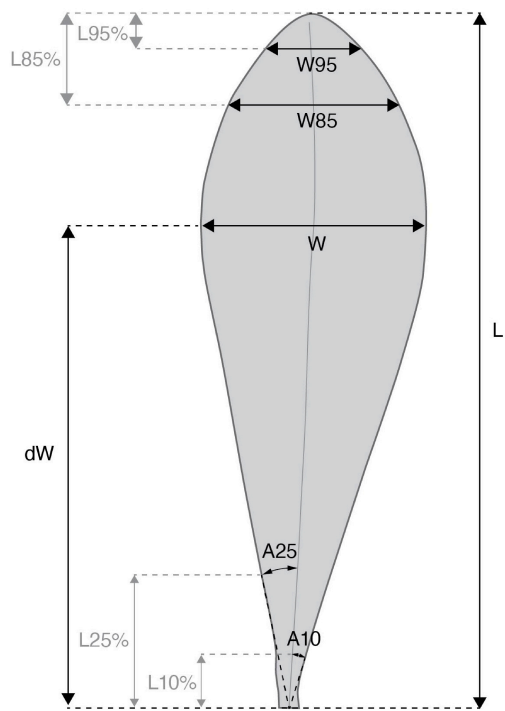


Figure 2. Measured leaf morphological traits (for description see Table 2, note: grey markings indicate auxiliary reference elements only).

2.3. Statistical Analyses

Because the number of leaves varied among shrubs, leaf measurements were first averaged at the shrub level. Shrub-level means were calculated as arithmetic means of all measured leaves from each shrub. Because each sampled shrub contributed at least three leaves, all sampled shrubs were included in these analyses. Shrub-level means were then used as the basic observational units for descriptive statistics, PCA, MANOVA, ANOVA and ESU-level comparisons. Descriptive statistics, including means and coefficients of variation (CV), were calculated for all analysed traits at the population level on the basis of

these shrub-level means. In contrast, nested linear mixed-effects models were fitted to the original leaf-level data to retain and estimate within-shrub variation.

Morphological variation was analysed using nested linear mixed-effects models according to the model:

$$Y = P + S(P) + \varepsilon$$

where P represents the fixed effect of population, $S(P)$ represents the random effect of shrub nested within population, and ε represents the residual variance component. The model accounted for the hierarchical structure of the dataset and was used to estimate the relative contributions of population-, shrub-level, and within-shrub components to total phenotypic variation. Variance percentages were derived from the marginal and conditional R^2 values of the mixed models: the population component was calculated as marginal R^2 , the shrub-level component as conditional R^2 minus marginal R^2 , and the within-shrub component as 1 minus conditional R^2 . All components were expressed as percentages.

Principal component analysis (PCA) was used as an exploratory ordination method to summarise the main axes of multivariate leaf morphological variation and to visualise morphological relationships among shrubs, populations and ESUs. Because several measured traits described partly overlapping aspects of leaf size and shape, variable selection was performed before PCA to reduce redundancy and improve biological interpretability. Pearson correlation coefficients were examined, and trait pairs with $|r| \geq 0.90$ were considered very highly correlated. This threshold was used as a diagnostic criterion, while final variable selection also considered biological interpretability, contribution to ordination structure, and representation of different aspects of leaf morphology. Preference was given to traits describing distinct components of leaf morphology, including leaf size, maximum width, distal narrowing and basal blade shape. Formal allometric correction was not applied; instead, size- and shape-related traits were analysed and interpreted separately. To further support variable selection, hierarchical clustering of traits was performed using a distance matrix derived from Pearson correlations ($1 - |r|$); the resulting dendrogram is provided in Appendix A Figure A1, and the full correlation matrix in Appendix A Table A1. Consequently, seven informative morphological variables were retained for PCA: W85, W, W95, L, A25, coef, and A10.

PCA was performed in IBM SPSS Statistics, version 29.0.2.0, using the Dimension Reduction–Factor procedure, with varimax rotation and Kaiser normalization. PCA scores were calculated for individual shrubs; for graphical presentation, population centroids were obtained by averaging shrub scores within each population and plotted in the ordination space, while convex hulls were used to illustrate the distribution and overlap of ESU groups. The suitability of the dataset for PCA was evaluated using Bartlett’s test of sphericity and the Kaiser–Meyer–Olkin (KMO) measure of sampling adequacy.

