Research

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A new dimension of leaf economic spectrum: temporal instability of relationships among genotypes

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Summary

- Leaf economic spectrum (LES) relationships have been studied across many different plant lineages and at different organizational scales. However, the temporal stability of the LES relationships is largely unknown. We used the wild blueberry system with high genotypic diversity to test whether trait–trait relationships across genotypes demonstrate the same LES relationships found in the global database (GLOPNET) and whether they are stable across years.
- We studied leaf structure, photosynthesis, and leaf nutrients for 16 genotypes of two wild blueberry species semi-naturally grown in a common farm in Maine, USA, across 4 yr.
- We found substantial variation in leaf structure, physiology, and nutrient traits within and among genotypes, as well as across years in wild blueberries. The LES trait–trait relationships (covariance structure) across genotypes were not always found in all years. The trait syndrome of wild blueberries was shifted by changing environmental conditions over the years. Additionally, traits in 1 yr cannot be used to predict those of another year.
- Our findings show that LES generally holds among genotypes but is temporally unstable, stressing the significant influence of trait plasticity in response to fluctuating environmental conditions across years, and the importance of temporal dimensions in shaping functional traits and species coexistence.

Introduction

The leaf economic spectrum (Westoby *et al.*, 2002; Wright *et al.*, 2004; Garnier & Navas, 2013) illustrates a strategy for either a fast or slow return on carbon investment in leaves, reflecting a trade-off between a long leaf lifespan (persistence and toughness) and high photosynthetic performance (Reich *et al.*, 1997; Wright *et al.*, 2004). This trade-off is demonstrated in the covariance of related functional traits (trait–trait relationships), often including photosynthetic rate (A_{max}) increasing with leaf nitrogen (N_m), A_{max} decreasing with leaf thickness (LMA), and N_m decreasing with LMA. This trade-off serves as a general principle governing the leaf design or development of leaf functional traits, which are structural and physiological features determining individual performance, species fitness, and plant response to environmental changes (McGill *et al.*, 2006). Variations in functional traits can be found at different scales and levels including across space (Asner *et al.*, 2016), through succession and time (R. Li *et al.*, 2015; X. Li *et al.*, 2015), across individuals of a species (Albert *et al.*, 2010a), within an organism, among species, and among communities (Messier *et al.*, 2010). These trait variations generally follow some well-known trait-trait relationships, suggesting common principles in leaf trait development. The LES (leaf economic spectrum) trait-trait relationships have been found on a global scale (Wright *et al.*, 2004), in different plant lineages (Edwards *et al.*, 2014; Bai *et al.*, 2015; Mason & Donovan, 2015; Zhang *et al.*, 2015; Martin & Isaac, 2021), and in various geographical regions (Wright *et al.*, 2005; Hu *et al.*, 2015; Asner *et al.*, 2016; Pan *et al.*, 2020). However, the existence of the LES has shown mixed results at small spatial scales, despite significant trait variation and the inclusion of many species, with some studies continuing to find the LES (Asner *et al.*, 2014; Thomas *et al.*, 2020) while others failing to find it (Albert *et al.*, 2010b; Messier *et al.*, 2010). Finding the temporal, environmental, and taxonomic determinants (when, where, and at what scale) of the existence (or not) of the LES trait–trait relationships, as well as the influence of scale, remains an open question. We seek to provide a new perspective (although not a final answer) on this question by examining the existence of the LES at small spatial scales across genotypes and by focusing on the relatively unknown temporal stability of trait–trait relationships.

