

Extent of intraspecific trait variability in ecologically central and marginal populations of a dominant alpine plant across European mountains

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- **Background and Aims** Studying trait variability and restricted gene flow between populations of species can reveal species dynamics. Peripheral populations commonly exhibit lower genetic diversity and trait variability due to isolation and ecological marginality, unlike central populations experiencing gene flow and optimal conditions. This study focused on *Carex curvula*, the dominant species in alpine acidic meadows of European mountain regions. The species is sparser in dry areas such as the Pyrenees and Balkans, compared to the Central–Eastern Alps and Carpathians. We hypothesized that distinct population groups could be identified based on their mean functional trait values and their correlation with the environment; we predicted that ecologically marginal populations would have stronger trait correlations, lower within-population trait variability (intraspecific trait variability, ITV) and lower genetic diversity than populations of optimal habitats.
- **Methods** Sampling was conducted in 34 populations that spanned the entire distribution range of *C. curvula*. We used hierarchical clustering to identify emergent functional groups of populations, defined by combinations of multiple traits associated with nutrient economy and drought tolerance (e.g. specific leaf area, anatomy). We contrasted the geographical distribution of these groups in relation to environment and genetic structure. We compared pairwise trait relationships, within-population trait variation (ITV) and neutral genetic diversity between groups.
- **Key Results** Our study identified emergent functional groups of populations. Those in the southernmost ranges, specifically the Pyrenees and Balkan region, showed drought-tolerant trait syndromes and correlated with indicators of limited water availability. While we noted a decline in population genetic diversity, we did not observe any significant changes in ITV in ecologically marginal (peripheral) populations.
- **Conclusions** Our research exemplifies the relationship between ecological marginality and geographical peripherality, which in this case study is linked to genetic depauperation but not to reduced ITV. Understanding these relationships is crucial for understanding the biogeographical factors shaping trait variation.

Key words: Alpine plants, cluster regression, ecological marginality, European mountains, functional biogeography, intraspecific trait variability, neutral genetic diversity.

INTRODUCTION

There is growing evidence that intraspecific trait variability (ITV), i.e. the variation of traits among individuals of the same species, plays a key role in determining individual and population performance, community structure and assembly (Westerband *et al.*, 2021). The distribution of trait values in a species' range is determined largely by environmental gradients, as changes in climate and local habitat factors mostly shape ITV (Albert *et al.*, 2010). Research into spatial trait variability is an essential approach that can elucidate the association

between plant species and their environment. This is due to the fact that plant functional traits, which encompass morphological, physiological and demographic features, play a crucial role in shaping plant fitness (e.g. Laforest-Lapointe *et al.*, 2014; Nolting *et al.*, 2021). However, further advancements in our understanding of how functional characteristics vary across a species' geographical distribution are currently hampered by a lack of data on intraspecific trait values (Moran *et al.*, 2016).

Trait variability in plants arises from phenotypic plasticity and genetic variation (Grassein *et al.*, 2010), and research on

species distributions at large scales has included exploration into the link between traits and environment (Das *et al.*, 2021; Laughlin *et al.*, 2021), as well as the genetic basis of phenotypic trait variation (Durufle *et al.*, 2019; Garzón *et al.*, 2019). The central–marginal hypothesis, a widely recognized concept in biogeography, proposes that peripheral populations located at the edge of a species' range exhibit lower performance and genetic variation compared to central populations (Pironon *et al.*, 2017). However, studies suggest that ecologically marginal populations may extend beyond the periphery of a species' range and exist across extensive spatial scales due to widespread ecologically marginal conditions in some regions (Kennedy *et al.*, 2020). In an evolutionary context, it has been hypothesized that the genetic differentiation of intraspecific traits represents a source of species adaptation capacity to climate change (Torres-Ruiz *et al.*, 2019). Studies have shown that ecologically marginal populations of species exhibiting marked functional differentiation may play an important role within the total ITV (Tonin *et al.*, 2020). Therefore, integrating information on trait and genetic variability within the context of broad-scale species ranges, with an emphasis on ecologically marginal conditions, can enhance our understanding of the historical factors that contribute to the present distribution patterns of ITV.

The observed variation of traits results from trade-offs among functionally related plant traits (Diaz *et al.*, 2016). Particular suites of traits may form syndromes that better reflect the coordinated evolution and function of plant characters as a response to selective pressures (Hayes *et al.*, 2019). For example, plants in insular systems are characterized by lower dispersal abilities and clonality compared to those in non-insular environments (Ottaviani *et al.*, 2020). Moreover, the intraspecific leaf economics spectrum (LES), which reflects a continuum of leaf trait syndromes going from short-lived leaves with fast metabolism to the reverse syndrome, has been associated with genetic differentiation in contrasted climates across the geographical distribution range of *Arabidopsis thaliana* (Sartori *et al.*, 2019). A study on *Helianthus anomalous* suggested that hot dry environments can selectively favour the correlated evolution of traits, indicating resource-conservative strategies, as shown by genetic neutral markers (Brouillette *et al.*, 2014). Estimates of ITV in association with neutral genetic variation could provide insights into gene flow, isolation by distance and species phylogeography (Gauzere *et al.*, 2020; López *et al.*, 2020). Intraspecific trait associations have been explored for some species across gradients and large geographical scales (e.g. Richardson *et al.*, 2013; Turtureanu *et al.*, 2020). However, more research is required to understand whether functional syndromes and population genetic structures, as indicated by neutral markers, show consistent spatial patterns, particularly in the context of ecologically central and marginal populations.

The notion of ITV encompasses both the examination of correlation strengths between traits and the analysis of trait variation within populations. First, it has been hypothesized that correlations (phenotypic integration) between traits reflecting key dimensions of plant ecological strategies (such as the LES) increase along gradients of environmental harshness to enhance fitness (Boucher *et al.*, 2013). Second, the variation of certain traits within populations may also improve our understanding of how plants cope with stressful conditions (Kuppler *et al.*, 2020). In *Trifolium montanum*, both local habitat heterogeneity

and genetic diversity were associated with within-population ITV (Karbstein *et al.*, 2020). Due to contrasting environmental regimes, alpine species exhibit large ITV, thus providing excellent opportunities to examine how ITV varies across environmental gradients (Albert *et al.*, 2010).

The central–marginal model suggests that populations situated on range margins tend to be more isolated and exhibit lower intrapopulation genetic diversity (Brussard, 1984), with lower levels of phenotypic variation. Moreover, the reduced genetic diversity in such populations was shown to correlate with shifts in trait values, although in certain regions, the decreased neutral genetic diversity at range margins may not hinder shifts in functional trait variation along environmental gradients (Kennedy *et al.*, 2020). Populations near the core of a species' range are expected to show continuity, high individual density, and increased levels of genetic and phenotypic variation, in contrast to those located on the margins or peripheries. To the best of our knowledge, no studies have been conducted to understand the association between within-population ITV and genetic diversity across a species' complete range, which includes ecologically marginal and peripheral environments. The study by Karbstein *et al.* (2020), for instance, focused on investigating the ecological marginal conditions within a comparatively small geographical zone. Integrating population-level measures of ITV and neutral genetic variation across the distribution area of a species could improve our understanding of how plants respond to environmental forcings in an evolutionary context.