ESUs were adopted from the phylogeographic structure identified by [23], who recognised three evolutionary lineages based on chloroplast DNA variation across the species’ distribution range. Morphological differentiation among the three ESUs was assessed using multivariate analysis of variance (MANOVA) based on all analysed leaf traits simultaneously. MANOVA was used as the primary multivariate test of ESU differentiation, while subsequent univariate ANOVAs and Bonferroni-adjusted pairwise comparisons were used as follow-up analyses to identify which traits contributed to the multivariate pattern. Statistically homogeneous groups were identified by shared letters. Assumptions of homogeneity of covariance matrices and homogeneity of variances were assessed using Box’s M test and Levene’s test, respectively. Because Box’s M test indicated unequal covariance matrices among ESUs, Pillai’s Trace was used as the primary MANOVA test statistic, as it is more robust to violations of the homogeneity of covariance matrices.

To further account for population-level structure in ESU comparisons, linear mixed-effects models were fitted for each trait using shrub-level mean values, with ESU as a fixed effect and population as a random intercept. Models were fitted by restricted maximum likelihood (REML) using the MIXED procedure in IBM SPSS Statistics, with Satterthwaite approximation for denominator degrees of freedom. Bonferroni-adjusted pairwise comparisons among ESUs were calculated from estimated marginal means.

The relationship between morphological differentiation and geographical distance among populations was evaluated using a Mantel test with 9999 permutations. Morphological distances were calculated as Euclidean distances between population centroids in the space defined by the first two principal components, whereas geographical distances were calculated from population coordinates. Statistical significance was evaluated at $p < 0.05$. A partial Mantel test was not applied because no independent genetic distance matrix was available. Furthermore, the strong spatial association between ESU membership and geographic position in the present dataset would not allow an unambiguous separation of geographic and evolutionary effects. Most statistical analyses were performed in IBM SPSS Statistics, version 29.0.2.0, while the Mantel test was performed in GenAlEx 6.51 [29].

3. Results

Descriptive statistics for all analysed leaf traits and populations are provided in Appendix A Table A2. Leaf size traits varied considerably among populations, with the largest leaves occurring mainly in northwestern populations (e.g., KP, GRA, SA), while populations from Bulgaria and Greece tended to have smaller and narrower leaves. Coefficients of variation were generally low to moderate across populations, indicating relatively low within-population variability, although some Romanian populations (especially BV) exhibited elevated variability for several traits.

Nested analyses of variance revealed significant differentiation among populations for all analysed leaf traits ($p < 0.001$; Table 3), indicating clear hierarchical variation in leaf morphology in *Daphne blagayana*.

Table 3. *F*-ratios for analysed traits from the nested analyses of variance and partitioning of total variance by hierarchical level for studied leaf morphological traits. Significance level: *** $p < 0.001$.

Trait	<i>F</i> -Ratios	Among Population (%)	Among Shrub (%)	Within Shrub (%)
A	24.8 ***	39.5	45.5	15.0
L	28.2 ***	41.9	42.5	15.6
W	22.7 ***	36.4	44.9	18.7
W/L	17.2 ***	29.2	46.6	24.2
coef	16.7 ***	27.9	45.8	26.3
dW	8.8 ***	10.7	25.9	63.4
W85	19.5 ***	31.9	45.2	22.9
W95	16.9 ***	26.9	42.4	30.7
A10	11.6 ***	18.1	39.7	42.2
A25	13.7 ***	22.0	42.1	35.9

Leaf size-related traits, particularly leaf area (A), leaf length (L), and leaf width (W), showed the strongest among-population variance components with a relatively large proportion of variance explained at the population level. In contrast, traits associated with finer aspects of leaf shape and distal morphology (especially dW, A10, and A25) showed substantially higher within-shrub variance.

Intermediate patterns were observed for general shape descriptors such as W/L ratio and form coefficient, which combined moderate population differentiation with relatively

consistent expression within shrubs. Overall, the hierarchical distribution of variance differed markedly among traits.

The suitability of the dataset for PCA was supported by a significant Bartlett’s test of sphericity ($\chi^2 = 7530$, $df = 21$, $p < 0.001$), while the KMO value indicated moderate but acceptable sampling adequacy (KMO = 0.660). Two principal components with eigenvalues greater than 1 were extracted, together explaining 91.8% of the total variance (Figure 3). PC1 accounted for 48.4% of the variance and was mainly defined by leaf size and width-related traits, particularly W85 (29.0%), W (28.2%), W95 (26.4%), and L (15.2%) (Table 4). Positive PC1 values therefore indicated broader and generally larger leaves, whereas negative PC1 values indicated narrower and smaller leaves. PC2 explained an additional 43.45% of the variance and was mainly associated with leaf shape traits, especially A25 (31.1%), coef (30.2%), and A10 (27.6%). Positive PC2 values indicated leaves with higher form coefficient and larger basal angles, corresponding to relatively broader or more rounded blade shape, whereas negative PC2 values indicate more elongated leaves with lower values of these shape descriptors.

