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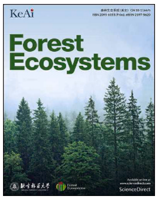

Leaf morphological trait integration and modularity provide insights into ecological adaptation in evergreen oaks

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ABSTRACT

The integration and modularity of leaf morphological traits are fundamental to plant adaptations, yet their responses to diverse environmental pressures remain unclear. In this study, we investigate the roles of leaf trait integration and modularity and how they interact with environmental factors. We analyzed geometric, traditional, and functional leaf traits across 908 individuals from 72 populations of two alpine evergreen oaks, *Quercus aquifolioides* Rehder & E.H. Wilson and *Quercus spinosa* David ex Franch., distributed throughout the Himalayan-Hengduan Mountains (HHM), employing genetic assignment as *a priori*. Multivariate and redundancy analyses revealed that *Q. aquifolioides*, which inhabits harsher environments, exhibits lower trait integration and greater morphological flexibility, allowing for dynamic adaptation to fluctuating conditions. In contrast, *Q. spinosa*, thriving in milder environments, demonstrates stronger integration and stability in leaf morphology, facilitating resource optimization and providing a competitive advantage. Notable differences in modularity between the two species were observed, particularly in specific leaf traits, as revealed by structural equation modeling (SEM) analysis. These results underscore the adaptive significance of leaf trait integration and modularity in extreme environments and highlight the critical role of leaf morphology in enhancing species resilience.

1. Introduction

Plants adapt to environment by balancing and coordinating multiple traits through process of integration and modularity (Berg, 1960; Pigliucci, 2003; Klingenberg, 2008; Li et al., 2024). Morphological integration refers to the strength and patterns of covariation of multiple traits (Olson and Miller, 1958; Klingenberg, 2008, 2014), allowing plants to maintain functional traits and a stable phenotype under varying environmental conditions (Hallgrímsson et al., 2009; Klingenberg et al., 2012; Murren, 2012; Felice et al., 2018). Morphological modularity, on the other hand, describes the independence between different modules and strong integration within modules (Klingenberg, 2008, 2014), enabling plants to rapidly adjust relevant traits in response to environmental changes and enhance overall adaptability (Wagner et al., 2007; Hallgrímsson et al., 2009). While numerous studies have investigated plant morphological variation (Hallgrímsson et al., 2009; Klingenberg et al., 2012), less attention has been given to the mechanisms of plant morphological integration and modularity under different environmental conditions (Wagner et al., 2007; Klingenberg, 2014).

Given the importance of morphological integration and modularity on plant adaptation, it is essential to understand how they manifest across various plant traits. Leaves are the major organs for physiological in plants and exhibit significant shape variation due to strong natural selection, allowing them to adapt to diverse environmental conditions (Nicotra et al., 2011; Ferris, 2019; Wang et al., 2022). Their primary functions, such as photosynthesis and transpiration, are influenced by temperature and water availability (Torres Jiménez et al., 2023), especially mean annual temperature (MAT) and mean annual precipitation (MAP), can significantly affect morphological traits and their adaptive capacities (Peppe et al., 2011; Fu et al., 2017; Wright et al., 2017). In addition, Lang's aridity index (AI_{LANG}: the ratio of the MAP and MAT, MAP/MAT) provides insight into a region's water resource status and climate aridity, serving as an important indicator for identifying areas most vulnerable (Quan et al., 2013; Alonso-Forn et al., 2020). Therefore, examining variations in leaf morphological traits under different

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environmental conditions is crucial for understanding plant adaptation mechanisms (Hermant et al., 2013; Kusi, 2013).

Traditional morphometrics, primarily focused on linear measurements, can distinguish species (Kremer et al., 2002) and reveal environmental effects on leaf morphology (Stephan et al., 2018). However, this approach often struggles to capture the complexity and variation in leaf shape (Rohlf and Marcus, 1993; Viscosi et al., 2009a). Emerging geometric morphometrics (GMMs) offer a more refined approach, can not only visualize differences in leaf morphology (Mitteroecker and Gunz, 2009; Rohlf, 2010; Klingenberg et al., 2012) but also uncover relationships between morphological traits (Viscosi et al., 2009b, 2012). Landmark-based GMMs, using the Procrustes approach, offer desirable statistical properties, provide robust statistical properties, and can be combined with other factors to analyze changes in leaf traits (Cardini and Marco, 2022). Additionally, functional traits such as leaf area and specific leaf area (SLA), which are related to resource acquisition and competition, can reveal the adaptive processes in response to varying environments (Liu et al., 2023). Recently, the integration of molecular markers with leaf shape has improved our understanding of the genetic correlations with morphology (Viscosi et al., 2009b; Rellstab et al., 2016; Liu et al., 2018; Li et al., 2021), enhancing insights into leaf morphological integration and modularity. For example, Klápště et al. (2021) identified multiple modules by integrating genetics and morphology, shedding light on the ecological preferences of oaks growing under different climatic conditions.

Trees, with their long-life cycles, exhibit significant leaf shape diversity due to ongoing adaptation to environmental pressures (Viscosi, 2015; Ferris, 2019; Klápště et al., 2021; Maya-García et al., 2020). The oak genus (*Quercus* spp.), with about 300–500 species, is one of the most ecologically important tree groups in the Northern Hemisphere, found across North America, Europe, and Asia (Zhou, 1993; Nixon, 1993; Manos et al., 1999; Denk et al., 2018). Due to extensive hybridization and gene flow, oaks exhibit significant variation in leaf morphology (Whittemore and Schaal, 1991; Bruschi et al., 2003a; Leroy et al., 2017; Du et al., 2022), which is also strongly influenced by varying climatic conditions (Richards et al., 2006; Viscosi, 2015; Solé-Medina et al., 2022). Given their wide distribution, diverse leaf forms, and important ecological roles, oaks serve as a model species for studying how leaf morphology variation adapts to environmental conditions (Cavender-Bares, 2019; Du et al., 2022).