In this study, we focused on the alpine sedge *Carex curvula*, which has been previously studied in relation to its post-glacial history and neutral genetic diversity (Puşcaş *et al.*, 2008b). Building on this significant knowledge and existing theoretical framework, we sampled populations across the Pyrenees, Alps, Carpathians and Balkan Peninsula mountains. We assume that ecological marginality is prevalent in peripheral regions such as the southernmost mountain ranges, as evidenced for example by a long-standing observation of reduced abundance of *C. curvula* in the South-Western Alps (Choler and Michalet, 2002). These regions are influenced by a Mediterranean climate, and the communities dominated by *C. curvula* are scattered and characterized by drought-tolerant alpine species because of severe summer drought. By contrast, the Central and Eastern Alps or the Carpathian Mountains are characterized by expansive alpine meadow ecosystems dominated by much denser communities (Puşcaş and Choler, 2012). Our previous studies on the phylogeographical structure of *C. curvula* provided evidence of two main lineages: a Western European (Alps and Pyrenees) and an Eastern European (Carpathians and Balkan Peninsula mountains), indicating an ancient isolation (Puşcaş *et al.*, 2008a). Long-distance dispersal and long-term persistence in isolated, island-like alpine habitats have been proposed to explain the impoverished genetic diversity in the Pyrenees and the Balkan Peninsula mountains, respectively (Puşcaş *et al.*, 2008a). In our study, we hypothesized that populations in the southernmost regions, assumed to experience ecologically marginal conditions, would exhibit distinguishable trait values for drought tolerance. We hypothesized that these emergent functional groups of populations (Kleyer *et al.*, 2012) would possess a distinct genetic signature. We also predicted that populations situated in ecologically marginal areas would exhibit stronger trait correlation (coordination) and

lower levels of within-population ITV and genetic diversity. To test these hypotheses, we measured plant height and leaf traits related to nutrient economy [e.g. leaf carbon-to-nitrogen ratio, specific leaf area (SLA)] and to drought tolerance (e.g. histological traits) (Grigore and Toma, 2017). More specifically, we addressed the following questions: (1) Can emergent functional groups of populations be identified by utilizing mean functional trait values and their optimized association with the environment? (2) Do emergent functional groups coincide with a central–peripheral geographical distribution and display a specific genetic structure? (3) Do ecologically marginal populations exhibit greater trait coordination and lower ITV and genetic diversity within populations?

MATERIALS AND METHODS

Study species and sampling design

The present study focuses on the crooked sedge *C. curvula*, which dominates one of the most characteristic alpine meadows of the higher mountains in temperate Europe. These grasslands can be found in the Pyrenees, the Alps, the Carpathians and in some of the Balkan Peninsula mountain ranges, where they are a typical representative of late-successional alpine belt communities on acidic substrates (Choler and Michalet, 2002; Puşcaş et al., 2008a). *Carex curvula* is more frequent and abundant in the central parts of its distribution (the Carpathians, the eastern and central parts of the Alps) and occurs in more fragmented and sparsely vegetated situations in the peripheral southern areas under Mediterranean influence: the Pyrenees, the Balkan region and the South-Western Alps (Puşcaş et al., 2008a; Puşcaş and Choler, 2012). *Carex curvula* s.l. was divided by Gilomen (1938) into two infrataxa based on their edaphic preferences: *C. curvula* subsp. *curvula* (calcifuge and present throughout the species' range), and *C. curvula* subsp. *rosae* (confined to the Alps and Pyrenees and favouring calcicole soils, according to Chater, 1980). This investigation exclusively examined *C. curvula* subsp. *curvula*.

Sampling covered the whole distribution of *C. curvula* (Fig. 1) using an 80 × 80-km grid (as per the European Terrestrial Reference System – ETRS 89) and was conducted within the framework of the ODYSSEE long-term monitoring initiative (<http://odyssee.granturi.ubbcluj.ro/>). In each grid cell where *C. curvula* occurs, we sampled at least one 100-m² site in a meadow dominated by this species (cover >25 %; Fig. 1C). A total of 34 sites were sampled (Supplementary Data Table S1).

Functional traits and environmental variables

Based on evidence showing that above-ground traits display a stronger and more consistent response to drought compared to root traits (Lozano et al., 2020), we have excluded the latter in this study. This decision was made in order to concentrate on critical above-ground traits that directly impact plant adaptation to drought conditions. Twenty shoots of *C. curvula* (Fig. 2A) were collected at random from each 100-m² plot, storing the leaf material in wet tissues for no more than one day. (1) Vegetative height, i.e. the distance between the top of the photosynthetic tissue and the ground, was

measured for all individuals in an upright position. (2) Leaf carbon and nitrogen content per unit of dry leaf mass (in mg g⁻¹) were determined on dried leaf samples from five individuals in each population, ground with an oscillating ball mill before using the same elemental analyser that was used for soil (Flash EA1112, Thermo Fisher Scientific, USA). (3) SLA, the ratio of leaf area to leaf dry mass (cm² g⁻¹), was measured using leaves collected from ten individuals. The samples were dried in an oven at 60 °C for 48 h. (4) Leaf tensile strength (in N mm⁻¹), also known as resistance to tearing, was measured on the same ten individuals using a portable lab-made tearing device (tensometer) on which the leaf samples were stretched by a handcrafted dynamometer until breaking. (5) To derive traits at the leaf anatomical level, the middle lamellae of the last fully mature leaves were collected from five individuals in each population and preserved in a mixture of formaldehyde:ethanol:acetic acid (final concentration 10 %:50 %:5 %). Leaf segments were dehydrated by immersion in baths of increasing concentrations of ethanol and butanol before embedding them in paraffin. We prepared cross-sections 15 µm in width and photographed them through an Olympus BX51 microscope equipped with a CoolSnap Pro-colour camera. Images were converted to black and white before estimating the total cross-sectional area and the area occupied by bulliform cells, which are found in monocotyledonous families such as Cyperaceae where they are involved in rolling and unrolling leaves under drought (Grigore and Toma, 2017) (Fig. 2B). The ‘bulliform cell ratio’ was calculated as the ratio of the area occupied by bulliform cells to the total leaf cross-sectional area. The trait ‘circularity’, which indicates how circular or elongated the leaf cross-section is, was calculated using the formula: $4\pi \frac{\text{area}}{\text{perimeter}^2}$ which yields the circularity index (roundness) from within (0,1] where a value near zero indicates a high level of distortion while 1 corresponds to a perfect circle (Niinemets et al., 2004). In leaf blades, ‘roundness’ is a shape parameter that relates to the evaporative and heated areas of the leaf (Sosnovsky et al., 2021); it is therefore expected to increase under drought-stress conditions. Table 1 provides a summary of the traits used in this study. Images were processed using ‘raster’ (Hijmans et al., 2022) and ‘tiff’ (Urbanek and Johnson, 2013) R packages. The circularity index was calculated using the ‘stars’ (Pebesma et al., 2021b), ‘sf’ (Pebesma, 2018), ‘sp’ (Pebesma and Bivand, 2005) and ‘lwgeom’ (Pebesma et al., 2021a) R packages.