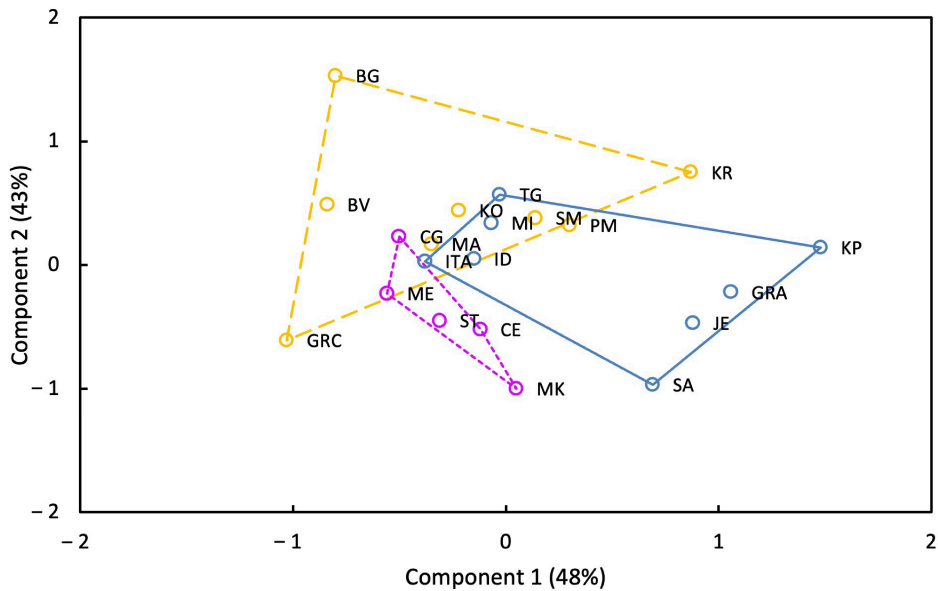


Figure 3. Principal component analysis (PCA) of population centroids based on leaf morphological traits of *Daphne blagayana*. Population grouping follows the three evolutionarily significant units (ESUs) previously identified from chloroplast DNA variation by [23]: northwestern (solid blue line), central-southeastern (dashed yellow line), and central (dotted purple line).

Table 4. Rotated component loadings of leaf morphological traits on the first two principal components (PC1 and PC2) and percentage contribution of each trait to the corresponding component.

Trait	PC1	PC2	Contribution * to PC1 (%)	Contribution to PC2 (%)
W85	0.991	−0.050	29.0	0.1
W	0.977	−0.020	28.2	0.0
W95	0.946	0.006	26.4	0.0
L	0.718	−0.579	15.2	11.0
A25	−0.092	0.972	0.2	31.1
coef	0.102	0.959	0.3	30.2
A10	−0.147	0.916	0.6	27.6

* Trait contributions were calculated from squared rotated loadings and expressed as percentages of the total squared loadings for each component.

Population centroids showed partial grouping according to the previously defined ESUs. Considerable overlap remained, but the combined PC1-PC2 morphospace revealed clearer differentiation than either axis alone. Populations of the northwestern ESU were mainly positioned toward positive PC1 and negative PC2 values, corresponding to generally larger and broader leaves with comparatively lower shape-related scores. The central-southeastern ESU occupied a broader and partly intermediate position in the ordination space, with substantial overlap with both the northwestern and central ESUs. In contrast, the central ESU was positioned mainly toward negative PC1 and negative PC2 values, indicating generally smaller or narrower leaves with lower scores for the shape-related traits associated with PC2.

Box's M test indicated heterogeneity of covariance matrices among ESUs (Box's $M = 357.290$, $p < 0.001$); therefore, Pillai's Trace was used for interpretation of the MANOVA. MANOVA revealed significant multivariate morphological differentiation among the three phylogeographically defined ESUs (Pillai's Trace = 0.461, $F = 25.161$, $p < 0.001$). Levene's tests indicated unequal variance for some traits. Accordingly, univariate ANOVAs were interpreted as follow-up analyses to the primary MANOVA. Univariate analyses confirmed significant differences among ESUs for all examined leaf traits (Table 5).