As trait–trait relationships are influenced by environmental conditions (Turnbull *et al.*, 2016; Cui *et al.*, 2020; Fyllas *et al.*, 2020; Zhang *et al.*, 2022), there could be temporal shifts in these relationships. However, most studies only encompass a single year, and then one time point data are used to analyze temporal and spatial patterns of trait-trait relationships, species performance, community structure, and ecosystem functioning (Swenson *et al.*, 2017). While collecting trait data at a single point in time may be pragmatic, traits are known to change plastically in response to the environment (Moran, 1992; Baythavong, 2011), and the influence of traits on individual performance is also environment-dependent (Lande & Arnold, 1983; Wade & Kalisz, 1990). How trait values and trait–trait relationships change over time are not well-understood, although the environment is always changing. Revealing the temporal dynamics of trait values can help us understand changes in individual performance and ecosystem processes over time (Swenson *et al.*, 2017). Further, although the effect of environmental factors on LES trait–trait relationships has been explored by studying trait–trait relationships in contrasting ecosystems (e.g. Zhang *et al.*, 2022) or under different controlled environments (e.g. Cui *et al.*, 2020), the novel temporal dimension has not been conceptualized and studied. Studying the temporal dynamics of the same system excludes the confounding effects of variations among individuals or ecosystems and reflects *in situ* responses of the same individuals to natural changes in a combination of environmental factors.

Plant functional traits are an important ecological tool used to investigate the individual performance, species fitness, community structure, ecosystem functioning (Albert *et al.*, 2010a), and biodiversity (de Bello *et al.*, 2009). Plant functional traits can indicate ecological strategies (Wright *et al.*, 2004; Reich, 2014) and physiological functions (Reich, 2014), providing a useful and quantitative tool to study physiological and ecological questions (McGill *et al.*, 2006). Differentiation or overlap in functional traits could be used to study niche differentiation (niche: a set of conditions, resources, and interactions that a species needs to carry out its ecological role) or species/genotype coexistence, but how temporal changes in traits over the years will affect niche overlap or differentiation has rarely been tested. Two co-occurring species in the same environment that often show substantial niche overlap are able to coexist if they show equal or similar competitive capacities (Mahdi *et al.*, 1989; Miller & Spoolman, 2009; Berdugo *et al.*, 2019). As a result, co-occurring species in a given habitat could exhibit similar ecological strategies and share similar traits (Cornwell *et al.*, 2006), leading to the trait convergence within these species (Lavorel & Garnier, 2002; Lebrija-Trejos *et al.*, 2010). Meanwhile, if they show high niche overlap, the competition can also be intensified due to their reliance on the same resources (Simberloff & Dayan, 1991), which may lead to trait divergence. Yet, we have very little understanding of how temporal dynamics in functional trait overlap and differentiation may contribute to coexistence mechanisms among species and/or genotypes.

The semi-natural wild blueberry agricultural system is unique, with diverse genotypes naturally growing in a single field and is managed to promote high yield. This system provides a model system to monitor the temporal dynamics of traits and trait–trait relationships. The two common species of wild blueberry typically found in managed fields in North America are the sweet blueberry (*Vaccinium angustifolium* Aiton) and the sour top or velvetleaf blueberry (*Vaccinium myrtilloides* Michx) (Hall *et al.*, 1979). *Vaccinium angustifolium* and *V. myrtilloides* have a unique growth habit (Bell *et al.*, 2009); they both grow vegetatively by rhizomes and have a 2-yr production cycle that alternates between a crop year (reproductive year) and a vegetative year. Managed wild blueberry fields, first developed by Indigenous people in North America, contain a large number of genotypes, which have been coevolving for thousands of years (Borns *et al.*, 2004; Drummond *et al.*, 2009). They show highly pronounced inter-genotype and intra-genotype variation within each species in morphology, phenology, and yield (Kloet, 1978; Smagula *et al.*, 1997). Due to the large number of naturally co-occurring genotypes within a farm, the wild blueberry agricultural system provides an ideal model system to explore intra-genotype and inter-genotype trait variation, and trait–trait relationships among genotypes as well as the responses of these relationships to temporal changes in environmental conditions. The objectives of this study were to (1) quantify the temporal contribution (across years) to leaf trait variations in the wild blueberry system and demonstrate the influence of temporal changes in environmental conditions; (2) assess whether LES relationships (functional trade-offs) exist in this unique agricultural system with high intergenotype and intra-genotype variation; and (3) test whether LES relationships are stable across years. To address the above objectives, we studied traits commonly included for LES analyses (A_{max}, $\mathrm{N_m}$, LMA) and other structural and functional traits that influence the above LES traits, such as leaf size, plant height, and Chl concentration.