Several soil parameters were measured by collecting soil cores from five randomly chosen locations at each site, from the top 15 cm of the soil profile. This yielded composite soil samples which were sieved and air-dried before laboratory analyses. An elemental analyser (Flash EA1112, Thermo Fisher Scientific) was used to measure the total carbon and nitrogen content of ground material. Using a pH-meter (inoLab 7110, WTW, Germany), soil pH was determined in a 1:4 (w/w) mixture of soil and distilled water. Organic matter content was measured by loss on ignition analysis performed for 4 h at 550 °C. The soil thermal regime was monitored during the period 2014–2021 using miniaturized and standalone temperature data loggers (Hobo pendant UA, Onset Computer Corporation, Bourne, MA, USA) installed within the ODYSSEE field campaign. Loggers were buried at a depth of 5 cm to record

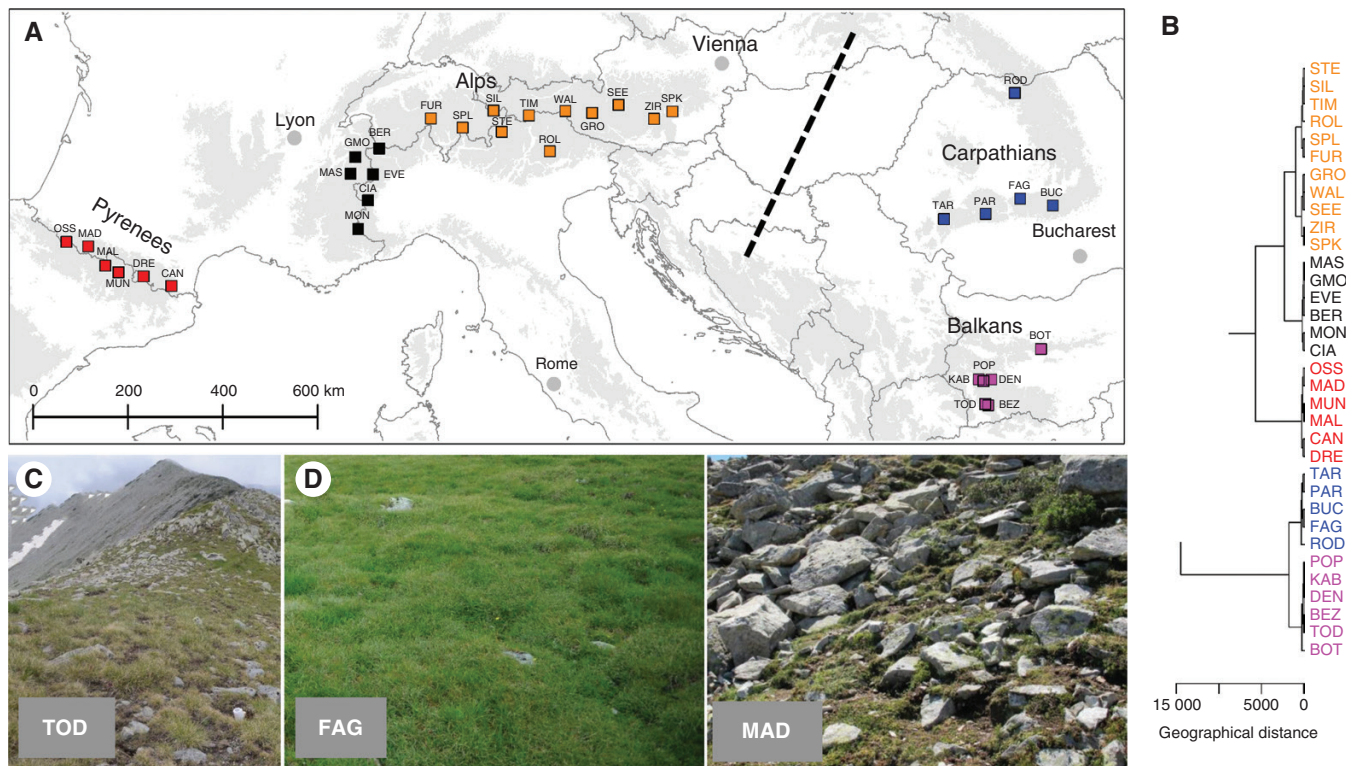


FIG. 1. (A) Distribution of the sampling locations across the temperate European mountains. Symbols have been colour coded based on geographical region. The dashed line separates the two main genetic lineages (Western and Eastern clades) according to [Puşcaş et al. \(2008a\)](#). Population details can be found in [Supplementary Data Table S1](#). (B) Hierarchical clustering of the sites based on geographical distances. (C) Sparse *Carex curvula* meadows in the Balkan region (Todorka, Bulgaria). (D) Two contrasting situations: a site with high cover of *C. curvula* (FAG – Făgăraş Mountains, Southern Carpathians, Romania) and a site with low vegetation cover (MAD – Pic de Madamète, Pyrenees, France).

near-surface ground temperatures every 2 h. Despite some incomplete time series owing to unexplained cessation of loggers or depletion of battery power, we were able to collect sufficient data to cover both winter and summer conditions for at least 1 year at each site. We used the number of days from January to March with average soil temperatures below -1°C to characterize winter thermal conditions (freezing season length; FSL). A previous study ([Choler, 2018](#)) highlighted the relevance of this thermal indicator for characterizing alpine habitats. Soil summer thermal conditions were characterized by the mean soil temperature in July ($^{\circ}\text{C}$; Soil.Temp.J).

The onset of snowmelt (Onset) was derived from MODIS (Moderate Resolution Imaging Spectroradiometer) Collection 6 remote sensing data with a resolution of 250 m ([Myneni et al., 2002](#)). The timing of the first day of snowmelt is critical for alpine species as it determines water availability, plant growth and productivity. The 8-d temporal resolution of the MODIS product allowed a fair estimate of Onset in snow-covered ecosystems ([Choler, 2015](#)). Collection 6 of the MOD09Q1 products was downloaded from the Land Processes Distributed Active Archive Center (<https://e4ftl01.cr.usgs.gov/>). We used the topographic position index (TPI) ([De Reu et al., 2013](#)) to describe the mesotopographical conditions, which play a crucial role in determining the hydrological processes that govern water balance in alpine habitats. TPI was derived from a 25-m-resolution Digital Elevation Model over Europe (EU-DEM, <https://www.eea.europa.eu/>

[data-and-maps/data/eu-dem](#)) from the GMES RDA project with a focal window size of 3×3 pixels. A high/low TPI indicates a local topographic convexity/concavity (peak/valley) while a TPI near zero indicates a flat or near-continuous slope.

We used the Normalized Difference Vegetation Index (NDVI) as a remotely sensed proxy of plant cover ([Fig. 1D](#)). NDVI was estimated based on the high-resolution (10 m) images provided by the Sentinel-2 mission and processed through the MAJA algorithm (<https://www.connectbycnes.fr/en/maja>). The calculation was based on the NIR (band 8) and RED (band 4) reflectance. To mitigate the effect of cloud cover, which is very frequent in the alpine zone, we selected several clear images from the summer months (July–August) of the years 2015–2019 to calculate an averaged NDVI at a resolution of 3×3 pixels. NDVI heterogeneity was assessed through the standard deviation of NDVI over a 30×30 -m area (NDVI.var).