Table 5. Mean values ($\pm$ SD) of leaf morphological traits in the three evolutionarily significant units (ESUs) of *Daphne blagayana*. Different letters indicate significant differences among ESUs according to Bonferroni post hoc comparisons ($p < 0.05$).

Trait	ESU1 ($n = 206$)	ESU2 ($n = 234$)	ESU3 ($n = 156$)	F ($df = 2, 593$)	p
L	4.30 $\pm$ 0.71 a	3.93 $\pm$ 0.73 b	3.50 $\pm$ 0.66 c	71.4	<0.001
W	1.49 $\pm$ 0.25 a	1.30 $\pm$ 0.20 b	1.30 $\pm$ 0.26 b	41.3	<0.001
coef	0.506 $\pm$ 0.057 a	0.492 $\pm$ 0.061 a	0.544 $\pm$ 0.067 b	39.2	<0.001
W85	1.12 $\pm$ 0.21 a	0.97 $\pm$ 0.16 b	0.98 $\pm$ 0.21 b	34.5	<0.001
W95	0.63 $\pm$ 0.14 a	0.53 $\pm$ 0.10 b	0.55 $\pm$ 0.14 b	31.5	<0.001
A10	18.46 $\pm$ 3.55 a	16.52 $\pm$ 3.65 b	19.53 $\pm$ 4.41 c	27.3	<0.001
A25	17.05 $\pm$ 3.04 a	16.52 $\pm$ 2.90 a	18.94 $\pm$ 3.57 b	31.9	<0.001

The northwestern ESU was characterized by significantly larger and wider leaves, showing the highest values for leaf length (L), width (W), W85, and W95. In contrast, differentiation between the central-southeastern and central ESUs was expressed primarily through leaf shape rather than overall leaf size. Although these two groups did not differ significantly in width-related traits, they showed clear differences in form coefficient, A10, and A25, indicating differences in leaf elongation and distal shape. Overall, the central ESU had more elongated and distally expanded leaves, whereas the central-southeastern ESU showed generally intermediate leaf size and lower values of most shape-related parameters.

Additional mixed-effects models, with ESU as a fixed effect and population as a random intercept, showed that ESU effects were reduced when population-level structure was explicitly accounted for (Appendix A Table A3). ESU remained significant for leaf length, coef and A25, whereas width-related traits showed only marginal or non-significant ESU effects. These results indicate that part of the ESU-related differentiation reflects population-level structure, but the main pattern of differentiation, especially in leaf length and selected shape-related traits, remained detectable.

The Mantel analysis revealed a weak but significant positive correlation between geographic and morphological distances among populations of *Daphne blagayana* ($r = 0.234$, $p = 0.028$, 9999 permutations). Geographically more distant populations tended to show greater morphological differentiation, although the relationship remained weak.

4. Discussion

Leaf morphological variation in *Daphne blagayana* was clearly structured across hierarchical levels, but the pattern differed between size- and shape-related traits. Size-related traits showed stronger differentiation among populations and lower variation within shrubs, whereas finer aspects of leaf shape varied more strongly among leaves of the same shrub. Leaf morphology also partly reflected the previously identified phylogeographic structure, although considerable variation remained among populations and within ESUs. The relationship between morphology and evolutionary lineages thus differed among traits and hierarchical levels.

The present study extends the earlier phylogeographic study of [23], in which leaf morphology was included only as a supplementary dataset to support the plastid DNA analyses. The larger morphometric dataset and hierarchical sampling design used here allowed variation to be analysed across hierarchical levels (leaf, individual, population and ESU) rather than only among populations. This approach enabled a more detailed assessment of morphological differentiation within and among the previously identified evolutionary lineages and a clearer evaluation of the relationship between leaf morphology and phylogeographic structure.

Traits associated with overall leaf size and width were comparatively stable within shrubs, while their mean values differed considerably among populations. Similar geographic differentiation in leaf dimensions has been reported in European woody species. In European oaks, traits describing leaf size, lobe morphology and leaf proportions showed clear differentiation among populations and taxonomic groups despite substantial intraspecific variation [30]. Leaf morphological differentiation may also be detectable when correspondence with molecular genetic structure is incomplete, as reported for closely related *Quercus* taxa, where several leaf shape traits discriminated taxa more clearly than neutral molecular markers [31]. In widely distributed tree populations, leaf size and growth-related traits were associated with geographic and climatic gradients, while genetic population structure also contributes to the observed pattern [32]. The results for *D. blagayana* are consistent with this broader pattern: size-related traits show clear population and geographic structure but are not direct markers of evolutionary lineages.