Quercus aquifolioides Rehder & E.H. Wilson and *Quercus spinosa* David ex Franch. (subgenus *Cerris*, section *Ilex*) are the most widely distributed oak species throughout the Himalayan-Hengduan Mountains, thriving at elevations from 2,000 to 4,500 m (Zhou, 1993; Huang et al., 1999; Denk et al., 2018). *Q. aquifolioides* is adapted to cold regions and *Q. spinosa* thrives in warmer areas (Meng et al., 2017; Liu et al., 2022). Both species exhibit diverse leaf morphology (Chai et al., 2015; Li et al., 2021), with experience asymmetric gene flow (Du et al., 2017, 2020; Liu et al., 2022). As typical alpine trees, they exemplify adaptive strategies in extreme environments (Feng et al., 2016; Du et al., 2020). Notably, leaf trait integration has not been previously studied in these two species, making them ideal for exploring how leaf integration and modularity relate to ecological adaptation. Investigating these relationships can enhance our understanding of how local environmental factors experience leaf morphology and contribute to adaptive strategies in specific habitats. This regional focus addresses the limitations of generalization often encountered in global studies, providing insights that are more applicable to real-world conditions.

In this study, we use the oak species *Q. aquifolioides* and *Q. spinosa* as model species to explore the relationship between leaf traits and ecological adaptation. We hypothesize that *Q. aquifolioides* and *Q. spinosa* have adopted different ecological strategies to adapt to distinct environmental conditions through the regulation of leaf trait integration and modularity. Specifically, we investigated how variations in leaf morphology relate to the genetic differentiation between these species, how integration and modularity of leaf traits differ between *Q. aquifolioides* and *Q. spinosa*, and how environmental conditions influence these traits and their adaptive strategies. To address these questions, we analyze a large dataset comprising 908 individuals from 72 populations, measuring leaf morphological traits, including both geometric traits and traditional traits, while using genetic assignment as *a priori*. We analyze these relationships using structural equation modeling (SEM) and gather environmental data to correlate with leaf traits and assess how these factors affect adaptation. This study enhances our understanding of how leaf traits contribute to ecological adaptation in oaks and provides insights into the broader mechanisms of adaptation in tree species, highlighting the significance of leaf morphology in responding to environmental challenges across diverse habitats.

2. Materials and methods

2.1. *Field sampling*

We sampled the natural populations throughout the entire geographic range of the two evergreen oak species in mainland China. In total, we sampled 72 populations, including 29 populations of *Q. aquifolioides* and 43 populations of *Q. spinosa*, respectively, and each population was separated at least 30 km (Fig. 1a). We collected a total of 908 individuals, therefore seven to 22 individuals for each population. To prevent collecting cloned samples we ensured a minimum distance of 10 m between individuals. We collected five intact mature leaves from each adult individual for morphological analyses. One leaf was dried and stored in silica gel for DNA isolation and microsatellite genotyping. All sampled specimens were deposited in the archives of the Molecular Ecology Laboratory of Beijing Forestry University, China. The detailed sampling information of each population is listed in Table S1.

2.2. *Landmark configuration and leaf traits*

We flattened, dried, and scanned 4,508 leaves from 908 individuals at 600 dpi resolution using a CanoScan 5600 F scanner (Canon Inc., Japan). We marked a total of 13 landmarks by IMAGEJ v.1.5 and these landmarks constituted the geometric configuration of oak leaf (Figs. 1b and S1) (Abràmoff et al., 2004; Viscosi, 2015; Liu et al., 2018; Li et al., 2021). We organized the 13 landmarks of each leaf into 13 pairs of Cartesian coordinates (X, Y), and then we imported the coordinates into MORPHO J v.1.06 (Abràmoff et al., 2004).

We performed generalized Procrustes analysis (GPA) (Rohlf and Slice, 1990) to minimize differences between landmark configurations by translation, scaling, and rotation to maximize the coincidence of leaf coordinate data using the MORPHO J v.1.06 (Klingenberg, 2010, 2011). Leaf size and leaf shape information were separated, and covariance matrices of tree level were created for subsequent multivariate statistical analysis.

We also measured four traditional morphological leaf traits (Fig. 1b), petiole length (PL), length of the lamina from the lamina base to the widest part (WP), lamina width (LW), and lamina length (LL), which were considered as classical oak leaf traits (Kremer et al., 2002). We utilized the “vegan” R package (Oksanen et al., 2022) to calculate PC1 through principal component analysis (PCA) for the four traditional morphological traits. Furthermore, we measured the dry-weight leaves and determined the area of each fully extended leaf. We then calculated the SLA which is defined as the ratio of leaf area to dry leaf mass (leaf area per unit of dry leaf weight; $cm^2 \cdot g^{-1}$). A total of seven leaf traits (geometric leaf morphological traits: leaf size, leaf shape; traditional leaf morphological traits: PL, WP, LW, and LL; functional leaf traits: SLA) and traditional leaf traits PC1 were used for further analysis (Table S1).