Climate variables were estimated from gridded datasets of air temperature, moisture and solar irradiance, and from our own *in situ* records of soil temperature. The summer daily average temperature (TempAir.JJA; June–August; $^{\circ}\text{C}$) and daily average minimum temperature during the growing season (from vegetation onset to end of July) were retrieved from WorldClim v1.4 ([Hijmans et al., 2005](#)) at a resolution of 30 arcsec (~ 1 km) for the period 1960–1990. Summer actual evapotranspiration (ET.JJA; June–August; mm) was obtained

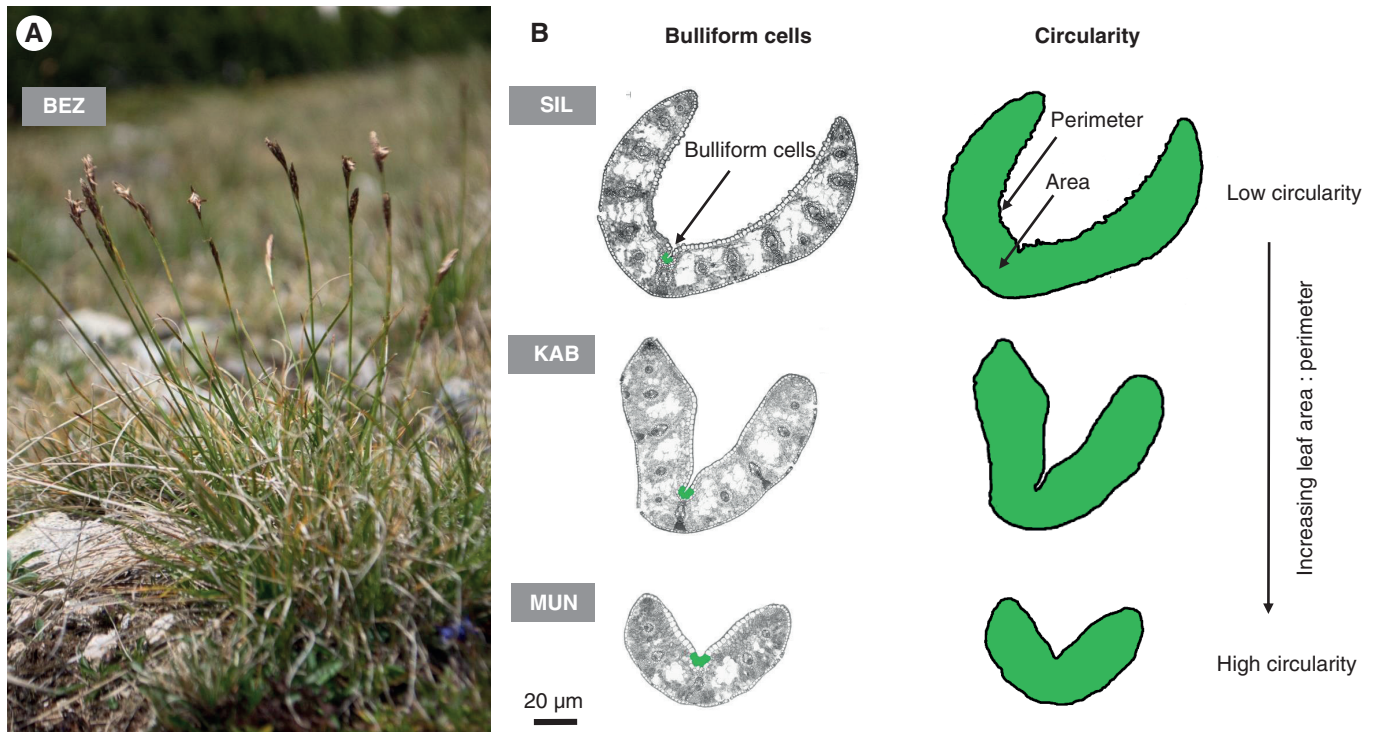


FIG. 2. (A) Tussock of *Carex curvula* from the Balkan region (BEZ – Bezboğ). (B) The two anatomical traits used in this study. Three examples are shown to illustrate both (1) the increasing proportion of bulliform cells forming the total leaf cross-sectional area and (2) the increasing circularity of the leaf cross-section. These examples represent samples from the Alps (SIL – Silvretta), Balkan Peninsula mountains (KAB – Kabul) and Pyrenees (MUN – Muntanyó). Population details can be found in [Supplementary Data Table S1](#).

TABLE I. Overview, summary and variability of functional traits measured in this study.

Trait	Description	Mean	Range	CV (%)
Height	Vegetative height (cm)	10.00	3.90–17.15	33.97
SLA	Specific leaf area ($\text{cm}^2 \text{g}^{-1}$)	11.48	7.87–14.94	15.44
Leaf strength	Leaf tensile strength (N mm^{-1})	286.45	209.46–407.98	18.80
LCC	Leaf carbon concentration relative to dry-mass (%)	45.00	43.50–46.61	1.45
C:N	C:N ratio	22.10	15.38–29.24	15.04
Bulliform cells	Ratio of bulliform cell area to leaf cross-sectional area (%)	0.78	0.38–1.43	27.73
Circularity	Circularity index, i.e. ratio of leaf cross-sectional area to the area of a circle with the same convex perimeter	0.20	0.13–0.27	14.41

CV, coefficient of variability at the population level.

from Terraclimate at ~ 4 -km spatial resolution for the period 1981–2010 ([Abatzoglou et al., 2018](#)). Potential evapotranspiration (PET.J; mm) was obtained from the E-OBS dataset at a resolution of 0.1° (monthly sum for July; 1980–2017; [Haylock et al., 2008](#)). The incoming shortwave radiation (Rad.J; J m^{-2}) was obtained from the HelioClim-1 database at a resolution of ~ 20 km (monthly sum for July; 1985–2005; [Lefevre et al., 2007](#)). Although these gridded climate products have different spatial and temporal resolutions, they provide daily coverage of the entire study area and provide useful information about regional-scale climate variability. The habitat and climate variables used in this study are summarized in [Supplementary Data Table S2](#) and [Fig. S1](#).

Neutral genetic variation

To assess neutral genetic variation, we used simple sequence repeat (SSR) fingerprinting. We randomly chose 15 individuals in each population and sampled healthy, green leaves and stored them in tubes with silica gel until DNA extraction. Genomic DNA was extracted using a NucleoSpin[®] 96 Plant II (Macherey-Nagel, Germany) according to the manufacturer’s protocol. The DNA quality was estimated on a 1 % agarose gel stained with ethidium bromide, and the concentration was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). We amplified 13 microsatellite loci specifically designed for *C. curvula* ([Şuteu](#)

et al., 2023). The 4× diluted genomic DNA was subjected to PCR amplification in a 10-μL volume containing 1× KAPA Taq Buffer A, 0.25 mM of each dNTP, 0.2 μM of each primer, 0.1 mg mL⁻¹ BSA, and 0.4 U of KAPA Taq DNA Polymerase (Kapa Biosystems, USA). The following PCR programme was used: 94 °C for 3 min, 94 °C for 1 min, primer-specific annealing temperature for 1 min, 72 °C for 2 min, 45× steps –2–4, and final elongation at 72 °C for 7 min. The PCR products were purified on a mix of Sephadex–Sephacryl (1:1) (GE Healthcare Bio-Sciences AB) and then 10× diluted. Then, 1.5 μL from this dilution was added to a 10-μL mix (10:0.1) of HiDi formamide and GeneScan™ 500 ROX™ Size Standard (Applied Biosystems, Thermo Fisher Scientific), and then subjected to capillary electrophoresis on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific) using a 36-cm capillary and POP-7™ polymer. GeneMapper v.4.0 software (Applied Biosystems, Thermo Fisher Scientific) was used for allele scoring. Data reliability was tested by comparing duplicates (ratio of replicates to samples was 1:10). The analysis of the 13 microsatellite polymorphic loci detected 284 alleles in 510 individuals and the number of alleles per sample and per locus ranged between one and six.