Materials and Methods

Study sites and plant materials

This work was carried out on Blueberry Hill Farm in Jonesboro, Maine, USA (Supporting Information Fig. S1; 44.6439°N, 67.6465°W, elevation 257 m), which has an average annual temperature of 6.3°C and average annual precipitation of 1230 mm (United States Climate Data, 2022). We measured the physiological and morphological traits of two species of wild blueberries, *V. angustifolium* Aiton and *V. myrtilloides* Michx (Table S1), for 4 yr from 2017 to 2020 when species were in the vegetative (vegetative) phase in 2017 and 2019, and in the crop (reproductive) phase in 2018 and 2020. Wild blueberries are deciduous species showing leaf unfolding in late April/early May, and leaf dropping in late September/early October. Thus, all the physiological measurements were implemented in July and August while the leaves were fully mature. Climatic conditions were recorded on-site using an automated weather station (Davis Instruments, Hayward, CA, USA). Total precipitation during the months of May through August was 365.1 mm in 2017, 266.6 mm in 2018, 530.7 mm in 2019, and 245 mm in 2020 and temperature was 15.5°C in 2017, 16.64°C in 2018, 15.73°C in 2019, and 16.68°C in 2020 (Table S2), indicating extreme wet conditions in 2019, and dry and high temperatures in 2018 and 2020.

The study site holds well-drained sandy loam acidic soils with pH ranging from 4.6 to 5.2 and organic matter content from 4.5% to 9.5%. Although the field has a long history of management including pesticide applications and occasional fertilization during the study period, the plants were not irrigated or fertilized.

Sixteen genotypes (eight each from *V. angustifolium* and *V. myrtilloides*) of wild blueberries were selected randomly from the 10-ha farm in 2017. Efforts have been made to have a sample size of eight genotypes per species, but due to weather and logistic constraints, it varies from 6 to 8, and in one case $n = 4$ for photosynthesis data of *V. myrtilloides* in 2017. A sample size of four to six is common for ecophysiological studies, and here, we want to test whether this sample size could capture trait variations and trait–trait relationships. To select these genotypes, the farm was divided into *c.* 200 grid cells, and each grid cell was assigned a number. Then, random numbers were used to select 16 grid cells and one genotype per cell. When more than one genotype was present in a selected cell, the genotype in the middle was selected. The genotypes could be visually distinguished from each other based on the differences in leaf form, color, size, and morphology (Fig. S2), and their genetics were tested. Individual genotypes or 'clones' make up a visually distinctive mosaic patchwork with identical genetic information (Bell *et al.*, 2009; Barai *et al.*, 2022). We use the term genotypes throughout our study because each mosaic patchwork of wild blueberry plants is a different genotype selected for our study. Six representative plant stems from within the genotypes were used to quantify within-genotype variations and were marked with a ribbon for the morphological, phenological, and physiological throughout the study. Due to the small plant size, we used different individual plant stems each year to assess all the traits. For the measurement of plant physiological traits such as plant photosynthesis, stomatal conductance, LMA, leaf size, plant height, stem diameter, and Chl concentration, we measured six individual plant stems within a genotype. For the measurement of leaf nutrient analysis, we used 40–60 leaves, oven-dried, and ground into powder for leaf nutrient analyses. All traits measured were listed in Table S3.

Phylogenetic analysis

In order to understand the genotypic differences and relatedness between the studied wild blueberry genotypes, phylogenetic analysis was carried out using DNA sequence data from Alaba (2023). Briefly, total genomic DNA from 50 mg of fresh and young leaf tissues was extracted for each genotype using the cetyltrimethylammonium bromide extraction methods (Doyle & Doyle, 1987). Then, the Quant-iT PicoGreen dsDNA assay (Invitrogen) was used to quantify the DNA, and samples were normalized to 20 ng μl^{-1} before the OmeSeq-qRRS library preparation. Afterwards, NGSCOMPOSER, an automated pipeline (Kuster *et al.*, 2021; https://github.com/bodeolukolu/ngsComposer) was used to demultiplex and quality-filter the Illumina short reads. For full details on DNA isolation, extraction, and sequencing, please refer to Alaba (2023).