The PCA indicated that differentiation among ESUs was not based only on overall leaf size but also on differences in leaf shape. The northwestern ESU was characterised by larger and broader leaves, whereas the central ESU generally had smaller and narrower leaves. In contrast, the central–southeastern ESU showed intermediate leaf morphology, overlapping with both other ESUs. These patterns indicate that morphological differentiation among ESUs involved both leaf size and leaf shape rather than a single morphological gradient.

Larger and broader leaves in the northwestern ESU were among the clearest morphological patterns in the study and may reflect the combined influence of phylogeographic history and local environmental conditions. However, this interpretation remains hypothetical because environmental variables were not included in the present analyses. This hypothesis is nevertheless consistent with the fact that the northwestern ESU represents the most distinct plastid DNA lineage identified in the previous phylogeographic study [23] and also shows the clearest morphological differentiation in the present analysis. Similar concordance between phylogeographic structure and phenotypic differentiation, together with the modifying influence of local environmental conditions, has been reported in other plant species [33]. Although the distinct plastid lineage suggests a genetic contribution to this pattern, the larger leaves of northwestern populations should not be interpreted as purely genetically determined. Leaf morphology can respond strongly to local environmental heterogeneity [9]. Light availability is one possible factor, as substantial plasticity in leaf traits in response to contrasting light environments has been reported in both trees [34]

and shrubs [35,36]. Water availability and temperature may also influence leaf size through their effects on heat balance and boundary-layer properties [5]. One possible explanation is that larger leaves in northwestern populations may be associated with more mesic, cooler or shaded habitats, while altitude, soil depth, water availability and soil conditions may also contribute [2]. The contrasting hierarchical structure of size- and shape-related traits may reflect differences in their sensitivity to environmental and developmental influences. Leaf size may respond more consistently to broad-scale environmental variation, whereas finer aspects of leaf shape may be influenced more strongly by developmental processes and microenvironmental variation within shrubs. Nevertheless, the shape-related traits examined here are not entirely independent of leaf size, as indicated by the weak but non-zero correlations between some shape variables and leaf area (Table A1). This interpretation is consistent with broader studies showing that leaf size is closely associated with climatic variables such as temperature and precipitation, whereas different aspects of leaf shape may respond differently to environmental gradients and reflect the combined effects of climate, developmental constraints and evolutionary history [37–39]. These hypotheses should be tested in future studies incorporating environmental variables.

The contrast with shape-related traits was clear. Traits associated with leaf apex shape and leaf proportions, particularly dW , A10 and A25, showed substantially higher within-shrub variation. Variation among leaves of the same plant may result from developmental differences, leaf position and local microenvironmental conditions. Leaf shape reflects interacting developmental and environmental influences in addition to evolutionary history [7]. Strong within-plant variation in leaf shape has also been documented in woody plants, particularly where developmental stage or local growing conditions affect leaf development [40,41]. In these studies, developmental changes primarily involved transitions in leaf proportions and overall leaf shape during plant ontogeny. Although our study did not examine heteroblasty, these studies illustrate how strongly leaf shape can vary within woody individuals. The high within-shrub variation in dW , A10 and A25 observed in *D. blagayana*, all describing leaf proportions or the position of maximum leaf width, is consistent with greater variability in these finer aspects of leaf shape.

Shape-related traits were nevertheless not entirely unstructured. PC2 separated combinations of A25, form coefficient and A10, and differences between the central–southeastern and central ESUs were associated mainly with shape rather than width-related traits. The mixed-effects models also retained significant ESU effects for form coefficient and A25. Thus, high within-shrub variation does not mean that shape-related traits lack geographic or evolutionary information. Some shape-related traits combine substantial local variation with differentiation among broader groups. Analyses based only on population or ESU means would not reveal how differently size- and shape-related variation is distributed among leaves, shrubs and populations. One of the main strengths of the present study is its hierarchical sampling design, which enabled phenotypic variation to be quantified at each nested biological level. Intraspecific trait variation can occur at all these levels, and their relative contributions differ among traits [3,8,12]. Such stratified analyses provide biological insight beyond simple comparisons of group means by helping to distinguish developmental and microenvironmental variation within individuals from population differentiation and broader evolutionary structure.

