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RESEARCH

Are leaf traits and genetic differentiation patterns divergent in endemic vs widespread congeneric plant species? Insights from the Mediterranean forest species pair *Aegonychon calabrum-A. purpurocaeruleum* (Boraginaceae)

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Abstract
Understanding trait and genetic variability within endemic plant species is key to elucidate their plasticity, resource-use attitudes and adaptive responses in variable habitat conditions. However, no studies have focused on both variability dimensions and their relationship in narrow Mediterranean endemics of the forest understorey. We investigated this topic in the species pair *Aegonychon calabrum*, a rare herb in southern Italy, and its related widespread congener *A. purpurocaeruleum* (Boraginaceae). After assessing intraspecific variation in Leaf Area (LA), Specific Leaf Area (SLA), Leaf Dry Matter Content (LDMC), and population genetic structure by AFLP fingerprinting, we used P_{ST}–F_{ST} comparisons to infer the proportion of genotypic and phenotypic differentiation driven by directional vs. stabilizing selection. We also examined whether individuals exhibit similar trait values due to their genetic proximity. Lower LA suggested lower resource-acquisitive ability in *A. calabrum*, but variation in traits and CSR scores was comparable to its widespread congener. Population heterozygosity was slightly lower than in *A. purpurocaeruleum,* not supporting genetic erosion in the endemic species. In both species, phenotypic differentiation was higher than genetic differentiation suggesting that directional or divergent selection is potentially acting on the examined leaf traits and thus on their resource use attitudes. Trait values positively correlate with genetic proximity in a clade formed by two of the three *A. calabrum* populations, a pattern consistent with population-level niche conservatism. The case study of *A. calabrum* supports that, while generally fitting the "refuge" model, populations of endemic understory herbs of Mediterranean forests can be subject to non-stabilizing selection.

Keywords Mediterranean endemics · Understory herbs · Leaf traits · Ecological strategies · Genetic structure · Non-stabilizing selection

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Introduction

Understanding intraspecific variation in endemic plant species in terms of level and patterns of differentiation within and among populations is a central issue in plant biogeography, evolutionary ecology and diversity conservation (Thompson 2020). While many studies have focused on the genetic aspects of this differentiation (Arafeh et al. 2002; Ellegren and Galtier 2016), little is still known about intraspecific variation in functional traits related to resource use and ecological strategies (Martín-Hernanz et al. 2019), especially for taxa of forest habitats in the Mediterranean region (Selvi et al. 2023). Even less understood are the relationships between genetic and functional trait variation in these plants, especially when compared to widespread congeners inhabiting similar habitats.

A substantial body of literature suggests that endemic plant species undergo stabilizing selection, resulting in a limited range of variation in functional traits that ensures adaptation to their usually narrow ecological niches and geographic distribution (Behroozian et al. 2020; Nery et al. 2023). Consequently, stabilizing selection favours individuals with intermediate, rather than extreme trait values, thus maintaining the species fitness within a specific set of environmental conditions (Behroozian et al. 2020). Stabilizing selection does not tend to change the mean trait values, reducing variation in a population by disfavouring individuals in the tails of the trait's distribution (Joel and Kingsolver 2007). In some cases, however, endemic species may also be subject to directional selection, particularly in response to environmental heterogeneity within their limited range or to gradual shifts in climatic or ecological conditions (Colas et al. 1997). Directional selection could drive the population's traits in a specific direction over time, potentially expanding or shifting their range, though it tends to reduce variation within populations (Joel and Kingsolver 2007). In principle, divergent selection could also take place in endemic species when different populations growing across variable microhabitats are affected by different selective pressures. In this case, the populations under divergent selection might exploit trait values optimised for each of these habitats. So far, however, the latter mechanism has not been clearly demonstrated in studies on narrow endemic species, though some of these show a certain degree of adaptability to different ecological conditions. To determine how much genotypic and phenotypic differentiation is caused by selective versus neutral processes, previous studies have compared intraspecific phenotypic divergence (P_{ST}) and neutral loci differentiation (F_{ST}), referred to as P_{ST}–F_{ST} comparisons (Brommer 2011; Leinonen et al. 2013). If phenotypic traits evolve neutrally, the proportion of their variation among populations should match the variation in allele frequencies at neutral loci ($P_{ST} = F_{ST}$), suggesting divergence due to genetic drift. However, if P_{ST} is greater or lower than Fst, this indicates that phenotypic differentiation is likely driven by natural selection. Directional or divergent selection occur when $P_{ST} > F_{ST}$, favouring "extreme" phenotypes, while stabilizing selection occurs when Pst < Fst, favouring similar phenotypes across populations (Brommer 2011; Leinonen et al. 2008, 2013; Marin et al. 2020; Martin et al. 2008; Merilä & Crnokrak 2001; Seymour et al. 2019; Whitlock 2008). According to Leinonen et al. (2006) and Brommer (2011), Pst serves as a proxy for the quantitative genetic differentiation index (Q_{ST}) when additive genetic variance cannot be easily quantified, such as in field studies. Since estimating Q_{ST} would require individuals from different populations to be grown in a common garden experiment (Leinonen et al. 2008), P_{ST} represents a practical alternative to approximate Q_{ST} in studies of populations from different sites with potential local adaptations (Franzoni et al. 2023).

The variation in functional traits driven by natural or neutral selection within a population is likely to reflect variations in the resource use strategies and fitness among individuals. This, in turn, can explain the adaptive trade-offs that individuals adopt in allocating resources, for instance, toward vegetative development or reproductive capacity (Grime and Pierce 2012). Among the many functional traits, leaf traits are relatively easy to measure and convey key ecological information (Puglielli et al. 2024). Leaf traits can be used to infer Grime's CSR plant strategies (Pierce et al. 2017), which represent the main trade-offs faced by plants between competitive ability (C), stress-tolerance (S) and ruderal behaviour (R). The competitive dimension is represented by the investment in biomass production and growth, which is feasible in stable, productive habitats. The stress-tolerance consists of the maintenance of metabolic function in variable and limiting environments, whereas ruderality consists in the rapid investment in regeneration and reproduction in productive but disturbed habitats (Grime 1977; Pierce et al. 2017). These dimensions are generally assumed to be related to the main axes of plant trait variability (Garnier et al. 2015), representing the investments of nutrients and mass in leaves (Castro-Díez 2012).

The hypothesis that endemic plant species differ in leaf traits and strategies with respect to widespread congeners was recently supported for taxa of Mediterranean forest habitats (Selvi et al. 2025), though previous studies in taxa of mostly open habitats did not provide evidence for such divergence (Lavergne et al. 2004; Hand et al. 2017). Also, endemics can be assumed to be prone to local adaptation, reaching some degree of functional convergence in leaf traits, and to be characterized by the retention of ecological niche-related traits over time, a process known as "niche conservatism" (Barros et al. 2020). Niche conservatism has been largely discussed at a broad phylogenetic scale, but in recent years, studies (Barros et al 2020; Gouveia et al. 2014) have moved the interest to a finer scale, such as among haplotypes or populations. Indeed, similar to the rules for plant community assembly, the geographical co-occurrence of two or more individuals results from their historical origin and dispersal, shared genetic characteristics, and adaptations to both past and current climates (Ackerly 2009; Villalobos et al. 2017; Barros et al. 2020).