Emergent functional groups of populations

We performed trait-based hierarchical clustering to identify functional groups of populations, i.e. groups of populations that show similar suites of trait values. We followed the procedure of [Kleyer et al. \(2012\)](#) to cluster all trait combinations and to select a composite of several statistically stable, informative traits that exhibit a strong response to the environment. Ward's hierarchical clustering was repeated for all combinations of traits (i.e. combinations of one to six traits). We calculated the cophenetic correlation to quantify how the clustering results correspond to the original pairwise distance matrix.

In a second step, we modelled the responses of emergent groups to the environmental variables by cutting each tree into three emergent groups of sufficient robustness to examine the response to the environment. For each group and environmental factor, univariate generalized linear models were fitted to determine the shape of the relationship (linear or hump-shaped) and its significance. We kept all significant univariate models and calculated R^2 for each. Each cluster was rated for its overall responsiveness to the environment, expressed as the average goodness of fit (R^2) of the significant models for all three groups of a certain trait combination. As clustering selection does not necessarily lead to a single best solution ([Kleyer et al., 2012](#)), a subset of the more suitable clusterings was chosen based on the following criteria: (1) the clustering should be based on at least three traits to capture the different dimensions of trait variation, (2) the clustering exhibits a cophenetic correlation coefficient >0.6 and (3) the mean R^2 across groups was >0.2. Since we intended to focus on a single clustering that maximizes the number of traits, i.e. that combines traits characterizing both plant stature and foliage properties including leaf anatomical traits – the most innovative facet of this work – we sorted the

table in descending order based on the response to environment (R^2) and chose the first clustering comprising anatomical traits, height, as well as a morphological and a chemical trait. To evaluate the goodness of fit of each model, we used the Hosmer–Lemeshow test ([Hosmer et al., 1997](#)), as implemented in the pROC package ([Robin et al., 2021](#)). A P -value <0.05 suggests a lack of fit.

Spatial distribution of functional groups and genetic variation

We employed a hierarchical cluster analysis on geographical locations, population-mean trait values and genetic (microsatellite) data at the population level. This analysis was based on the Euclidean distance and was carried out with Ward's linkage algorithm using the 'stats' R package ([R Core Team and contributors worldwide, 2022](#)). We evaluated the spatial distribution of the functional groups within the clustering trees by examining the placement of mountain ranges labelled with different colours and used tanglegrams to summarize the topological similarities among the trees. In addition, we calculated the cophenetic correlation between distance matrices pertaining to functional, genetic, geographical, climate and habitat dendrograms. Both the tanglegrams and the cophenetic correlations were produced with the 'dendextend' R package ([Galili, 2015](#)).

ITV and genetic diversity in relation to ecological marginality

To assess the strength of trait coordination among populations, we fitted pairwise trait relationships (standardized major axes; SMAs) for both the pre-defined functional groups of populations and the full dataset. To evaluate within-population ITV, we first calculated the coefficient of variation (CV) for each trait using the values measured for individuals in the same population. Then, we assessed population-wise ITV as the mean of CV from all traits ([Karbstein et al., 2020](#)). Finally, we compared the distribution of ITV of individual traits and population-wise ITV across the emergent functional groups.

Within-population genetic diversity was assessed using Nei's genetic diversity index ([Nei, 1987](#)). We examined the distribution of Nei's index among the emergent functional groups and constructed the phylogenetic tree with Ward's hierarchical clustering based on the genetic distances between populations using the 'ade4' R package ([Jombart, 2008](#)).

All statistical analyses for this study were performed using R.

RESULTS

Emergent functional groups of populations

Nineteen clusters of trait values met our criteria of high cophenetic correlation and strong response to the environment. The chosen clusters comprised both anatomical traits, height, SLA, leaf strength and C:N ([Table 2](#)). The relative positioning of chosen clustering is shown in [Supplementary Data Fig. S2](#). Other combinations did not show distinct patterns. In addition, we identified the clusters showing the highest response of groups to the environment ([Table S3](#); [Fig. S3](#)).

TABLE 2. Subset of clusterings used to select the trait combination discussed in this study (in bold type). These were filtered from a full list of all possible trait combinations to comprise: at least three functional traits, cophenetic correlation coefficient >0.6, and average group $R^2 > 0.2$. A combination involving all trait types (size, structural, chemical, anatomical) was assumed to be the most informative. Abbreviations of traits as in Table 1.

No. of traits	Involved traits	Cophenetic correlation	No. of stable clusters	Average R^2
4	SLA, Height, Leaf strength, Circularity	0.76	2	0.34
3	SLA, Height, Circularity	0.76	4	0.34
6	C:N, LCC, SLA, Height, Leaf strength, Bulliform cells	0.61	2	0.33
5	LCC, SLA, Height, Leaf strength, Circularity	0.65	2	0.32
3	C:N, SLA, Leaf strength	0.69	2	0.30
3	SLA, Leaf strength, Circularity	0.61	4	0.30
5	C:N, SLA, Leaf strength, Bulliform cells, Circularity	0.61	2	0.29
4	C:N, SLA, Height, Leaf strength	0.66	2	0.28
6	C:N, SLA, Height, Leaf strength, Bulliform cells, Circularity	0.63	2	0.27
3	LCC, SLA, Circularity	0.63	2	0.25
3	SLA, Height, Leaf strength	0.61	2	0.25
5	C:N, SLA, Height, Leaf strength, Bulliform cells	0.64	2	0.24
5	C:N, SLA, Height, Leaf strength, Circularity	0.7	2	0.24
4	SLA, Height, Bulliform cells, Circularity	0.69	3	0.24
3	C:N, LCC, Leaf strength	0.67	2	0.24
5	C:N, LCC, SLA, Height, Leaf strength	0.66	2	0.23
4	C:N, Height, Leaf strength, Circularity	0.67	2	0.22
4	C:N, SLA, Leaf strength, Circularity	0.63	2	0.21
4	C:N, Leaf strength, Bulliform cells, Circularity	0.62	2	0.20

The trait values of F3 were the most divergent among the functional groups: statistically significant lower height (6.3 ± 2.1 cm) compared to F1 (12.7 ± 2.1 cm; Tukey's, $P < 0.001$), lower SLA (9.1 ± 1.3 cm g⁻¹) compared to F1 (12.3 ± 1.4 cm² g⁻¹; Tukey's, $P < 0.001$) and F2 (11.8 ± 1.0 cm; Tukey's, $P < 0.001$), higher leaf strength (351 ± 47 N mm⁻¹) compared to F1 (266 ± 35 N mm⁻¹; Tukey's, $P < 0.001$) and F2 (275 ± 52 N mm⁻¹; Tukey's, $P < 0.01$), higher C:N (26 ± 2.6) compared to F1 (22 ± 2.6 ; Tukey's, $P = 0.022$) and F2 (19 ± 2.3 ; Tukey's, $P < 0.001$), higher bulliform cell proportion (0.87 ± 0.28 %) compared to F1 (0.64 ± 0.14 %; Tukey's, $P < 0.019$), and slightly elevated circularity indices (0.22 ± 0.03) compared to F1 (0.20 ± 0.03 ; Tukey's, $P = 0.16$) and F2 (0.19 ± 0.02 ; Tukey's, $P = 0.12$).