2.3. *Data analysis*

2.3.1. *Microsatellite genotyping*

We used nuclear microsatellites to estimate pairwise relationship

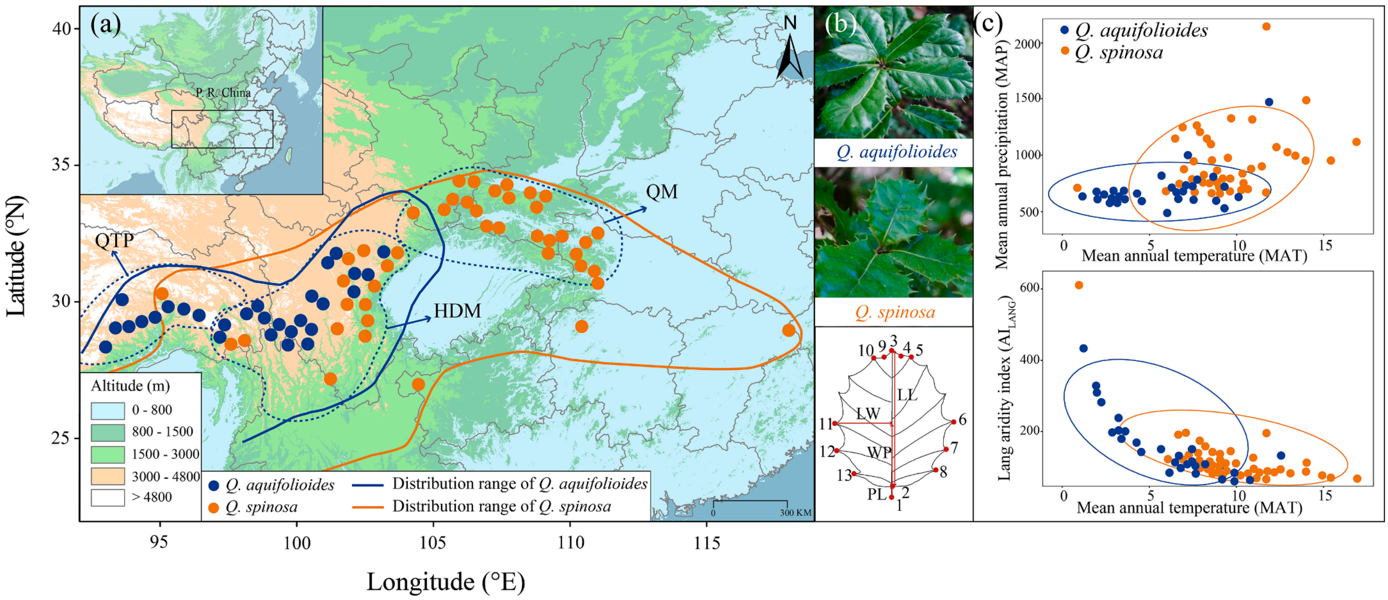

Fig. 1. Geographical distribution, leaf morphology, and climate factors of *Q. aquifolioides* and *Q. spinosa*. **(a)** Species range and sampling sites. The map displays the geographical range of *Q. aquifolioides* (blue) and *Q. spinosa* (orange) across the Qinghai-Tibet Plateau (QTP), HDM, and Qinling Mountains (QM). Curved lines indicate the species' ranges, and dots mark the sampling locations for each species. The black rectangle in the top left corner denotes the study region. **(b)** Leaf morphology: representative leaves of *Q. aquifolioides* (top) and *Q. spinosa* (middle), with 13 GMMs landmarks and four traditional leaf traits (bottom). Landmark descriptions are detailed in Fig. S1. Traditional leaf traits include lamina length (LL), petiole length (PL), lamina width (LW) and length of lamina from the lamina base to the widest part (WP). **(c)** Climate variables: MAP (top) and AI_{LANG} (MAP/MAT, down) of *Q. aquifolioides* and *Q. spinosa* across a gradient of MAT.

coefficients among individuals. 15 simple sequence repeats (*n*SSR) originating from *Q. macrocarpa*, *Q. robur*, *Q. petraea*, and *Q. mongolica* showed high polymorphism in *Q. aquifolioides* and *Q. spinosa* (Li et al., 2021; Liu et al., 2022) were used for genotyping (Table S2). We found the number of genetic markers is adequate to assign the individuals to lineages as *a priori* and investigated the large population size. We estimated the observed heterozygosity (H_O), mean expected heterozygosity (H_E), mean effective population size (N_E), mean Shannon index (*I*), and inbreeding coefficient (F_{IS}) by GenAlEx v.6.5 (Peakall and Smouse, 2006). We evaluated the significance of genetic diversity by *T*-test implemented in SPSS v.22 (SPSS Inc, Chicago, IL, USA) with a significance level of 0.05.

We utilized the Bayesian cluster analysis implemented in STRUCTURE v.2.3.4 (Pritchard et al., 2000) to identify population genetic structure. The program was run with the number of clusters (*K*) varied from one to ten, and 20 independent replicates were conducted for each *K*-value. The burn-in period was set to 100,000 steps, followed by the number of Markov chain Monte Carlo (MCMC) after the burn-in of 100, 000. We selected the optimal *K*-value by $\triangle K$ statistics performed in the web-based program Structure Harvester (Earl and Vonholdt, 2012). Threshold *Q* values of 0.9/0.1 were selected to achieve the best balance of efficiency and accuracy when assigning individuals to lineages (Vähä and Primmer, 2006; Lepais et al., 2009; Viscosi et al., 2012; Lyu et al., 2018). Individuals with *Q* values between 0.1 and 0.9 were interpreted as representing "mixed" ancestry.

We further conducted a hierarchical analysis of molecular variance (AMOVA) to estimate the genetic differentiation of *Q. aquifolioides* and *Q. spinosa* in Arlequin v.3.5 (Excoffier and Lischer, 2010). The significance of genetic differentiation was evaluated using 10,000 permutations.

2.3.2. Morphological discrimination, integration, and modularity analysis

We compared the differences in leaf traits between and within species using the "ggplot2" R package (Wickham, 2016). Then we performed discriminant analysis (DA) and canonical variate analysis (CVA) to investigate differences in geometric leaf morphological traits. DA provides reliable information on the differences between and within species using a cross-validation procedure with the T^2 statistic (*P* value for tests with 1,000 permutations <0.0001), focusing primarily on two groups (Klingenberg, 2011). CVA enhances the separation of specified groups through ordination analysis permutation tests using Mahalanobis and Procrustes distances, focusing on three or more groups. In this study CVA was performed to enhance separation among predefined groups, focusing on species, lineages, and mixed individuals.

We performed PCA to assess leaf shape integration at the individual level (Klingenberg, 2011). Strong morphological integration can constrain leaf variation (Klingenberg et al., 2012). Leaf shape integration existed when the proportion of a PC was significantly higher than others, indicating concentrated leaf variation (Schluter, 1996; Klingenberg, 2010; Klingenberg et al., 2012). Two-block partial least-squares (2 B-PLS) analysis is used to examine the covariation of shapes, especially to identify allometric patterns between leaf size and shape (Rohlf and Corti, 2000; Klingenberg, 2010, 2011). Allometry, which studies these size-shape relationships, is known to contribute to morphological integration (Klingenberg, 2009, 2012). Covariance was measured using the RV (square Pearson correlation) coefficient, and its significance was evaluated using 10,000 permutations.