The multivariate analyses showed significant morphological differentiation among the three ESUs, but the mixed-effects models gave a more conservative result. In the initial MANOVA and follow-up ANOVAs, all analysed traits differed significantly among ESUs. When population was included as a random intercept, ESU effects were reduced and width-related traits became marginal or non-significant, while significant effects remained mainly for leaf length, form coefficient and A25. Part of the apparent ESU-related differen-

tiation reflects differences among populations within the geographically structured ESUs. However, the remaining effects show that the correspondence between leaf morphology and ESU structure is not entirely explained by population clustering.

This is particularly important because the ESUs are geographically structured. The northwestern ESU is geographically separated from the other groups, whereas the other two ESUs cover a broad and partly overlapping part of the species range. ESU membership, geographic position and local environmental conditions are spatially confounded in the present dataset and therefore cannot be statistically separated. Comparable patterns occur in other widespread woody species, where phenotypic variation reflects the combined influence of environmental gradients, phenotypic plasticity and genetic population structure [2,32]. Common-garden or reciprocal-transplant studies would be needed to separate inherited differentiation from phenotypic plasticity and test local adaptation directly.

The weak but significant Mantel correlation is consistent with this interpretation and suggests that geographic distance contributes to morphological differentiation, but represents only one of several interacting processes. Similar patterns are commonly observed in widespread plant species, where phenotypic variation reflects the combined effects of geographic isolation, environmental heterogeneity and historical population processes [42]. A contrasting pattern was found in Balkan populations of *Juniperus oxycedrus* L. subsp. *oxycedrus*, where extensive leaf morphological variation showed no clear geographic structure [27]. In *D. blagayana*, isolation by distance alone cannot explain the observed variation, and phylogeographic history, population differentiation and environmental heterogeneity probably all contribute.

The three ESUs were identified independently of the present morphometric dataset and were based primarily on two chloroplast DNA regions [23]. However, plastid markers represent only one component of population history and do not provide a genome-wide measure of genetic differentiation [43]. Consequently, complete correspondence between plastid lineages and quantitative morphological traits should not be expected, particularly for traits that are also influenced by environmental conditions and phenotypic plasticity. Similar patterns have been reported in European oaks, where clear leaf morphological differentiation may coexist with incomplete differentiation in molecular markers [31]. Accordingly, leaf morphology should be regarded as complementary rather than alternative evidence for identifying evolutionary lineages within the species.

The observed pattern is also consistent with the earlier conclusion that the previously proposed infraspecific taxa of *D. blagayana* (*D. blagayana* subsp. *lerchenfeldiana*, var. *kellereri* and subsp. *zogovicii*) are not supported as distinct evolutionary or morphological groups [23]. Rather than supporting discrete infraspecific entities, the present results indicate that morphological variation is largely continuous across populations and only partly structured by phylogeographic history. Although the present study was not designed as a taxonomic revision, the substantially larger morphometric dataset provides no additional evidence supporting the recognition of these taxa as distinct infraspecific units.

Several limitations should be considered. Environmental variables such as temperature, precipitation, altitude, soil properties, soil moisture and light availability were not included, so their effects cannot be separated from geographic and phylogeographic structure. Clonal independence of sampled shrubs was inferred from spatial separation but was not genetically verified. Size- and shape-related traits were analysed separately and the two main PCA axes largely separated these components. Formal allometric correction was also not applied, and some shape-related differences may partly reflect scaling with leaf size. These limitations argue against interpreting morphological differentiation as direct evidence of genetic or adaptive divergence.

From a conservation perspective, the results support the biological relevance of the previously identified ESUs, but also show considerable morphological variation among populations and within shrubs. Conservation focused only on major phylogeographic units could overlook part of the phenotypic variation within the species. This highlights the value of combining molecular and morphological information in forest genetic studies, where both evolutionary history and phenotypic diversity are relevant for identifying conservation priorities. For a woody species with a fragmented distribution and many small, isolated populations, maintaining populations across the distribution range may help preserve both historical differentiation and locally expressed phenotypic variation. This is also relevant to forest genetic conservation, where genetic lineages, population differentiation and phenotypic responses to local conditions do not necessarily follow the same spatial patterns [1,16].

Overall, leaf morphology in *D. blagayana* was associated with the previously identified phylogeographic structure of the species, but the relationship differed among traits and was strongly influenced by population-level variation. Because phylogeographic structure, geographic position and environmental heterogeneity are spatially confounded in the present dataset, their individual contributions to leaf morphological variation cannot be statistically separated. Size-related traits showed clearer population and geographic differentiation, whereas finer aspects of leaf shape varied more strongly within shrubs while still contributing to differences among some ESUs. Leaf morphology therefore provides valuable complementary information on evolutionary and geographic differentiation, but represents only one part of the variation within the species.