Using the sequenced genome data, we constructed a phylogenetic tree for the 16 selected genotypes. The V.PHYLOMAKER2 package was used to generate the phylogenetic tree for 13 wild blueberry genotypes (Jin & Qian, 2022). Two genotypes (G2 and G3) from *V. myrtilloides* species and one genotype (G5) from *V. angustifolium* species were missing from the phylogenetic analysis due to low-quality genome sequencing data. For full details on phylogenetic analysis, refer to Alaba (2023). We also plotted the relative size of trait values alongside the phylogenetic tree in order to visualize and compare how different LES traits vary among the genotypes of two different species over the years.

Morphological measurements

Six individual stems within each genotype were marked using a permanent marker for stem length and stem diameter measurements. Plant height (from the ground surface to the tallest bud) was measured using a ruler, and stem diameter was measured using a vernier caliper at the location 2 cm above the ground surface. Leaf thickness of fully mature and healthy leaves was measured using a digital micrometer (Mitutoyo, digital micrometer, 0.0001 mm accuracy) for six individual stems per genotype. For leaf size measurements, fully mature and healthy leaves from six different individual stems of each genotype were collected from unmarked stems. The total area of the leaves from each individual stem was determined by a leaf area meter (LI-3000A area meter; Li-Cor, Lincoln, NE, USA), and the average leaf size was calculated by dividing the total leaf area by the total leaf number. Then, the leaf samples were oven-dried at 70°C for 48 h, and dry mass was measured using a precision balance. Leaf mass per area (LMA) was calculated as leaf dry mass divided by leaf area.

Gas exchange measurements

The light-saturated net CO_2 assimilation rate per area (A_a, $\mu mol\ m^{-2}\ s^{-1}$) and stomatal conductance (g_s; $\mu mol\ m^{-2}\ s^{-1}$) were measured with a portable photosynthetic system LI-6400 (Li-Cor) in the year 2017 and 2018 and LICOR-6800 (Li-Cor) in the years 2019 and 2020. A small chamber of 2 cm^2 area was used to place at the middle section of the leaf for the above measurement. The leaves were measured on sunny days between 08:30 and 10:30 h solar time subjected to 10 min of constant saturating light intensity with the photosynthetic flux density (PPFD) at 1500 $\mu mol\ m^{-2}\ s^{-1}$, ambient temperatures (28–35°C), CO_2 concentration (*c.* 420 ppm), and relative humidity (40–60%). Light-saturated net CO_2 assimilation rate per mass (A_m; $\mu mol\ g^{-1}\ s^{-1}$) was determined as A_a divided by LMA for that plant. Intrinsic water use efficiency was calculated by dividing the light-saturated CO_2 assimilation per leaf area by stomatal conductance (Hatfield & Dold, 2019).

Leaf nutrient and Chl concentrations

For leaf nutrient concentrations, *c.* 40–60 fully mature and healthy leaves from the unmarked stems of each genotype were collected and oven-dried at 70°C for 48 h at the end of the growing season in August. After oven drying, leaves were ground into a fine powder and sent as one homogenized sample per genotype to the University of Maine Soil and Plant Tissue Testing Laboratory in Orono, ME, USA, for leaf nutrient analysis. Total leaf carbon (C) and nitrogen (N) were quantified using a Leco Tru-Mac CN analyzer (Midland, ON, Canada). Leaf macronutrients (P, K, Ca, and Mg) and micronutrients (Al, B, Cu, Fe, Mn, and Zn) were quantified using a Thermo-Fisher model iCAP 6300 radial view ICP-OES (Waltham, MA, USA). From the results obtained from the plant tissue analysis, photosynthetic Nitrogen use efficiency (A_N) and Phosphorus use efficiency (A_P) were determined as A_m divided by mass-based concentrations of N and P (N_m and P_m), respectively. Leaf Chl concentration was measured every year with a Chl meter (SPAD 502 DL meter; Konica-Minolta, Tokyo, Japan) by placing the chamber at the middle section of the leaves for six sun-exposed mature leaves from six stems of each genotype that were marked with a ribbon. Leaf C and N ratio was determined by dividing carbon per mass by nitrogen per mass.