We divided the landmarks into multiple modules to evaluate the optimal modularity by generating 12 modular partitions (Fig. S2). We compared the covariant strength among these hypotheses and all possible partitions. If the RV coefficient is close to the lower limit of the coefficient distribution, it indicates that the hypothesis is consistent with the true modular partition (Klingenberg, 2009).

In addition, we used an SEM using the "lavaan" package in R 4.2.2 (Rosseel, 2012) to explore the relationships among the leaf morphological traits. A meta-model was developed based on assumed correlations between the geometric leaf morphological traits (leaf size and leaf shape) and traditional leaf morphological traits (PL, LL, LW, and WP), as well as correlations among traditional leaf morphological traits (Fig. S3). SEM was fitted using a maximum likelihood approach and evaluated using Bentler's comparative fit index (CFI) and *P*-value as recommended by Hoyle (2012). The cutoff values for goodness-of-fit were CFI >0.9 and $P > 0.05$.

Because the two species co-occurred at some sites in sympatry

(Table S1), we aimed to test for ecological character displacement (ECD), defined as greater divergence in sympatry than in allopatry, as observed in deciduous oaks (Du et al., 2022; Qi et al., 2025). To assess this, we estimated the mean leaf shapes of the two species in both sympatric (D_{sym}) and allopatric (D_{allo}) populations and calculated the difference between these values ($D_{sym\text{-}allo}$) (Adams, 2004). ECD exists when a greater divergence in sympatry than in allopatry: yields a positive value for $D_{sym\text{-}allo}$ (Du et al., 2022).

2.3.3. Correlations among leaf traits, environmental, and genetic factors

We obtained 19 bioclimatic variables of the current conditions (approximately 1970–2000) at a resolution of 30 s (roughly 1 km^2) resolution from WorldClim Version 2 raster layers (Fick and Hijmans, 2017). Numerous studies have shown that leaf traits are closely related to MAT, MAP, and AI_{LANG} (Peppe et al., 2011; Quan et al., 2013; Fu et al., 2017; Alonso-Forn et al., 2020). To examine the influence of these climatic factors on leaf traits, we employed two approaches: representative key climatic factors (MAT, MAP, and AI_{LANG}) and climate PCA (based on the 19 bioclimatic variables) for further analysis. We extracted climate PC1 (bio01-bio19), temperature PC1 (bio01-bio11), and precipitation PC1 (bio12-bio19) using the "vegan" R package (Oksanen et al., 2022). First, we depicted the differences in MAT, MAP, and AI_{LANG} between *Q. aquifolioides* and *Q. spinosa* using the "ggplot2" package in R v.4.2.2 (Wickham, 2016; Fig. S4 and Table S3). Then, we constructed two scatter plots with MAT as the *x*-axis: One with MAP as the *y*-axis and one with AI_{LANG} as the *y*-axis. For each plot, we marked the data points for the two species and added 95% confidence ellipses around them (Fig. 1c). Next, we used linear mixed modeling to investigate the response of leaf traits to climatic, temperature, and precipitation PC1 for all and pure individuals (genetically purebred, *Q* values greater than 0.9 or less than 0.1) with the "lme 4" package in R v.4.2.2 (Adams, 2004; Bates et al., 2015). The significance of the effect was assessed using an analysis of variance test from the "car" R package (Fox et al., 2013) and visualized with the "ggplot2" package (Wickham, 2016).

We also conducted marginal (full) and conditional (partial) distance-based redundancy analyses (dbRDA) using the *capscale* function in the R package "vegan" (Oksanen et al., 2022). We divided all the variables into three variable sets: genetics, climate, and geography. The marginal dbRDA models included all variables, whereas the conditional models tested the significance of each variable set, controlling the other two variable sets. The significance of each factor in the dbRDA was determined using the ANOVA function in the "vegan" package, which calculated pseudo-*F* ratios, variance components, and *P* values (Oksanen et al., 2022).

To minimize the influence of introgression, we focused on genetically purebred individuals in this study unless specified otherwise. For GMM analysis, however, we include both pure and mixed individuals to present a comprehensive view of leaf morphology. The workflow, presented in Fig. 2, offers a streamlined and adaptable framework for analyzing trait variation in relation to genetic, geographic, and climatic factors, and may serve as a reference for future studies in similar systems.

3. Results

3.1. Genetic diversity and differentiation

The genetic diversity was higher in *Q. aquifolioides* than in *Q. spinosa* (H_O: 0.47 vs. 0.36, H_E: 0.48 vs. 0.41, F_{IS}: 0.02 vs. 0.11; Table S4). The genetic differentiation of *Q. spinosa* was higher than that of *Q. aquifolioides* (F_{ST}: 0.24 vs. F_{ST}: 0.08), and most of the genetic variation occurred within populations (Table S5). Bayesian clustering identified $K = 2$ as the optimal number of clusters for intra- and inter-species (Fig. S5). A total of 211 mixed individuals were detected by a strict threshold of *Q* values between 0.1 and 0.9 (Fig. S5a and Table S1): 69 mixed individuals were identified in *Q. aquifolioides*, with 31 from the Tibet lineage and 38 from the Hengduan Mountains-Western Sichuan Plateau (HDM-WSP) lineage (Fig. S5b and Table S1); 142 mixed individuals were identified in *Q. spinosa*, with 46 from the Tibet-HDM lineage and 96 from the QM-east China lineage (Fig. S5c and Table S1).