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Appendix A

Table A1. Pearson correlation coefficients among the analysed leaf morphological traits of *Daphne blagayana*.

Trait	A	L	W	W/L	coef	dW	W85	W95	A10
L	0.914								
W	0.938	0.758							
W/L	−0.064	−0.423	0.248						
coef	−0.193	−0.541	0.084	0.947					
dW	0.097	0.162	0.085	−0.17	−0.336				
W85	0.880	0.704	0.952	0.260	0.063	0.314			
W95	0.770	0.578	0.861	0.316	0.110	0.389	0.96		
A10	−0.299	−0.539	−0.158	0.621	0.779	−0.478	−0.207	−0.163	
A25	−0.279	−0.572	−0.089	0.757	0.901	−0.521	−0.156	−0.120	0.913

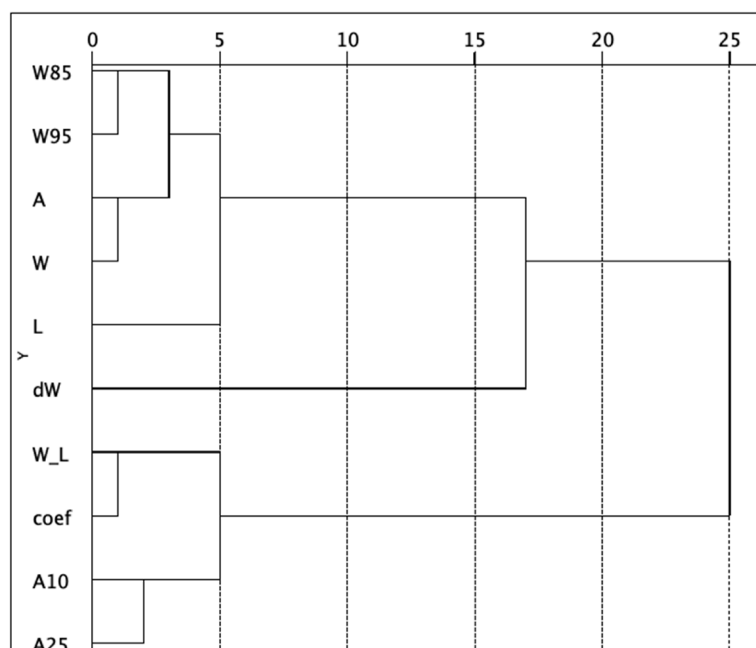


Figure A1. Hierarchical clustering of leaf morphological traits based on Pearson correlation similarity and average linkage.

Table A2. Mean values and coefficients of variation (CV, %) for leaf morphological traits across studied populations of *Daphne blagayana*.

Pop			A	L	W	W_L	coef	dW	W85	W95	A10	A25
1	ITA	mean	2.93	3.63	1.24	0.35	0.49	0.63	0.95	0.55	19.32	18.33
		CV (%)	22.53	14.33	12.10	14.29	12.24	4.76	11.58	14.55	17.65	14.62
2	TG	mean	3.28	3.75	1.40	0.39	0.56	0.58	1.00	0.53	20.12	19.79
		CV (%)	26.22	13.33	14.29	2.56	3.57	5.17	16.00	16.98	8.35	6.52
3	ID	mean	3.36	4.02	1.34	0.35	0.50	0.61	0.98	0.54	20.84	17.55
		CV (%)	16.67	10.45	9.70	5.71	6.00	3.28	9.18	11.11	10.99	9.06
4	SA	mean	4.29	4.79	1.54	0.34	0.46	0.64	1.18	0.66	15.14	13.82
		CV (%)	19.58	8.77	13.64	11.76	10.87	3.13	13.56	18.18	17.04	18.81
5	JE	mean	4.53	4.72	1.56	0.35	0.49	0.64	1.20	0.70	17.04	15.74
		CV (%)	28.04	15.25	14.10	11.43	12.24	4.69	17.50	21.43	20.95	20.20
6	GRA	mean	4.60	4.57	1.62	0.37	0.51	0.63	1.26	0.71	17.31	16.39
		CV (%)	28.91	16.41	14.81	13.51	11.76	3.17	16.67	21.13	18.95	16.60
7	KP	mean	5.05	4.66	1.76	0.39	0.53	0.62	1.31	0.77	18.44	17.50
		CV (%)	20.40	12.23	10.80	7.69	7.55	3.23	11.45	14.29	17.57	14.06
8	MI	mean	3.37	3.92	1.37	0.36	0.53	0.61	1.00	0.54	20.85	19.00
		CV (%)	17.51	11.99	10.22	11.11	9.43	4.92	14.00	20.37	14.58	13.42
9	SM	mean	3.14	3.63	1.39	0.39	0.55	0.64	1.07	0.60	19.14	18.42
		CV (%)	23.57	16.25	12.23	7.69	10.91	4.69	13.08	13.33	27.64	24.16
10	MA	mean	2.82	3.50	1.23	0.36	0.52	0.65	0.97	0.56	19.30	18.30
		CV (%)	26.95	14.00	14.63	8.33	7.69	4.62	16.49	21.43	16.79	13.83
11	KO	mean	2.67	3.29	1.26	0.39	0.55	0.65	1.01	0.58	19.74	18.50
		CV (%)	30.71	15.81	16.67	10.26	9.09	4.62	18.81	24.14	17.93	14.86
12	KR	mean	4.35	4.16	1.62	0.40	0.57	0.61	1.19	0.65	20.59	20.32
		CV (%)	25.75	13.22	15.43	10.00	8.77	4.92	17.65	21.54	16.85	14.27