In the present work, we attempted to bring light on these questions by adopting trait-based and population genetics approaches in the narrow endemic *Aegonychon calabrum* (Ten.) Holub and its widespread congener *A. purpurocaeruleum* (L.) Holub (Boraginaceae). These two taxa are sympatric, share a common life-form, main morphological characters, reproductive systems and preference for forest habitats (Cecchi et al. 2014; Selvi in Pignatti et al. 2017), thus offering an optimal model to compare intraspecific trait variation, ecological strategies and genetic structure in relation to their contrasting range size.

Using samples from different populations, we investigated whether the two species differ in the range and patterns of intraspecific variation in core leaf traits (LA, SLA, LDMC), ecological strategies and genetic structure as resulting from AFLP fingerprinting. To this purpose, we compared genetic and phenotypic intraspecific trait variation and tested the hypothesis that individuals of different populations tend to exhibit similar trait values due to genetic proximity. This could support that endemic and widespread related species from a similar forest habitat are similarly prone to niche conservatism. Finally, P_{ST}–F_{ST} comparisons allowed us to get insights into the relative roles of genetic drift and natural selection in shaping population differentiation in the two species.

Materials and methods

The species model system

Our model system consists of the endemic/non-endemic species pair *A. calabrum* and *A. purpurocaeruleum* (Fig. 1A, B) two members of Boraginaceae tribe Lithospermeae with a contrasting range size. The former is a narrow endemic restricted to a few mountain sites of the southern Apennines (Sila, Monte Pollino, Monte La Spina), with a single northernmost population in Campania (Pignatti et al. 2017). The latter is distributed across most of southern Europe eastwards to Iran and is frequent across all Italian regions (except forValle d'Aosta and Sardinia). The ranges of the two species overlap in southern Italy, though they usually do not coexist at the same sites, likely due to different habitat requirements.

Together with the Asian *A. zollingeri* (A.DC.) Holub, the two species form a well-supported clade within *Lithospermum* L. s.l., sister to *Buglossoides* Moench (Cecchi et al. 2014; Chacón et al. 2019). Both species are perennial hemicryptophytic herbs of the forest understory, usually found in the semi-shaded conditions of either the interior of open woods or at the edges of stands with a denser canopy. While *A. purpurocaeruleum* is mostly found in thermophilous deciduous forests up to 1000 m a.s.l., *A. calabrum* occurs in either mixed holm oak-broadleaf or pine forests (*Pinus nigra* J.F.Arnold subsp. *laricio* Palib. ex Maire), up to 1400 m a.s.l. (Selvi et al. 2023).

The ability for vegetative propagation through creeping sterile stems is displayed by both taxa, which also share a similar growth habit, floral and fruit morphology. The clonal ability, however, is stronger in *A. calabrum*, in which the lateral stems grow longer and allow a more effective lateral spread than in *A. purpurocaeruleum*. Outcrossing is

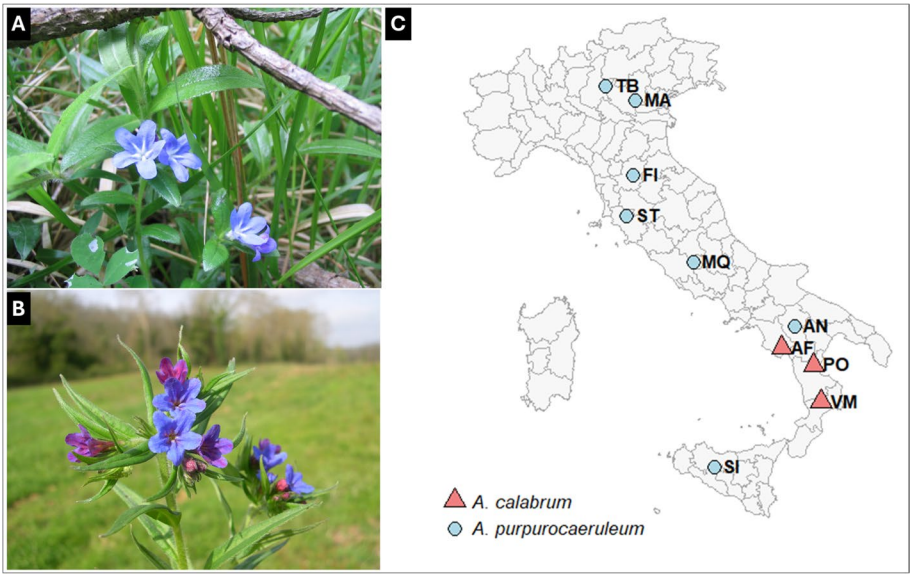

Fig. 1 Field photos of (**A**) *Aegonychon calabrum* (Tuscany, population ST; photo by F. Selvi) and (**B**) *A. purpurocaeruleum* (Calabria, population VM; photo by A. Coppi); **C**) Geographic distribution of the sampled populations

the prevalent mating system. The showy blue or purple flowers of both species are borne on bracteate cymes and visited by a range of insects, including long-tongued Diptera, territorial Hymenoptera or Lepidoptera, in search of sugar-rich nectar. The fruit, a dry schizocarp often consisting of a single mericarpid (nutlet), instead of four as usual in Boraginaceae, is rounded and with a white, smooth surface, without any obvious adaptation for zoochorous or anemochorous dispersal.

Morphologically, *A. calabrum* differs from its sister species mainly by the shorter and usually simple cymes, rather than 2–3 forked, and with less numerous flowers. The corolla is uniformly sky blue, without an early-anthesis stage of purple colour as in *A. purpurocaeruleum*. Karyologically, the two species are both diploid but with different chromosome numbers, *A. calabrum* showing the number 2n = 20 and *A. purpurocaeruleum* 2n = 16 (Pignatti et al. 2017).

No hybrids are known, suggesting the existence of effective interspecific reproductive barriers.