3.2. Leaf morphological discrimination, integration, and modularity

All leaf traits differed significantly between and within the species ($P < 0.05$; Fig. S6). Compared to *Q. aquifolioides*, *Q. spinosa* showed significantly larger leaf size, SLA, LL, and LW, but significantly smaller

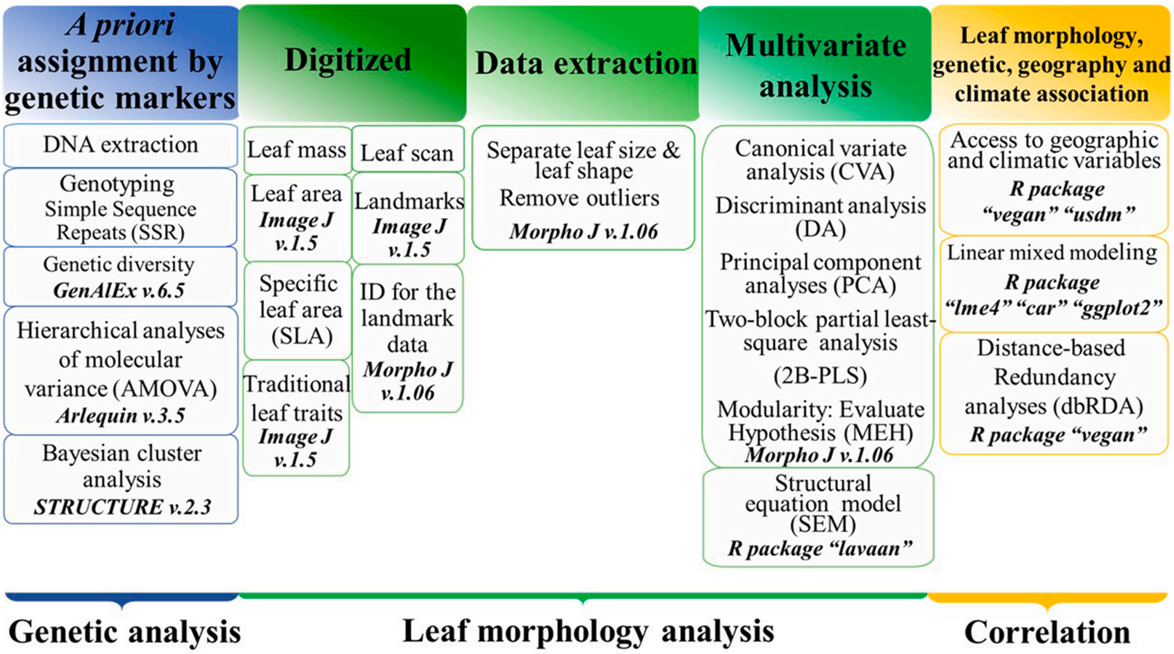

Fig. 2. Workflow for analyzing leaf morphological traits in relation to genetic, geographic, and climatic factors. The software and R package used are indicated in bold italics.

PL and WP (Fig. S6). DA indicated a higher discrimination proportion for *Q. spinosa* compared to *Q. aquifolioides* (92% vs. 83%; Table S6). Additionally, the west lineages (*Q. aqu*-Tibet lineage and *Q. spi*-Tibet-HDM lineage) showed higher discrimination proportion compared to the east lineages (*Q. aqu*-HDM-WSP lineage and *Q. spi*-QM-east China lineage) (87% vs. 80%, 80% vs. 77%; Table S6).

CVA revealed a distinct separation between inter- and intraspecific individuals of *Q. aquifolioides* and *Q. spinosa* (Figs. 3a, S7a, and S7b). Through the transformation grids, the results show that the interspecific mixed individuals exhibited leaf morphologies more aligned with *Q. spinosa* (Fig. 3a), whereas mixed individuals between east and west lineages within each oak species leaned towards the east lineages of two oak species (Figs. S7a and S7b). PC1 explained a larger proportion of leaf morphology (49%) than PC2 (22%) (Figs. 3b, S7c, and S7d), highlighting greater variation in leaf shape within *Q. aquifolioides* along PC1 than *Q. spinosa*. Leaf morphology integration was observed along the PC1 direction, with *Q. spinosa* showing greater integration than *Q. aquifolioides*. Additionally, 2 B-PLS analysis revealed a significant allometric pattern between leaf shape and leaf size for both the inter- and intraspecific levels of the two species ($P < 0.0001$; Fig. S7). In *Q. aquifolioides*, the optimal modular partition included the petiole, lower part, and upper middle part of the leaf, with the landmarks at the widest part of the leaf grouped into the upper middle section (Fig. 3c and Tables S7 and S8). For *Q. spinosa*, the optimal modular partition included the petiole, lower middle part, and leaf tip, with the landmarks at the widest part assigned to the lower middle section (Fig. 3d and Tables S7 and S8). SEM revealed correlations among six leaf morphological traits (CFI = 1, $P > 0.05$; Figs. 3e and f, S8, and S9). In *Q. aquifolioides*, leaf size and leaf shape showed the strongest correlation with LW, whereas interactions among WP, PL, LW, and LL, exhibited relatively weak path coefficients. Conversely, in *Q. spinosa*, leaf size and leaf shape showed a strong correlation with LL, with larger path coefficients among WP, PL, LW, and LL. The correlations between traditional leaf morphological traits were stronger in *Q. spinosa* compared to *Q. aquifolioides*, indicating stronger trait integration in *Q. spinosa*, potentially possibly reflecting adaptive strategies in different ecological environments (Fig. 3e and f). Additionally, we found greater morphological divergence in allopatry than in sympatry, indicating no evidence of ECD (Table S9).

3.3. Environmental and genetic influences on leaf trait integration and modularity

Q. aquifolioides occupies regions with lower yearly precipitation and temperature compared to *Q. spinosa*, positioning it on the left side of the plot (Fig. 1c). Additionally, *Q. aquifolioides* exhibits a higher Lang's aridity index, positioning it above *Q. spinosa* (Fig. 1c). Specifically, *Q. aquifolioides*, with less integration of leaf traits, exhibited a stronger response to climate gradients (Figs. 4 and S10). In contrast, the leaf traits of *Q. spinosa* exhibited a weaker response to climate gradients, with selective traits primarily responding significantly to precipitation (Fig. S10). Especially, the SLA of *Q. spinosa* remained stable across temperature and precipitation, while *Q. aquifolioides* displayed greater variability in SLA (Figs. 4 and S10). These contrasting responses highlight distinct adaptative strategies between the two oak species, a pattern that remained consistent when genetically admixed individuals were included in the analysis (Fig. S11).