Table A2. Cont.

Pop			A	L	W	W_L	coef	dW	W85	W95	A10	A25
13	ST	mean	3.30	3.98	1.31	0.34	0.49	0.62	0.96	0.52	16.61	16.60
		CV (%)	28.79	16.33	16.79	11.76	12.24	6.45	19.79	23.08	24.44	19.88
14	CE	mean	3.16	3.85	1.33	0.36	0.50	0.64	1.02	0.57	15.13	15.80
		CV (%)	17.72	9.87	11.28	11.11	10.00	3.13	11.76	14.04	19.89	16.71
15	ME	mean	2.67	3.48	1.21	0.36	0.51	0.64	0.93	0.52	17.10	16.73
		CV (%)	26.97	15.23	15.70	11.11	9.80	4.69	17.20	21.15	20.12	15.00
16	CG	mean	2.64	3.44	1.21	0.37	0.53	0.63	0.93	0.53	19.14	18.60
		CV (%)	29.17	17.15	14.05	10.81	11.32	4.76	13.98	15.09	19.49	14.46
17	MK	mean	4.21	4.77	1.40	0.30	0.44	0.62	1.02	0.54	15.29	15.43
		CV (%)	22.33	12.79	15.00	13.33	13.64	4.84	15.69	22.22	18.64	16.53
18	GRC	mean	2.54	3.58	1.11	0.32	0.47	0.62	0.82	0.44	16.32	16.29
		CV (%)	30.71	15.36	18.02	12.50	10.64	3.23	19.51	22.73	16.42	12.22
19	BG	mean	2.10	2.79	1.14	0.42	0.62	0.61	0.85	0.47	24.12	22.93
		CV (%)	25.71	13.62	13.16	9.52	8.06	3.28	15.29	19.15	13.43	11.51
20	BV	mean	2.43	3.19	1.19	0.39	0.56	0.60	0.84	0.44	19.76	19.60
		CV (%)	40.33	22.26	19.33	10.26	10.71	5.00	19.05	20.45	28.90	19.80
21	PM	mean	3.33	3.70	1.41	0.40	0.55	0.63	1.10	0.64	18.15	18.38
		CV (%)	37.54	18.92	19.86	12.50	12.73	3.17	19.09	20.31	23.36	19.04

Table A3. Results of conservative linear mixed-effects models testing ESU effects on leaf morphological traits, with population included as a random intercept.

Trait	F	df	p	Bonferroni Pairwise Significant Differences
L	5.679	2, 17.939	0.012	ESU1–ESU3
W	3.430	2, 18.042	0.055	none
coef	4.154	2, 17.842	0.033	ESU2–ESU3
W85	2.916	2, 17.916	0.080	none
W95	2.695	2, 17.775	0.095	none
A10	3.398	2, 17.844	0.056	none
A25	3.584	2, 17.701	0.049	none

Models were fitted using REML on shrub-level mean values. ESU was included as a fixed effect and population as a random intercept. Numerator *df* = 2 for all tests; denominator *df* were estimated using the Satterthwaite approximation. Pairwise comparisons among ESUs were Bonferroni-adjusted.

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