Sampling design and plant material

Aegonychon purpurocaeruleum was sampled at seven distant Italian sites, from Veneto (Garda Lake) at the north (45.6N) to Sicily at the south (37.6N), thus covering the entire latitudinal gradient in the country. *Aegonychon calabrum* was sampled at three sites, including one close to the southern limit in Calabria and one close to the northern limit in Campania (Fig. 1C); geographical details and major environmental variables of the sampling sites are provided in Table 1. At each site, three leaves for trait and molecular analyses were gathered from the middle portion of the stem of each of ten fully developed individuals in good health status. Distance among sampled individuals was at least 10 m to avoid taking samples from "ramets" of the same individual, and to represent as much as possible the variability within the populations. The two leaves used for trait analysis were placed in airtight bags containing water and stored in refrigerated bags to maintain

Table 1 List and ID codes of the examined populations of *Aegonychon purpurocaeruleum* and *A. calabrum*, with indication of Italian region of origin, forest type, elevation a.s.l. (m), latitude and longitude

Species, ID	Region	Forest type	Elevation (m a.s.l.)	Lat °N	Long° E
A. purpurocaeruleum					
TB	Veneto	mixed *Quercus pubescens*	140	45.6334	10.7054
MA	Veneto	mixed *Q. cerris*	78	45.4186	11.2759
ST	Tuscany	mixed *Q. cerris*	85	42.9529	11.1570
MQ	Latium	mixed *Q. pubescens*	390	41.9915	13.0083
AN	Basilicata	mixed broadleaf	740	40.6503	15.8289
SI	Sicily	mixed conifer	860	37.7091	13.5866
FI	Tuscany	mixed *Ostrya-Q.pubescens*	490	43.8113	11.3466
A. calabrum					
AF	Campania	*Q. ilex*	905	40.1308	15.6984
PO	Calabria	mixed *Q.ilex*-broadleaf	950	39.8916	16.1460
VM	Calabria	*Pinus laricio*	1260	39.0691	16.5706

leaf moisture until weighing (Pérez-Harguindeguy et al. 2013). The third leaf sample was placed in drying silica-gel bags for genetic analyses.

Leaf trait analysis

Trait measurements were performed on two leaves per individual. These were blotted dry with adsorbent paper and immediately weighed using a precision balance (with a sensitivity of 0.001 g). The upper surfaces of all sampled leaves were then scanned at high resolution to allow use of LAFORE (Veiko Lehsten, University of Oldenburg) to calculate the leaf area (LA, mm^2). Subsequently, the leaves were oven-dried at 40 °C for approximately ten days to determine dry weight (DW). LA and DW were then used to calculate two core traits, leaf dry matter content (LDMC) and specific leaf area (SLA). Trait values for each individual were obtained by averaging the values of the two analysed leaves.

Separate linear mixed models were fitted for each leaf trait (LA, SLA and LDMC) and for each CSR score, with species as a fixed factor and population as a random factor. The models were implemented using the *lmer* function in the *lme4* package (Bates et al. 2003). After calculating the mean, standard deviation and coefficient of variation across the populations of both species, significance of the differences was then determined using the Kruskal–Wallis test implemented with the *kruskalmc* function from the pgirmess package (Giraudoux et al. 2018). Next, ordination by Principal Component Analysis was performed on the trait dataset to determine and visualize the functional space exploited by the populations and the major axes of variation, using the Vegan package in R (Oksanen et al. 2007). CSR percentages were then assessed for each leaf sample using the Stratefy calculator (Pierce et al. 2017), and differences between populations tested as above for the leaf traits. Grime triangles were then obtained using the *ggtern* function from the *ggtern* package (Hamilton and Ferry 2018), to visualize the relative position of each population in the CSR scheme.

AFLP fingerprinting, genetic structure, PST-FST comparisons and niche conservatism analyses

AFLP-fingerprinting has been widely used to infer genetic variation in plant populations because of reproducibility, robustness and resolving power of polymorphism (Nybom 2004). Thanks to this tool, patterns of genetic structure and differentiation have been elucidated also in taxa of Boraginaceae with fragmented and/or restricted distribution (Coppi et al. 2008, 2014; Mengoni et al. 2006; Stehlik 2003). Despite the inability to identify specific genomic regions associated with the detected polymorphisms, this technique remains useful especially when investigating "non-model" species, for which a reference genome sequence is not available, as in the case of *Aegonychon* Gray.

Genomic DNA extraction from each leaf sample was performed with the 2×Cetyl-Trimethyl-Ammonium-Bromide (CTAB) protocol (Doyle & Doyle 1990), and the extracted DNA was quantified using a biophotometer (BioPhotometer Eppendorf). AFLP fingerprinting was assessed on 100 individual samples following the standard procedure described in Vos et al. (1995), with minor modifications (Coppi et al. 2018, and references therein). A preliminary test on the reproducibility of the AFLP protocol was performed as described in Coppi et al. (2018). The final primer combinations were hex_EcoRI-CGT/MseI-CTC and fam_EcoRI-CAG/MseI-AGG. The AFLP profiles obtained by capillary electrophoresis were analysed with GeneMarker v. 1.5 (SoftGenetics LLC, PA USA),

using a cut-off value of 5% of the maximum fluorescence peak. Genetic variation was assessed as within-population heterozygosity (He) based on Nei's metrics (Nei 1987) as implemented in Arlequin v. 2.000 (Schneider et al. 2000). Outlier loci in each AFLP profile were performed to identify the number of detected loci under potential selection pressure (Beaumont and Nicholas 1996; Beaumont and Balding 2004). This metric assumes that the locus frequencies within a population follow a multivariate β-distribution as a function of the Fixation Index values, and the mean frequencies of locus between populations (Rannala 1996; Rannala & Hartigan 1996; Burr 2000). The analysis of outlier loci was performed using BayeScan software v.2.1 (Foll and Gaggiotti 2008), keeping the number of pilots run at 20 and 10,000 iterations each (Yang et al. 2016; Coppi et al. 2018). We considered the outlier loci substantial, falling over a threshold value set on the logarithm of Posterior Odds values (LogPO), as determined in Foll and Gaggiotti (2008).

To infer the genetic structure of *A. calabrum* and *A. purpurocaeruleum*, a model-free multivariate analysis was conducted using Discriminant Analysis of Principal Components (DAPC; Jombart et al., 2010), implemented in the Adegenet package v2.1.x for the R environment. The presence/absence data of the AFLP bands were treated as dominant markers. To identify the optimal number of genetic clusters (*K*) without a priori assumptions, the find.clusters function based on the *K*-means algorithm was performed. To reduce background noise while retaining the maximum biological variance, the first 30 Principal Components (PCs) were retained. The most parsimonious and optimal number of clusters was selected by evaluating the trend of the Bayesian Information Criterion (BIC), testing a range of *K* from 1 to 11. The BIC analysis identified $K=7$ as the optimal number of genetic clusters (Appendix 1.1). Subsequently, the DAPC analysis was performed by retaining 15 PCs (corresponding to approximately 70% of the cumulative variance explained by the PCA) to prevent overfitting, and retaining 6 discriminant functions (equal to $K-1$). Finally, the individual membership probabilities for each of the identified genetic groups were graphically summarized using a bar assignment plot, integrating the information about the populations of origin of the samples.

Analysis of Molecular Variance (AMOVA; Excoffier et al. 1992) and the calculation of population genetic distances (F_{ST} values; Slatkin 1995) were performed in Arlequin v. 2.000 (Schneider et al. 2000) by comparing AFLP profiles between species and among populations within the species.