Marginal dbRDA, a multivariable regression, showed that geographic and climatic factors significantly influenced variation in leaf size and the traditional leaf traits PC1 for *Q. aquifolioides* (Table 1). For *Q. spinosa*, leaf size, leaf shape, and traditional leaf traits PC1 were jointly explained by geographic, genetic, and climatic factors (Table 1). These patterns were further supported by partial RDA analyses (Table S10). Overall, climatic factors emerged as key drivers of leaf trait variation, but *Q. spinosa* showed greater stability under climatic fluctuations compared to *Q. aquifolioides*.

4. Discussion

The observed differences in leaf morphological traits between *Q. aquifolioides* and *Q. spinosa* revealed distinct levels of leaf trait integration and modularity in response to their respective environments. *Q. aquifolioides*, which inhabits harsher environments, exhibited lower trait integration and greater responsiveness to climate. This suggests that less integrated traits allow greater flexibility and adaptability to environmental fluctuations, offering a critical advantage in more extreme conditions. In contrast, *Q. spinosa*, thriving in relatively milder environments, displayed stronger trait integration and greater stability

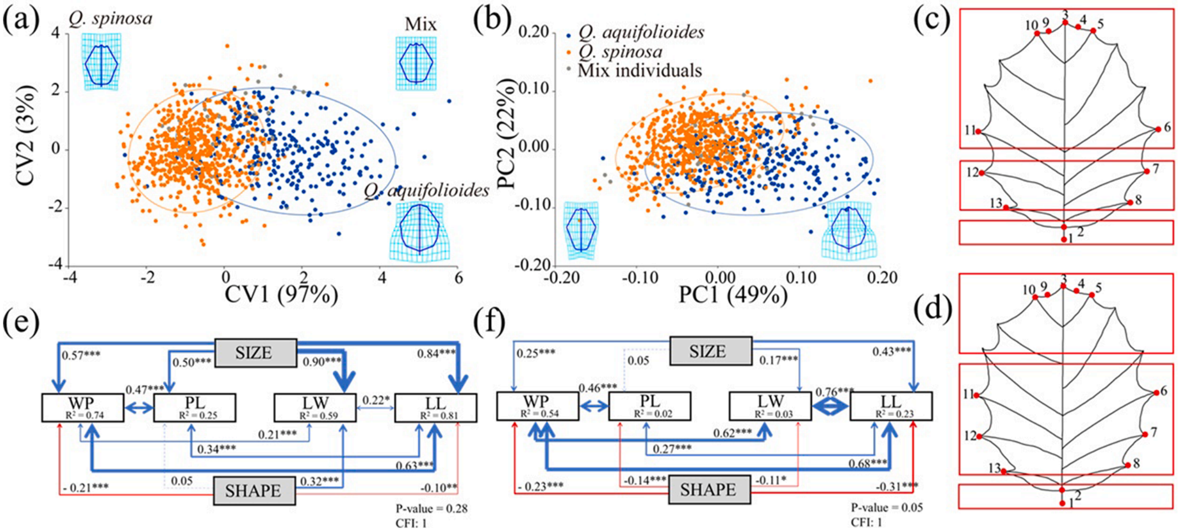

Fig. 3. Morphological analysis of *Q. aquifolioides* and *Q. spinosa*. **(a)** CVA and **(b)** PCA for *Q. aquifolioides* and *Q. spinosa*, with 95% confidence ellipses. Transformation grids illustrate the average leaf shapes of each group and shapes with extreme negative (−) and positive (+) PC scores. **(c, d)** Optimal modularity for leaves of *Q. aquifolioides* and *Q. spinosa*. Detailed modular partitions are shown in Fig. S1. (**e**, **f**) SEM depicting relationships between traditional leaf morphological traits (white box) and geometric leaf morphological traits (gray box) in genetically pure individuals of *Q. aquifolioides* and *Q. spinosa*. Standardized path coefficients are provided alongside arrows, indicating effect size. Solid and dashed arrows represent significant and non-significant relationships, respectively with blue and red colors representing positive and negative associations. The arrow width reflects the strength of the path coefficients or effect size. The proportion of variance explained (R^2) is indicative above each response variable. Significance levels: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