The response of functional groups to environmental variables is summarized in Table 3 (see also Supplementary Data Fig. S2). F3 was associated with higher summer soil temperatures (TempSoil.J) and summer evapotranspiration (ET.JJA), F2 was linked to lower minimum air temperatures during the growing season (TempAirMin.GS), while F1 was associated with a decrease in potential evapotranspiration (PET.J) (Table 3). Overall, the emergent functional groups of populations responded more to regional variables than to local factors. This was also observed in the clustering based on the combination of traits with the highest response to the environment which even showed no response at all to local factors (Table S3.). The populations from the Pyrenees (F3 group) were highly associated with TempSoil.J and ET.JJA (Table S3).

Spatial distribution of functional groups and genetic variation

The dendrogram based on geographical distances revealed clusters that closely match the main mountain ranges, i.e. the Pyrenees, Alps, Carpathians and Balkan Peninsula. The Alps were divided into two distinct groups, with the South-Western Alps being separated from the remaining areas of the range (Fig. 1A, B).

The trait structure also displayed a well-defined spatial pattern (Fig. 3A). The three emerging functional groups (F1–F3, Fig. 3A) exhibited a clear geographical structure. While F1 comprised only populations from the Alps and Carpathians, and a few from the South-Western Alps, F2 comprised all the Balkan Peninsula populations and the remaining populations from the South-Western Alps. The most distinguishable group was F3, which included all Pyrenean populations.

The genetic structure displayed a marked west–east gradient (Supplementary Data Fig. S4) with populations from the Balkan Peninsula and Carpathians grouped together, while the Pyrenean populations had a clearly distinct genetic signature within the cluster including the populations from the South-Western and Central Alps, indicating a notable contrast between the Pyrenees and Balkan Peninsula in terms of genetic structure.

The tanglegram showed a strong correspondence between the functional and genetic structure for the Pyrenean populations (Supplementary Data Fig. S4). Overall, the functional structure had the highest values of cophenetic correlation with the genetic structure and climate (Supplementary Data Fig. S5).

TABLE 3. Environmental response of the functional groups (F1–F3) that emerged from the clustering of selected trait combinations. The coefficient of determination (R^2) is provided only for the significant univariate models along with the direction of either linear (+, –) or hump-shaped relationships (\cap). Abbreviations of environmental variables as in Supplementary Data Table S2. Non-significant relationships are denoted by n.s. For the following models, the Hosmer–Lemeshow goodness-of-fit test (HL; d.f. = 9) suggested a lack of fit: F1–Soil.OM (HL: 21.53; $P = 0.01$), F1–ET.JJA (HL: 17.33, $P = 0.04$).

	F1	F2	F3
Habitat variables			
FSL	n.s.	n.s.	n.s.
Onset	n.s.	n.s.	n.s.
Soil.pH	n.s.	n.s.	n.s.
Soil.OM	0.17 (+)	n.s.	n.s.
Soil.CN	0.31 (+)	n.s.	n.s.
TPI	n.s.	n.s.	n.s.
NDVI	0.24 (+)	0.14 (–)	n.s.
NDVI.var	n.s.	n.s.	n.s.
Climate variables			
TempSoil.J	0.25 (–)	n.s.	0.55 (+)
TempAir.JJA	n.s.	0.15 (–)	0.18 (+)
Rad.J	0.26 (–)	n.s.	n.s.
TempAirMin.GS	n.s.	0.40 (–)	0.18 (+)
ET.JJA	0.33 (\cap)	n.s.	0.48 (+)
PET.J	0.40 (–)	0.17 (+)	n.s.

ITV and genetic diversity in relation to ecological marginality

The results of pairwise SMAs between traits within the functional groups F1 and F2 + F3 provided little evidence for higher coordination in marginal populations. Only three out of 15 trait pairs showed significant SMAs for F2 + F3 but not for F1 (Fig. 4): height–C:N (slope: -0.60 , $P = 0.040$), SLA–leaf strength (slope: -0.02 , $P = 0.018$) and leaf strength–proportion of bulliform cells (slope: -310.00 , $P = 0.045$).

In terms of ITV in marginal populations, the only notable difference observed between the groups was in the CV of height. The mean CVs of height in F3 (1.19 ± 0.59) and F2 (0.87 ± 0.23) were higher compared to that of F1 (0.57 ± 0.11), as indicated by Tukey's $P < 0.001$ and $P = 0.045$, respectively. None of the traits showed any significant within-population CV for F3 and F2, nor were there any population-wise ITV differences (Fig. 5A).

Population genetic diversity was strikingly depauperate in the Pyrenean populations, i.e. F3 (Nei's genetic diversity index = 0.028 ± 0.024) compared to F1 (0.081 ± 0.011 ; Tukey's, $P < 0.001$) and F2 (0.073 ± 0.011 ; Tukey's, $P < 0.001$). However, there were no significant differences in ITV for this functional group of populations, as shown in Fig. 5B.

DISCUSSION

Our study provided a unique evaluation of the ITV of a key-stone species, performed comprehensively across its entire continental-scale distribution range, and in a well-defined phylogeographical context. First, our research revealed the emergence of unique functional groups of populations characterized by multiple trait values. Notably, the proportion of bulliform cells, a syndrome of leaf traits, exhibited an increase

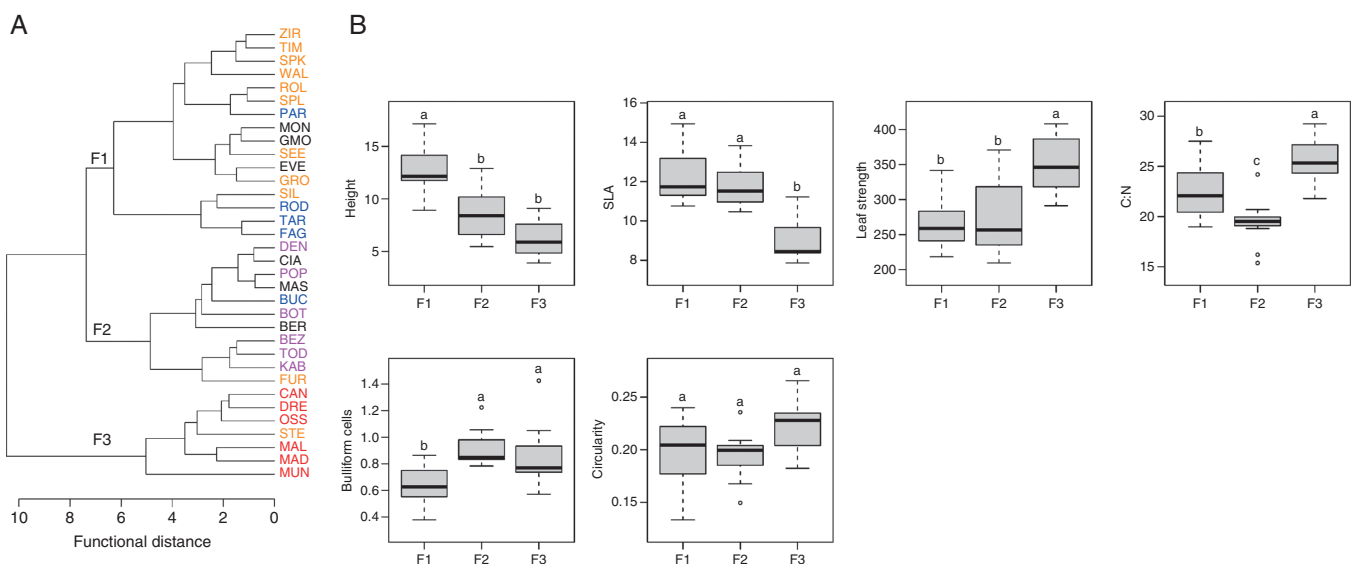


FIG. 3. (A) Hierarchical clustering of the sites based on the selected combination of traits, namely height, SLA, leaf strength, C:N, bulliform cells and circularity. Three functional groups (F1–F3) were derived from the dendrogram to identify the main trait syndromes. Colour codes as in Fig. 1. (B) Distribution of population means for different traits according to the three functional groups obtained from the hierarchical clustering. Different letters indicate significant differences between populations (*post hoc* Tukey's test; $P < 0.05$). Abbreviations of traits as in Table 1.

in marginal populations of the Balkan Peninsula and Pyrenees. These findings brought novel insights into the significance of underrepresented anatomical traits on a large biogeographical scale (Bachle and Nippert, 2021). Second, the outcomes of our study revealed the presence of a marked geographical signature expressed in the variation of trait values in *C. curvula*.