The degree of genetic differentiation (F_{ST}) among populations within each species was compared to that of differentiation at leaf traits (P_{ST}) using the *Pstat* R package (Da Silva and Da Silva 2018) to infer the relationship between phenotypic and genetic differentiation (P_{ST}-F_{ST}). F_{ST} values for each population from species *A. purpurocaeruleum* and *A. calabrum* were extrapolated from the molecular variance analysis. P_{ST} values and their bootstrapped distributions and confidence intervals were calculated with the Pstat package. Variations in P_{ST} were analysed as a function of the parameter c/h^2, where c is the proportion of the total variance attributed to additive genetic effects between populations and h^2 is the heritability, thus quantifying the incidence of phenotypic differences that can be attributed to additive genetic variance (see Brommer 2011; Castellani et al. 2023). Since narrow-sense heritability (h^2) and the proportion of total genetic variance due to additive effects (c) are unknown for wild populations, we assumed a null additive genetic effect ratio ($c/h^2=1$) as a robust baseline standard in evolutionary ecology. To test whether phenotypic divergence was correlated with neutral genetic differentiation, the matrices of pairwise P_{ST} values of for each trait were also correlated against that of F_{ST} values using standard Mantel tests (9,999 permutations, Pearson correlation method) using the vegan package in R.

The niche conservatism analysis at the population level was performed using the R package *Phylosignal* (Keck et al. 2016). Based on the concept of autocorrelation, first developed in spatial statistics, we used the Phylosignal tool to evaluate the tendency for conspecific populations to display similar trait values due to individuals' genetic similarity. Global autocorrelation measurement obtained by autocorrelograms provides valuable information about the occurrence of niche conservatism for a specific trait (Keck et al. 2016). In addition, using an adapted tool based on Local Indicators of Spatial Association (LISA; Anselin 1995), we evaluated the specific patterns in trait distribution among species and populations within species (Keck et al. 2016).To this purpose, the Local Moran's I index was computed using the function *lipaMoran* for each sample of the genetic dendrogram and for all considered traits (LA, LDMC and SLA). This function performs a non-parametric randomization test and detects significant positive or negative local trait autocorrelation among the tips of the dendrogram. All analyses were performed in R 4.4.2 (R Core Team 2023).

Results

Leaf trait variation

Mixed model results showed significant differences between the two species for LA, which was lower in *A. calabrum* (Appendix 1.2; Fig. 2), with $R^2m = 0.223$ and $R^2c = 0.629$. Although SLA and LDMC were also lower in the latter species, no significant differences occurred with respect to *A. purpurocaeruleum* ($R^2m = 0.002$, $R^2c = 0.729$ for SLA; $R^2m = 0.051$, $R^2c = 0.783$ for LDMC). Ordination by PCA accounted for 92.1% of the total variation and produced a partial separation of the two species along both components (PC1: 57.3%, PC2: 34.8%), mainly driven by LDMC and LA, respectively. Samples of *A. purpurocaeruleum* were more widely distributed especially towards the positive values of both axes (Fig. 3).

Means, standard deviations and coefficients of variation (CV%) for each trait across populations of the two species are given in Appendix 1.2. Overall intraspecific variation in LA was comparable in the two species, whereas SLA and LDMC showed slightly higher CV

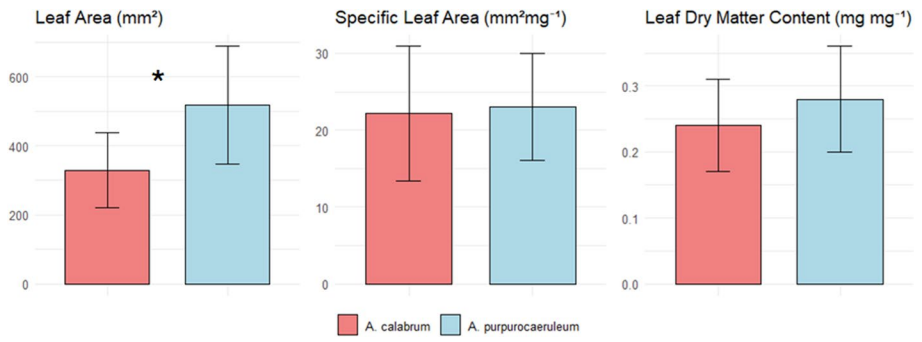

Fig. 2 Box plots showing variation in leaf area, specific leaf area, and leaf dry matter content in *Aegonychon calabrum* and *A. purpurocaeruleum*. Based on model results, only leaf area is significantly lower in *A. calabrum*. Significance levels: *p < 0.05; **p < 0.01; **p < 0.001

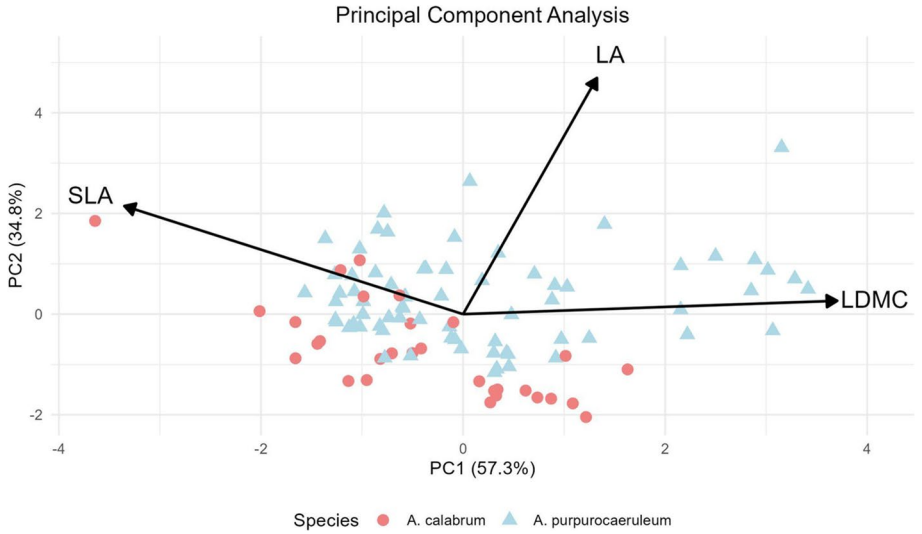

Fig. 3 Scattergram from PCA ordination of the individuals of *Aegonychon calabrum* (n = 30) and *A. purpurocaeruleum* (n = 70) based on leaf area (LA), specific leaf area (SLA), and leaf dry matter content (LDMC)

values in *A. calabrum*. Significant differences occurred in all traits between the respective populations of both taxa. In *A. calabrum*, population VM showed higher LA, while PO was characterized by lower SLA, and AF by lower LDMC. In *A. purpurocaeruleum*, the southernmost insular population (SI) was characterized by relatively high values of LA and LDMC, but a low SLA, whereas the northernmost population (TB) had low LA and a relatively high LDMC. At the intra-population level (among individuals of the same populations), variation of all three traits was considerably higher in VM than in PO and AF of *A. calabrum*. In *A. purpurocaeruleum*, the amount of intrapopulation depended on the trait and no consistent patterns occurred across populations.