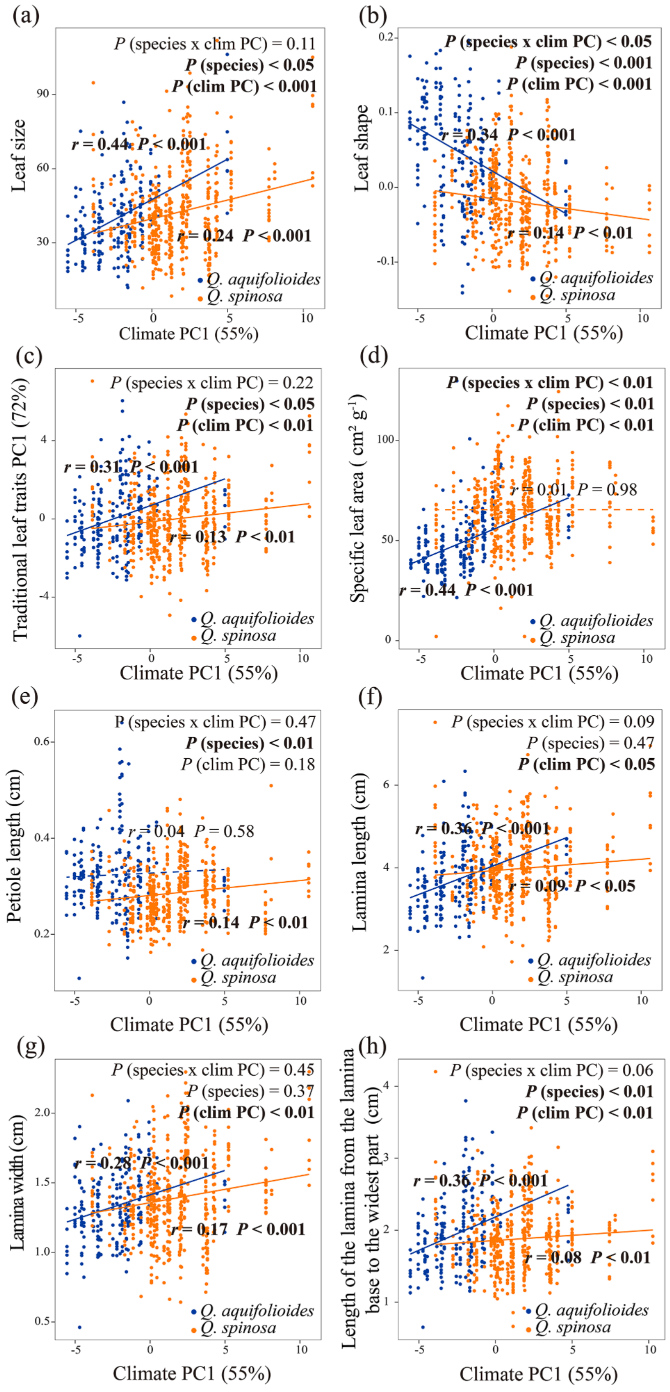

Fig. 4. Variation in leaf traits including leaf size, leaf shape, traditional leaf morphological traits PC1, SLA, and four traditional morphological leaf traits (lamina length, LL; petiole length, PL; lamina width, LW; length of lamina from the lamina base to the widest part, WP) of genetically pure individuals of *Q. aquifolioides* and *Q. spinosa*, shown in relation to climatic variation (climate PC1 representing bio01-bio19). Correlation coefficients (*R*) and significance values (*P*) are provided for each trait. *P*-values were calculated using a linear mixed-effect model.

in leaf morphology across various environmental conditions, maintaining its functional traits. These findings highlight how differing levels of trait integration contribute to the ecological adaptation of these oak species. The study enhances our understanding of how leaf traits interplay in shaping adaptive strategies, especially for species in extreme environments like the Himalayan-Hengduan Mountains.

4.1. Impact of genetic differentiation and geographic isolation on leaf morphology

Variation in leaf morphology can differ significantly within and between species (Blue and Jensen, 1988; Bruschi et al., 2003b). In this study, we observed notable differences in leaf morphology both between and within species. Such morphological variation might link to underlying genetic diversity, as species with higher genetic diversity may be more capable of adapting to varying environmental conditions (Nicotra et al., 2010; Kusi, 2013). A low F_{IS} indicates random mating of individuals within their populations, which helps maintain high genetic diversity (Wright, 1965; Hedrick, 2005) while a high F_{IS} indicates greater inbreeding within the population, which can reduce the genetic variation (Keller and Waller, 2002; Charlesworth and Willis, 2009). In addition, the complex orogenic history of the Qinghai-Tibetan Plateau and adjacent regions has resulted in long-term geographic isolation (Qiu et al., 2011; Wen et al., 2014), might also contributed to this morphological differentiation at species-level (Krauze-Michalska and Boratyńska, 2013; Favre et al., 2015). *Q. spinosa* exhibits a higher degree of genetic differentiation than *Q. aquifolioides*, and its leaf morphology is more distinct (as shown by DA), likely due to genetic divergence and geographic isolation.

We also found that the leaf morphology of mixed individuals tends to resemble one of the parent species or lineages. This tendency for similarity is consistent with the direction of gene flow: mainly from *Q. aquifolioides* to *Q. spinosa* at the interspecific level and west lineages to east lineages at the intraspecific level (Du et al., 2017, 2020; Liu et al., 2022).

ECD is a process that enhances phenotypic differences between species through resource competition in sympatry (Cavender-Bares, 2019; Du et al., 2022; Qi et al., 2025). It is reported that leaf traits in sympatric species may diverge more compared to their allopatric populations (Du et al., 2022). However, we did not detect this pattern in *Q. aquifolioides* and *Q. spinosa* (Table S9), suggesting the competition in harsh environments for these evergreen oaks may differ from that of their deciduous sister species in milder temperatures. In our study, the species with larger leaf area and higher SLA (*Q. spinosa*) may benefit from enhanced light capture and nutrient acquisition, thereby gaining a competitive advantage-a pattern consistent with previous research (Kunstler et al., 2016; Bennett et al., 2016; Wagg et al., 2017).

4.2. Morphological integration and modularity

The integration of leaf traits is a fundamental component of plant morphology (Lipson et al., 2002; Felice et al., 2018). In this study, we found both evergreen oaks demonstrate considerable leaf trait integration, indicative of coordinated morphological responses. Additionally, allometry, patterns of covariation between leaf size and shape, significantly contributes to leaf morphological integration (Klingenberg, 2009), explaining a substantial portion of the observed morphological variation in both species (Klingenberg, 1997a, 1997b, 1998; Viscosi et al., 2012). In both oaks, significant allometric relationships were detected, further supporting their role in shaping integrated leaf morphologies.

While integration of leaf traits can promote phenotypic stability by limiting potential variation in leaf shape under natural selection (Alberch, 1982; Hallgrímsson et al., 2009; Klingenberg et al., 2012), it may also introduce developmental constraints. Conversely, modularity allows for greater evolutionary flexibility in leaf morphology, enabling adaptations toward optimal forms and increasing the potential for variation (Wagner et al., 2007; Hallgrímsson et al., 2009). When the covariation of morphological traits aligns with natural selection, biological modularity facilitates the evolution of modular organs that enhance ecological adaptation (Schluter, 1996). However, when directional selection negatively impacts a trait, pushing it toward an optimal trade-off, modularity may act as an evolutionary constraint (Wagner and

Table 1
Results of dbRDA testing the association of leaf traits (leaf size, leaf shape, and traditional leaf traits PC1) with three sets of predictor variables. Marginal tests are shown on the left, assessing each variable set individually. Partial (conditional) tests, shown on the right, evaluate the significance of each variable set while controlling for the other two variable sets.