Our study is thoroughly documented from a phylogeographical perspective as it revealed a robust geographical pattern in the genetic structure of populations that corresponded to the trait structure. Third, we provided evidence that neither coordination between traits nor within-population ITV is higher in marginal conditions. Lower within-population ITV did not correspond to genetically depauperate populations.

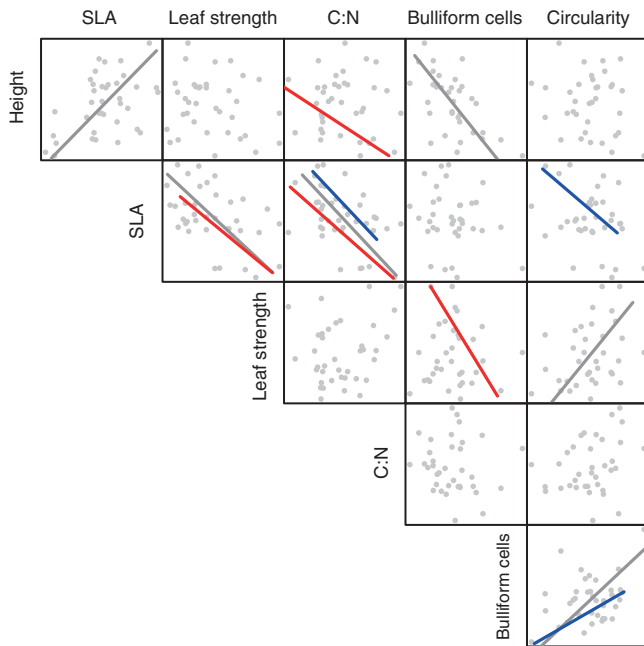


FIG. 4. Coordination among traits assessed by bivariate relationships using standardized major axis (SMA) regressions. Coloured lines indicate significant linear relationships for populations in functional group F1 (blue) or F2 + F3 (red). Grey lines indicate significant relationships for the full dataset. Abbreviations of traits as in Table 1.

Ecological marginality-associated trait syndromes coincide with geographical periphery and genetic depletion

We identified several factors indicative of ecologically marginal situations, such as higher summer soil temperatures, dry climate and reduced vegetation cover measured by the NDVI. Our results suggested a consistency between local- and regional-scale factors as indicators of ecological marginality at biogeographical scales. The severe summer drought, especially in the Pyrenees (Ninot et al., 2017), cascades into a less dense vegetation cover of communities dominated by *C. curvula*. Our results on ITV agreed with previous studies on *C. curvula* in the South-Western Alps showing that optimal conditions are infrequent in the southernmost regions where this taxon mostly occupies ecologically marginal habitats (Choler and Michalet, 2002). The optimal conditions for *C. curvula* are found in cold and humid mountain climates, e.g. in the Central and Eastern Alps or in the Carpathians, where it forms extensive swards (Puşcaş and Choler, 2012). In warmer climates, competition for light with taller low-alpine species such as *Carex sempervirens* is a limiting factor (Guisan et al., 1998).

In our study, we detected two distinct trait syndromes among the populations analysed. The populations in the Pyrenees region, compared to those in the Balkan region and South-Western Alps, exhibited a more pronounced conservative syndrome associated with marginal environmental conditions. Our

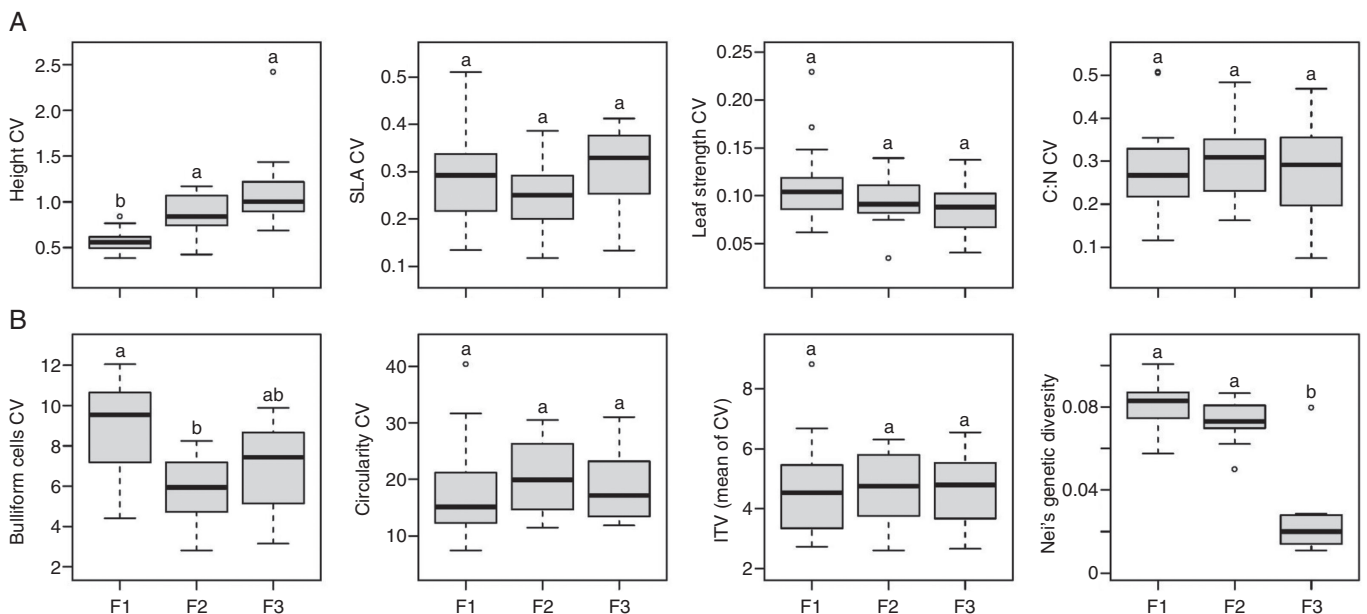


FIG. 5. (A) Distribution of the within-population coefficient of variation (CV) of traits according to the three functional groups obtained from the hierarchical clustering. Abbreviations of traits as in Table 1. (B) Distribution of within-population Nei's genetic diversity index across the three functional groups. The same letters indicate non-significant differences among groups (*post hoc* Tukey's test; $P < 0.05$).