In terms of CSR strategies, *A. calabrum* tended to show higher ruderality, lower stress tolerance and competitive ability than *A. purpurocaeruleum* (Appendix 1.3, Fig. 4). However, model results showed no significant differences between the two taxa. Indeed, species identity effect was negligible for all three strategies, as shown by R^2m values (C = 0.002; S = 0.019, R = 0.025), while population effects (R^2c values) accounted for 52% to 81% of the variation (C = 0.518, S = 0.791, R = 0.807). In fact, significant differences in the relative weight of the three strategies were detected between populations of both species, and especially in the degree of stress tolerance and ruderality in *A. calabrum* (Appendix 1.3).

Genetic structure

AFLP analysis was successfully performed on 94 samples and produced a total of 181 loci, 105 limited to the combination hex_EcoRI-CGT/MseI-CTC and 76 associated with the combination fam_EcoRI-CAG/MseI-AGG.

AMOVA results showed that most of the total genetic variation occurs at the intra-population level in both species, 99.12% and 90.81% in *A. calabrum* and *A.*

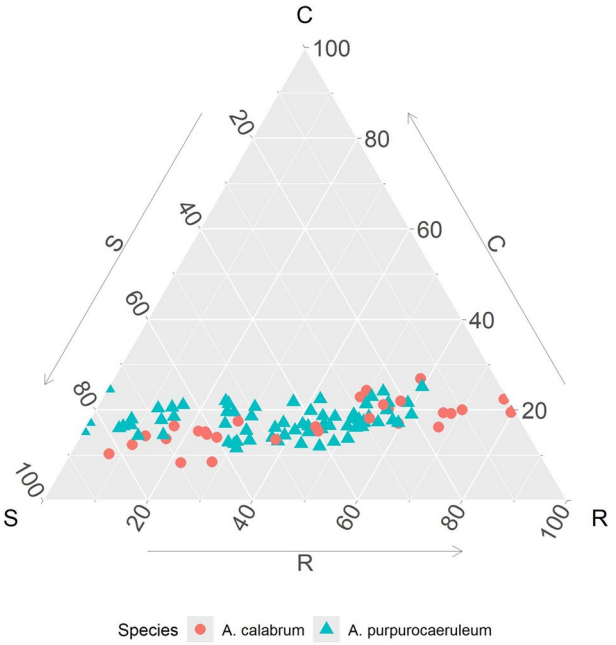

Fig. 4 Grime's CSR strategies triangle for the two species, based on values of leaf area, leaf fresh weight and leaf dry weight. The plot shows the position of each individual (mean of two leaves) based on the percentages of competitive ability (C), stress-tolerance (S) and ruderality (R)

Table 2 Results of AMOVA performed among and within populations of the narrow endemic *Aegonychon calabrum* and its widespread congener *A. purpurocaeruleum.* The table reports the degrees of freedom (d.f.), sum of squared deviations, variance components, and the percentages of total variance contributed by each component. P-values were estimated with 1000 permutations and were < 0.001 for both species

Source of Variation	d.f	Sum of squares	Variance components	Percentage of variation
A. purpurocaeruleum				
Among populations	6	323.714	2.798	9.190
Within populations	59	1630.165	27.630	90.810
Total	65	1953.879	30.427	
A. calabrum				
Among populations	2	58.124	0.240	0.880
Within populations	25	670.733	26.829	99.120
Total	27	728.857	27.069	

purpurocaeruleum, respectively (Table 2). The DAPC analysis (Appendix 1.1) revealed a clear genetic separation between the two species, driven by species-specific dominant genetic groups. *Aegonychon calabrum* was almost exclusively defined by two unique clusters (red and purple), differentially distributed among individuals across its three populations (AF, PO, and VM). The only notable exception was the VM population, which displays a minor fraction of genomic variation (in two individuals) assigned to a cluster (green) that is otherwise found, albeit at low frequency, in *A. purpurocaeruleum*. The latter species exhibited a higher genetic heterogeneity, partitioned across four species-specific clusters.

The F_{ST} values, quantifying the mean genetic distance among populations within each of the two species, were lower in *A. calabrum* (0.009 vs. 0.092 in *A. purpurocaeruleum*). In *A. calabrum*, the southern population VM was genetically more distant, with values of 0.020 and 0.004 from PO and AF, respectively. In *A. purpurocaeruleum*, the Sicilian accession showed the strongest divergence with respect to all the others, likely reflecting its insular position close to the southern limit of the species range (Appendix 1.4). Mean heterozygosity values were slightly lower in *A. calabrum* (mean He = 0.295 vs. 0.311 in *A. purpurocaeruleum*). Within *A. calabrum*, population heterozygosity varied from 0.270 (AF) to 0.331 (VM; Table 3), while a wider variability was detected in *A. purpurocaeruleum*, with heterozygosity ranging from 0.208 (SI) to 0.400 (ST). The BayeScan analysis identified nine outlier loci that had a posterior probability > 0.970 (at a log10 PO > 1.5 threshold) representing only 5% of all analysed loci. Two out of nine were specific for *A. calabrum*, whereas none was specific for *A. purpurocaeruleum* (Table 3).

PST-FST comparisons and niche conservatism analysis

The phenotypic differentiation in *A. calabrum* resulting from *Pstat* analysis varied from 0.788 for LDMC to 0.910 for SLA and from 0.849 for LA to 0.980 for LDMC in *A. purpurocaeruleum*. The P_{ST}-F_{ST} comparison for c/h^2 in both species (Figs. 5a-f and 6a-f) showed that phenotypic differentiation among populations (Appendix 1.5) was stronger than genetic differentiation ($P_{ST} > F_{ST}$ values), supporting that either directional or divergent selection are potentially acting on the examined leaf traits. Pairwise Mantel tests revealed contrasting patterns of correlation between neutral genetic divergence (F_{ST}) and quantitative phenotypic differentiation (P_{ST}) across the three leaf functional traits. A significant, positive correlation was only observed for Leaf Area (p-value = 0.0374).