Model	Variable set	Marginal tests			Model	Variable set	Conditional tests		
		F	%VAR	*P*			*F*	%VAR	*P*
Q. aquifolioides					***Q. aquifolioides***				
Leaf size					Leaf size				
Marginal (all variables)	Geography	7.12	4.33	**< 0.01**	Conditional (climate + genetics)	Geography	6.69	4.06	**< 0.01**
	Genetics	0.50	0.45	0.69	Conditional (geography + climate)	Genetics	0.50	0.45	0.68
	Climate	16.50	15.04	**< 0.001**	Conditional (geography + genetics)	Climate	9.22	8.41	**< 0.001**
Leaf shape					Leaf shape				
Marginal (all variables)	Geography	0.73	0.47	0.51	Conditional (climate + genetics)	Geography	1.09	0.70	0.35
	Genetics	0.73	0.71	0.52	Conditional (geography + climate)	Genetics	0.73	0.71	0.55
	Climate	13.75	13.35	**< 0.001**	Conditional (geography + genetics)	Climate	8.44	8.20	**< 0.001**
Traditional leaf traits PC1					Traditional leaf traits PC1				
Marginal (all variables)	Geography	5.28	3.32	**< 0.01**	Conditional (climate + genetics)	Geography	4.84	3.03	**< 0.05**
	Genetics	0.09	0.09	0.96	Conditional (geography + climate)	Genetics	0.10	0.09	0.95
	Climate	16.78	15.81	**< 0.001**	Conditional (geography + genetics)	Climate	10.31	9.72	**< 0.001**
Q. spinosa					***Q. spinosa***				
Leaf size					Leaf size				
Marginal (all variables)	Geography	14.30	3.58	**< 0.001**	Conditional (climate + genetics)	Geography	23.70	5.93	**< 0.001**
	Genetics	12.15	4.56	**< 0.001**	Conditional (geography + climate)	Genetics	12.15	4.56	**< 0.001**
	Climate	35.92	13.49	**< 0.001**	Conditional (geography + genetics)	Climate	17.83	6.70	**< 0.001**
Leaf shape					Leaf shape				
Marginal (all variables)	Geography	16.76	4.73	**< 0.001**	Conditional (climate + genetics)	Geography	22.61	6.38	**< 0.001**
	Genetics	9.13	3.87	**< 0.001**	Conditional (geography + climate)	Genetics	9.13	3.87	**< 0.001**
	Climate	7.34	3.10	**< 0.001**	Conditional (geography + genetics)	Climate	2.60	1.10	0.06
Traditional leaf traits PC1					Traditional leaf traits PC1				
Marginal (all variables)	Geography	6.73	1.72	**< 0.01**	Conditional (climate + genetics)	Geography	10.40	2.67	**< 0.001**
	Genetics	4.93	1.56	**< 0.01**	Conditional (geography + climate)	Genetics	4.94	1.90	**< 0.001**
	Climate	41.95	16.13	**< 0.001**	Conditional (geography + genetics)	Climate	28.45	10.94	**< 0.001**

Note: The marginal test included all variables: %VAR, percentage of variance explained by each variable; *F*, *F* values; Bold values indicate statistical significance at $P < 0.05$.

Zhang, 2011; Smith, 2016). Specifically, the differences in modularity between *Q. aquifolioides* and *Q. spinosa* are evident in LW (Fig. 3e and f). In *Q. spinosa*, LW shows significant variation with increasing LL, whereas *Q. aquifolioides* exhibits less variation in LW with increasing LL, resulting in a more elongated leaf morphology. Given that these traits have been shown to be highly integrated across multiple oak species (Viscosi et al., 2009b; Klápště et al., 2021), they likely reflect the influence of long-term stable selection patterns. The observed differences in morphological integration and modularity between *Q. aquifolioides* and *Q. spinosa* may enable these species to adopt distinct strategies and capacities for adaptation (Klingenberg, 2014; Klápště et al., 2021).

4.3. Adaptive roles of leaf trait integration and modularity

The integration and modularity of leaf morphology might reflect plant ecological strategies for adapting to specific environments and are crucial in responding to environmental changes (Pigliucci, 2003; Klingenberg, 2014; Klápště et al., 2021). The two studied species displayed notable differences in their integration: The species with a lower degree of leaf trait integration were more responsive to climate variation, while species with a higher degree of integration displayed greater stability. Specifically, *Q. aquifolioides*, which inhabits harsher environments such as tree line, exhibits weaker trait integration and greater variability. This flexibility may aid in adaptation to dynamic climates (Wright et al., 2005) and promote long-term survival. These traits likely reflect adaptations for water conservation and tolerance to temperature extremes (Du et al., 2020; Bhusal et al., 2021), enabling the species to endure water scarcity and extreme temperatures. *Q. spinosa*, which thrives in relatively mild conditions, shows greater leaf integration, reflecting a more stable adaptation, allowing for resource optimization and enhanced competitiveness (Chapin et al., 1993; Richards et al., 2006; Solé-Medina et al., 2022). In addition, the two species differ significantly in SLA (Fig. S6). SLA is a key functional trait reflecting the trade-off between resource capture and conservation, which also reflects this adaptive strategy (Wright et al., 2005). In resource-poor environments, plants often reduce water loss by decreasing SLA (thickening leaves, e.g., *Q. aquifolioides*), whereas in resource-rich environments, they tend to increase SLA (thinning leaves, e.g., *Q. spinosa*) to maximize photosynthetic efficiency (Knight and Ackerly, 2003; Poorter et al., 2009; Reich, 2014). The differences in leaf trait integration and SLA between the two species highlight how plants adjust traits, optimizing survival and adaptability across diverse environmental conditions.

5. Conclusions

This study demonstrates the significance of leaf integration and modularity in shaping plant ecological responses. Specifically, harsh environmental conditions diminish leaf morphological integration, enhancing their survival, while milder conditions foster greater integration, thereby improving resource acquisition. This knowledge offers profound insights into the evolutionary mechanisms of plants under varying ecological stresses. Future research should delve deeper into the interactions between genomic and phenotypic traits across different environmental contexts, establishing a more comprehensive theoretical framework for understanding plant adaptive evolution.

CRediT authorship contribution statement

Yi Zhang: Writing – original draft, Formal analysis, Conceptualization. **Yanjun Luo:** Writing – review & editing. **Min Qi:** Writing – review & editing. **Ying Li:** Formal analysis. **Fang K. Du:** Writing – review & editing, Funding acquisition, Data curation, Writing – original draft.

Data availability

Data will be made available on request.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fecs.2025.100350.

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