approach distinguished these two trait syndromes based on multiple trait values, including histological traits that are usually overlooked in such studies (Westerband *et al.*, 2021). Although there are previous investigations focusing on bulliform cells in *C. curvula* (Codignola *et al.*, 1987), none has explored this trait across large spatial scales. Second, the increased leaf circularity in the Pyrenees, but not in the Balkan Peninsula, may be considered a drought adaptation specific for this range. The closely related taxon *C. curvula* subsp. *rosae*, which occupies calcareous outcrops in the South-Western Alps and Pyrenees, but not in the Balkans, is characterized by similar rounded transverse leaf-sections (Gilomen, 1938; Choler *et al.*, 2004). Further studies should examine whether this trait similarity results from convergent evolution or phylogenetic conservatism. Thus, our results clearly indicate the relevance of measuring intraspecific anatomical traits to decipher alpine species' functional strategies (e.g. García-Cervigón *et al.*, 2021). Complementary developments in measuring less-reported anatomical traits at an intraspecific level are also promising to yield additional insights into the response of species to a different future climate. The current climatic trends call into question whether marginal populations of *C. curvula* will be able to cope with more frequent and more intense summer heatwaves in European mountains. Other traits, such as lower height, lower SLA, and higher leaf strength (particularly in the Pyrenean populations) are related to high investment in structural tissues, which allow plants to maintain leaf turgor under drought, as shown at the intraspecific level in alpine species for SLA (Scheepens *et al.*, 2010), or in other species for leaf strength (Kazakou *et al.*, 2019) and plant height (Tardella *et al.*, 2017).

The clustering of *C. curvula* populations based on the combination of five functional traits observed in our study concurs with previous research that identified geographically recognizable patterns of trait variation, such as in North American trees (Rueda *et al.*, 2018). Only a few studies have been able to identify spatially structured trait syndromes within species (e.g. Kennedy *et al.*, 2020; Camps *et al.*, 2021), while others did not find any marked differentiation due to an important proportion of ITV occurring at a local scale, particularly when the considered contrasting environmental conditions resulted from a disturbance (Kumordzi *et al.*, 2019). In our study, trait variation co-varies with a drought gradient that extends from the northern to the southern ranges, while incongruence between traits and geography is due to site-specific characteristics (Kempinen and Niittynen, 2022). As an example, the Bucegi Mountains (Carpathians) are home to a low-stature form of *C. curvula* referred to as *C. curvula* f. *pygmaea* (Șerbănescu and Nyárády, 1966), which is typically found on wind-exposed plateaus and on carbonate-rich bedrock, where it forms dry fell-field communities (our personal observations). These plants therefore exhibit traits (e.g. low height) that are more similar to those found in populations of the Balkan Peninsula or South-Western Alps.

With respect to the correspondence between the functional and genetic structures, we found that the Pyrenean populations are highly congruent and form a genetically distinct group (see also Puşcaş *et al.*, 2008a), probably due to a recent long-distance colonization from the South-Western Alps coupled with an overall decline in intrapopulation diversity.

ITV is not affected by ecological marginality

Contrary to our expectations, we found that coordination among traits was not lower in marginal populations. Earlier studies have found the within-species correlation among traits to be rather variable and to exhibit unexpected patterns. For example, Carvalho *et al.* (2020) found more integrated phenotypes in *Pinus sylvestris* at higher elevations in more stressful environments, while Boucher *et al.* (2013) found the strongest degree of phenotypic integration in *Polygonum viviparum* in sites with intermediate temperatures. In our case, marginal populations at range edges may be subject to genetic drift, leading to inefficient selection and thus low integration (Sexton *et al.*, 2009). However, for those trait pairs that contributed highly to the conservative syndrome, coordination was higher in marginal populations, suggesting either a high phenotypic plasticity or sufficient genetic variation for an efficient trait selection. The two factors cannot be mutually exclusive. While there were significant shifts in the mean trait values in marginal populations, within-population ITV was not lower. Harsher conditions are expected to provide fewer opportunities for individuals to occupy micro-habitats (i.e. decrease the breadth of the available niche). This has been shown for elevation (Boucher *et al.*, 2013; Carvalho *et al.*, 2020), although a different study found an increased functional trait diversity in alpine tundra (for some traits) at both ends of the elevation gradient (Spasojevic and Suding, 2012). Only a few studies have compared population trait means and ITV in marginal conditions. A notable exception is the study by Liu *et al.* (2019) who focused on *Brachypodium hybridum* and showed that plant geographical distributions could be shaped by interactions between ITV (rather than trait means) and environmental resource availability. Karbstein *et al.* (2020) showed that within-habitat heterogeneity was related to ITV in *Trifolium montanum*. In the present study, ITV was higher for height in marginal populations, which could suggest that plant communities are structured by differentiated interspecific competition (diversity of competitors) in contrasting within-site conditions, as confirmed by the increased number of vascular species (results not shown). Marginal habitats, displaying higher NDVI heterogeneity, are characterized by contrasting local conditions due to the presence of rocks and bare soil.

Inconsistency between ITV and genetic diversity

The lack of ITV differentiation in marginal populations was not mirrored by neutral genetic diversity, which was much lower in the Pyrenees, as previously outlined by Puşcaş *et al.* (2008a). Karbstein *et al.* (2020) found in *Trifolium montanum* that neutral genetic diversity was not as good a predictor of ITV as within-habitat heterogeneity at the plot scale. Since neutral markers used in our study are not related to genes responsive for traits, we could not have anticipated strong relationships with ITV. Neutral genetic diversity is expected to be driven by gene flow and/or drift, whereas phenotypic diversity is determined by local environmental factors (Fourtune *et al.*, 2018). A further inconsistency lies in the lower genetic diversity and higher species richness found in the Pyrenees and South-Western Alps, as observed previously (Puşcaş *et al.*, 2008b).

CONCLUSIONS

In contrast to prior studies that have predominantly investigated the correlation between ITV and the environment (e.g. Shimono and Kudo, 2003; Rixen *et al.*, 2022), our investigation provides a more in-depth understanding of the spatial patterns of ITV by integrating marginal and peripheral populations within a well-defined phylogeographical context across the European mountains. We highlighted the relevance of using a combination of morphological and anatomical traits across a range of genetic pools to seek patterns of plant adaptation to marginal conditions. Our study identified genetically distinct areas where populations of *C. curvula* were clustered according to trait values associated with drought tolerance. An important consideration arising from this research is that while population-mean trait shifts occur in these marginal situations, ITV and species richness remain high, even if genetic diversity is low. Therefore, the study of ITV, population genetic structure and habitat conditions may reveal response mechanisms that provide marginal populations with adaptive potential, and these factors should be considered to refine our current models of species distribution and to anticipate range shifts in response to global change.

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following.

Table S1: The sampled sites and functional traits used in this study. Table S2: Summary and descriptive statistics for the full set of environmental factors. Table S3: Environmental response of the functional groups that emerged from the clustering with the combination of traits that showed the largest response to the environment. Figure S1: Correlation matrices of the variables related to functional traits, habitat and climate. Figure S2: Distribution of the average R^2 of the clusterings that resulted from all trait combinations. Figure S3: Hierarchical clustering of the sites based on the combination of traits with the maximum responsiveness to the environment. Figure S4: Tanglegram showing the functional clustering based on the chosen trait combination and phylogenetic clustering. Figure S5: Correlation matrix of the cophenetic distance of the genetic, geographical, functional, climate and habitat trees.

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