The correlograms from analysis with Phylosignal confirmed no autocorrelation for any of the analysed traits, except for LA, which exhibited significant positive autocorrelation at intermediate lags of genetic distance and significant negative autocorrelation at higher distances (Appendix 1.6). The analysis for local trait patterns among populations within

Table 3 Estimates of genetic variation in *Aegonychon purpurocaeruleum* and *A. calabrum*. The following information is shown: N, number of analysed samples (individuals per population); He, expected heterozygosity within each population; POLYM and %POLYM, number and percentage of polymorphic loci in each population; %OUTL, percentage of outlier loci in each population. Population codes as in Table 1

Species/ID	N	He	POLYM	%POLYM	%OUTL
A. purpurocaeruleum					
TB	10	0.267	130	71.823	2.8
MA	10	0.348	152	83.978	2.8
ST	7	0.400	150	82.873	2.2
MQ	10	0.305	141	77.901	2.7
AN	10	0.269	127	70.166	2.2
SI	10	0.208	102	56.354	2.4
FI	9	0.379	158	87.293	2.6
Mean		0.311	137.1	75.770	2.5
A. calabrum					
AF	9	0.270	120	66.298	2.2
PO	9	0.284	129	71.271	2.3
VM	10	0.331	157	86.740	2.8
Mean		0.295	135.3	45.890	2.4

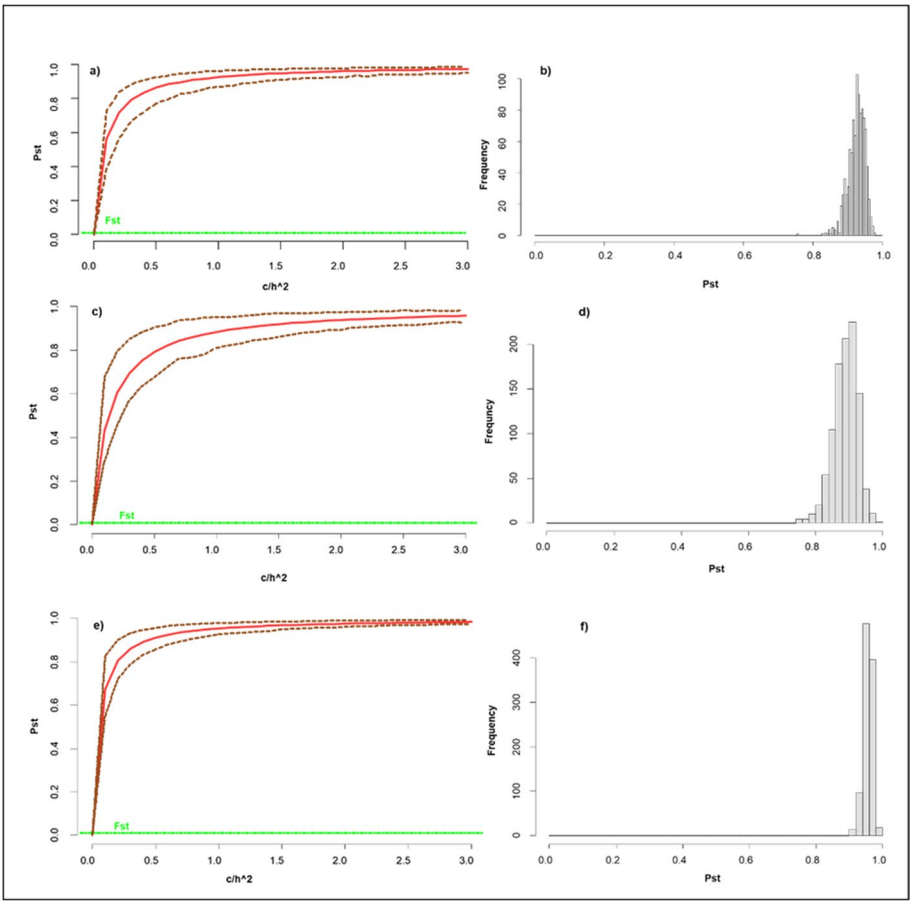

Fig. 5 a-f. Plots illustrating the relationship between F_{ST} and P_{ST} in *Aegonychon calabrum* for LA **a**, LDMC **c** and SLA **e**. The horizontal green line marks the value of F_{ST} among populations. P_{ST} values are shown as a function of c/h^2, which represent the ratio between the proportion of additive genetic variance among populations **c** and trait heritability (h^2). P_{ST} values and associated 95% confidence intervals are plotted in red and brown respectively. The P_{ST} barplot distribution for LA **b**, LDMC **d** and SLA **f** are also shown

species revealed significant local positive autocorrelation in a clade constituted by the two northern populations of *A. calabrum* from a similar habitat (PO and AF; Fig. 7).

Discussion

The lower leaf area and, to a lesser extent, specific leaf area, supported that the endemic *A. calabrum* is characterized by a more resource-conservative attitude than the widespread *A. purpurocaeruleum.* This finding fits evidence from a recent study on leaf trait variation in 27 pairs of endemic-non endemic species pairs of Mediterranean forests, showing lower resource acquisitive ability in the endemic taxa (Selvi et al. 2025). Unexpectedly, however, overall intraspecific variation in the three traits was comparable, and even slightly wider in *A. calabrum* than in its widespread congener,

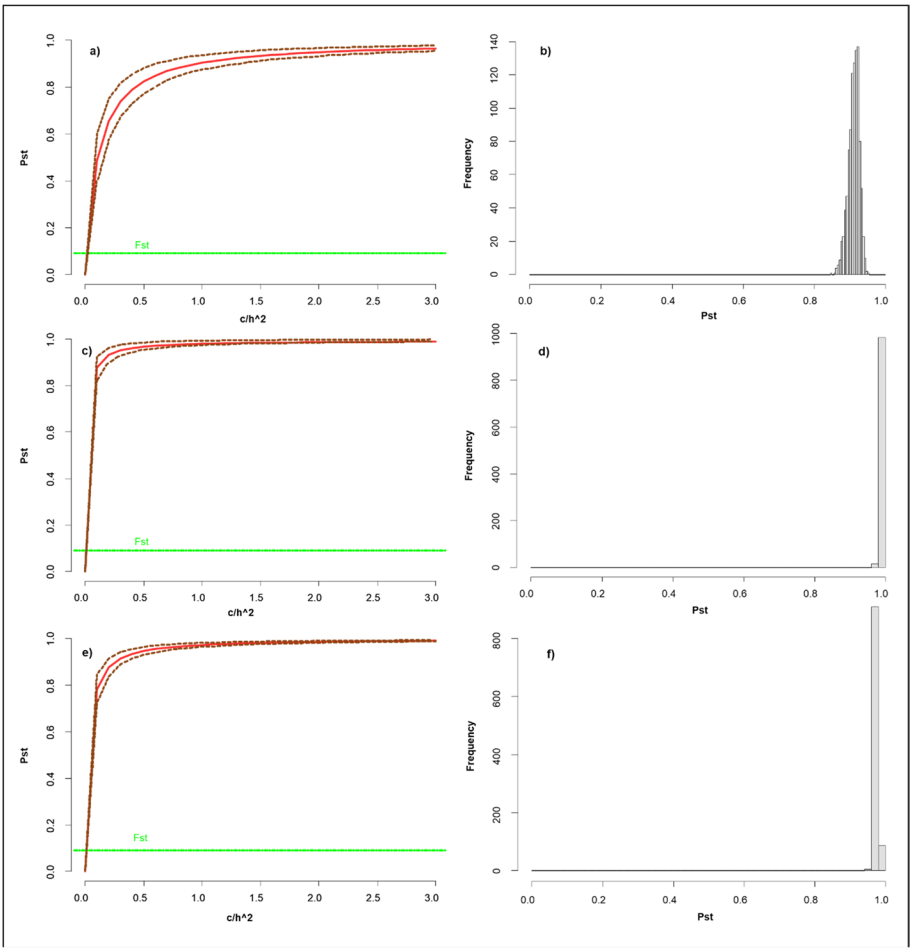

Fig. 6 a-f. Plots illustrating F_{ST}-P_{ST} comparisons in *Aegonychon purpurocaeruleum* for the traits LA **a**, LDMC **c** and SLA **e**. The horizontal green line marks the value of F_{ST} among populations. P_{ST} values are shown as a function of c/h^2, which represent the ratio between the proportion of additive genetic variance among populations **c** and trait heritability (h^2). P_{ST} values and associated 95% confidence intervals are plotted in red and brown, respectively. The P_{ST} barplot distribution for LA **b**, LDMC **d** and SLA **f** are also shown

despite the higher number of investigated populations of the latter and their distribution along a wider range of bioclimatic conditions from the Alpine to the Mediterranean region in Sicily. In spite of the narrow species range, the three populations of *A. calabrum* were located in heterogeneous climatic and edaphic conditions, potentially contributing to their phenotypic differentiation. The northern populations PO and AF from relatively low altitude, warm climate and calcareous substrate exhibited low LA and SLA values, two traits reflecting higher tolerance to water stress and a more resource-conservative strategy (Westoby et al. 2002; Garnier et al. 2015). Either drought, nutrient limitation or both can in fact explain low values of SLA and LA in species or populations of arid and/or oligotrophic environments (Poorter et al. 2009). On the contrary, the southern population VM from higher elevation, cooler climate and

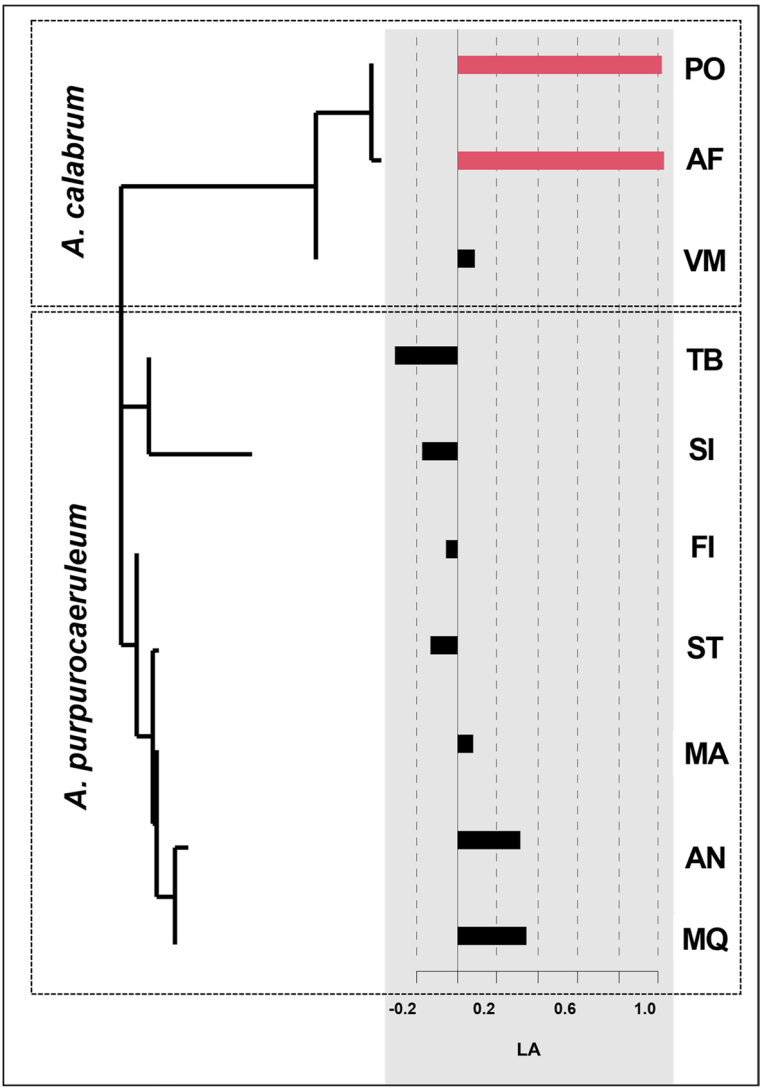

Fig. 7 Plot illustrating the Local Moran's I index values for trait leaf area (LA) in populations of *Aegonychon calabrum* and *A. purpurocaeruleum*. Red bars indicate significant local positive autocorrelation of the trait values

siliceous substrate was characterized by a more resource-acquisitive attitude (e.g. higher LA and SLA), likely thanks to more favourable conditions. Intraspecific trait variability translated into a relatively wide variability in ecological strategies, which was of similar magnitude to that in *A. purpurocaeruleum*. The relative contributions of the CSR strategies were in fact different between populations of *A. calabrum*, as VM resulted more ruderal to stress-tolerant, PO predominantly stress-tolerant, and AF ruderal.

Evidence about intraspecific variation in traits and ecological strategies can help to hypothesize the evolutionary model accounting for the restricted range and fragmented

distribution of *A. calabrum*. Two models can potentially explain the origin of narrow endemics, the "refuge" and the "specialist" model (Palacio et al. 2007; Matesanz et al. 2009). The former suggests that narrowly distributed species are stress-tolerant but have low competitive ability, thriving in marginal habitats due to competitive exclusion, while the latter posits that endemics may have adapted to narrowly distributed habitats dominated by abiotic constraints. Based on the specialist model, endemic species are generally believed to possess limited variability and to converge on narrow ranges of trait values, their rarity reflecting adaptation to specific environmental regimes and environmental tolerances. However, case studies refuting the hypothesis of rarity via specialization were detected in endemics with high intraspecific variation in leaf traits and CSR components, such as those in *Dianthus* L. (Behroozian et al. 2020). Moreover, other studies found that the ability of phenotypic plasticity to explain the rarity of endemics versus commonness of widespread congeners is limited (Boyd et al. 2023). Based on this evidence and coupled with a lack of clear divergence between *A. calabrum* and *A. purpurocaerulem* in ecological strategies, the former endemic species fits more the refuge than the specialist model.

Phenotypic plasticity in *A. calabrum* was paralleled by a relatively high level of genetic variability, only slightly lower than in *A. purpurocaeruleum*. Previous studies have suggested that plants with restricted distribution and occurring with small, fragmented populations are more prone to genetic erosion and have lower levels of variation than widespread species (Karron 1987; Arafeh et al. 2002; Edwards et al. 2014). This expectation was supported by significant, though small, differences in various genetic diversity measures between rare and widespread congeners (Gitzendanner & Soltis 2000). On the other hand, several studies showed that this is not always the case, such as those on endemics in *Petunia* Juss. (Turchetto et al. 2016) or *Helianthemum* Mill. in southern Spain (Martín-Hernanz et al. 2019). Within the Boraginaceae family, the taxa of *Anchusa* L. endemic to Sardinia also exhibited a relatively wide range of genetic diversity (0.135–0.391; Coppi et al. 2018). Such findings support that genetic diversity of endemics may be affected simultaneously by a large number of intrinsic and extrinsic factors, especially in Pleistocene glacial refugia in the ecologically heterogeneous mountains of the Mediterranean region (Martín-Hernanz et al. 2019), like those in Calabria where *A. calabrum* is discontinuously distributed. This evolutionary scenario was corroborated by the genetic structure analysis (DAPC), which revealed a clear genetic separation between *A. calabrum* and *A. purpurocaeruleum*, driven by species-specific genetic pools and a clear partitioning of clusters. Notably, differentiation at the population level matched previous phylogenetic evidence for the divergence between the two taxa, mirroring their morphological, palynological, and karyological differentiation (Cecchi et al. 2014; Chacón et al. 2019).

The intrapopulation genetic variability detected in *A. calabrum,* coupled with the weak differentiation among its populations, similarly to *A. purpurocaeruleum*, suggests the absence of genetic drift and a good status of genetic conservation for this species. In both species, moreover, the low proportion of outlier loci (< 3%), points to a similarly low impact of adaptive divergence among populations. Outlier proportions are in fact directly related to adaptation in different species to environmental pressures driving divergent selection, and indicating ecotype-level differentiation even when no divergence at neutral loci occurs (Russello et al. 2011; Feng et al. 2015; Coppi et al. 2020).

Comparing the levels of genotypic (F_{ST}) and phenotypic (P_{ST}) variation provided insights into the role of natural selection and genetic drift in the differentiation of populations (Brommer 2011; Leinonen et al. 2013), helping to unravel the role of adaptation to local habitat conditions (Merilä and Crnokrak 2001; Leinonen et al. 2006). Performing the P_{ST}-F_{ST}

comparison means to assume that F_{ST} reflects divergence caused solely by random genetic drift (Reynolds et al. 1983; Coppi et al. 2018), providing an estimate of what might occur in populations in the absence of selection (Merilä & Crnokrak 2001; Noguerales et al. 2016). Conversely, P_{ST} also accounts for the effects of natural selection on the phenotype (Castellani et al. 2023). In the present work, relatively low F_{ST} values were obtained at species level (*A. calabrum* = 0.009; A. *purpurocaeruleum* = 0.092) and significantly lower than P_{ST} values, even at a c/h2 ratio close to 0. The observed P_{ST}/F_{ST} ratio suggests the presence of directional or divergent selective pressure (Brommer 2011) on the functional traits examined (LA, LDMC, SLA) in both species, as lower critical c/h^2 values suggest that selection influences morphological traits and exclude the differentiation by sole genetic drift (Chavarria-Pizarro et al. 2019). Based on meta-analysis results (De Kort et al. 2013; Leinonen et al. 2008; see also Merilä and Crnokrak 2001), approximately 70% of the examined studies reported Q_{ST} values (here estimated as P_{ST}) exceeding F_{ST} values. These results are in line with evidence from the present investigation, supporting that divergent or directional selective pressures and local adaptation are common mechanisms in the differentiation of plant populations. Moreover, the average difference between P_{ST} and F_{ST} values observed in this study (range in *A. calabrum:* 0.507–0.691; *A. purpurocaeruleum:* 0.573–0.705) resulted significantly higher than those estimated for annual and perennial species (0.143 and 0.123, respectively; De Kort et al. 2013), corroborating a strong influence of divergent or directional selection compared to random genetic drift in both species. However, some methodological caveats regarding the use of P_{ST} estimates should be considered. As noted by some authors (Brommer 2011; Leinonen et al. 2013; Franzoni et al. 2023), P_{ST} relies on population-level variance components, which can oversimplify continuous individual morphological variation. Biologically, when intrapopulation phenotypic diversity is remarkably wide it can act as a strong confounding factor that inherently makes the P_{ST} index a highly conservative metric for detecting differentiation between populations. On the other hand, despite this limit and the potential confounding effects of phenotypic plasticity in natural populations, the observed P_{ST} values remained substantially and significantly higher than F_{ST} even under highly conservative c/h^2 scenarios. This clear decoupling strongly reinforces the hypothesis of divergent selection driving population differentiation, demonstrating that the signature of directional natural selection is powerful enough to overcome the extensive individual-level phenotypic variation within each population.

An additional aim of this work was to understand whether functional trait similarity among populations reflects genetic proximity or the effect of environmental constraints. Pairwise Mantel tests revealed contrasting patterns between F_{ST} and P_{ST} across the three traits, since a positive correlation was only observed for Leaf Area. This complex relationship between traits and genetic distance was further clarified by the correlograms from the Phylosignal analysis, which confirmed a general lack of autocorrelation for most traits, except for LA. Indeed, LA exhibited significant positive autocorrelation at intermediate values of genetic distance and significant negative autocorrelation at higher distances (Appendix 4). When analyzing local trait patterns within species, we found a significant local positive autocorrelation in a clade including the two northern populations of *A. calabrum* (PO and AF; Fig. 7). This pattern is consistent with the niche conservatism hypothesis at the population level, where genetically close populations tend to retain similar trait values due to shared ancestry, limited environmental divergence or stabilising selection, rather than random processes alone (Wiens et al. 2010; Peterson 2011).

Conclusions

This study provides insights into the relationships between a narrow endemic understory herb of Mediterranean forests and its widespread congener *A. purpurocaeruleum*, in terms of leaf traits and population genetic structure. Overall, differences in the level of intraspecific variability in both aspects were smaller than expected, with the two species showing similarly high levels of population heterozygosity, low distance among populations and comparable phenotypic plasticity in leaf area, specific leaf area and leaf dry matter content. Despite its more resource-conservative character, *A. calabrum* exhibited an unexpected differentiation between the examined populations in leaf traits and related CSR strategies, similarly to *A. purpurocaeruleum*. This points to a similar adaptive capacity to heterogeneous habitat conditions, without substantial genome-wide differentiation. The stronger among-population phenotypic differentiation, compared with the genetic one ($P_{ST} > F_{ST}$), supported that either directional or divergent selection are potentially acting on leaf traits. Trait autocorrelation in the northern populations of *A. calabrum* was consistent with the niche conservatism hypothesis, supporting the role of historical and genetic factors in population phenotypic differentiation. Distinct populations may therefore be subject to evolutionary processes, ranging from divergent selection to the retention of ancestral traits in genetically close groups. Both phenotypic plasticity and genetic variability, either within or among populations, are essential components of the biology of *A. calabrum*, likely making this endemic somewhat capable to adapt to changing habitat conditions.

Further investigations using other model systems and more advanced molecular approaches such as double-digest Restriction-site Associated DNA sequencing (ddRADseq) and genome-wide association (GWAS) will allow a more comprehensive characterization of genomic variation in endemic vs. related widespread species.

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Declarations

Conflict of interests The Authors declare no conflict of interests.

Competing interests The authors declare no competing interests.

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