Statistical analysis

To test whether traits differ among years, we used nested/hierarchical one-way ANOVAs for each trait using data for the two species combined and separately. In each model, a random intercept for genotype was nested within species. In each model, a different trait was set as the response variable and species and year were the fixed factors. To test the difference in functional traits between two species for each year, one-way ANOVA was used. The variance was calculated from the mean sum of squares using the hierarchical one-way ANOVA with measurements at individual levels nested within the genotypes under two species. Then, for each trait, this allowed us to separate stem/individual variance, genotype variance, species variance, and year variance using the 'varcomp' function (Messier *et al*., 2010).

The leaf (structural, photosynthetic, and nutrient) traits were averaged for each genotype of the two species for analyzing trait–trait relationships. Standardized major axis (SMA) tests were used to see the differences in slope and intercept of bivariate relationships (LMA, A_m, and N_m) between blueberries and the Glopnet (Wright *et al*., 2004) using the SMATR package in R (Warton *et al*., 2012). SMA regressions were calculated to determine whether there was a significant difference in slope between the genotypes of two lowbush blueberry species (*V. angustifolium* and *V. myrtilloides*) vs the Glopnet leaf economics dataset and the *Vaccinium* species in Glopnet dataset as previously described (Wright *et al*., 2004). A principal component analysis (PCA) was performed to summarize the joint variation of the functional traits for the two species (Pearson, 1901), with mean trait values of each genotype used for the analyses. To include environmental variation in our analysis, we assigned the growing season temperature and precipitation of a specific year of the study site to all genotypes in the PCA. All data, figures, and graphs were analyzed and produced using R v.4.0.3 (R Core Team, 2021).

In order to test whether the potential lack of trait–trait relationships was due to limited sample size, we constructed null distributions for the trait correlations of each species for each year based on the global trait distribution, randomly resampled 10 000 times with the *x* and *y* range and sample size of each species, centered on a randomly selected point (Anderegg *et al*., 2018). For instance, the within-*V. angustifolium* null models for LMA vs N_m relationships were generated by randomly drawing *V. angustifolium* mean trait values from the global distribution of mean LMA and N_m values of angiosperms and *Vaccinium* species, constrained to the same LMA and N_m trait range but centered on a randomly selected mean. The observed correlation between LMA vs N_m of *V. angustifolium* for each individual year was calculated. Null distribution was visualized using a histogram and then the observed correlation between the traits was marked on the same plot. The 2.5th and 97.5th percentiles of the null distribution were calculated and marked on the histogram. If the observed correlation fell outside this range (in the tails of the distribution), the correlation between LMA and N_m was considered to be significant, and it indicated a nonrandom pattern specific to *V. angustifolium* species.

In order to understand the overall temporal change of trait–trait relationships over time, we developed an integrated trait network and analyzed its temporal shift. Statistically significant trait-trait relationships (*P* value ≤ 0.05) were used to run the network analysis using the IGRAPH package. This method was also used by Messier *et al.* (2017).

Results

Across-year variation in leaf traits of wild blueberry

There were high variations in leaf morphology and trait values both within and between species (Figs 1, S2; Tables S4–S6). There were significant differences among years for all traits measured including leaf morphology and shape, photosynthetic traits, leaf nutrient concentration, and other leaf structural and functional traits for both species (*V. angustifolium* and *V. myrtilloides*), and when the two species are combined (Figs 1, S3; Table S4; Notes S1). For instance, photosynthetic rates per leaf area or dry mass of both species were significantly higher in 2019 than in other years (Table S4). The significant differences in some traits between the two species depended on the year (Table S5).

High year–year variation in relative leaf traits among genotypes was found (Fig. 1). For instance, G13 showed the highest A_m among genotypes in 2020 the lowest A_m in 2019 (Fig. 1). LMA was the highest in G13 and G16 among all genotypes in 2019, but in G8 in 2018 (Fig. 1). Overall, genotypes closer to each other in the phylogenetic tree did not show closer trait values (Fig. 1).

Variance partitioning across stems, genotypes, species, and year

Variance partition analysis revealed that trait variation within lowbush blueberry was the largest across years and across stems

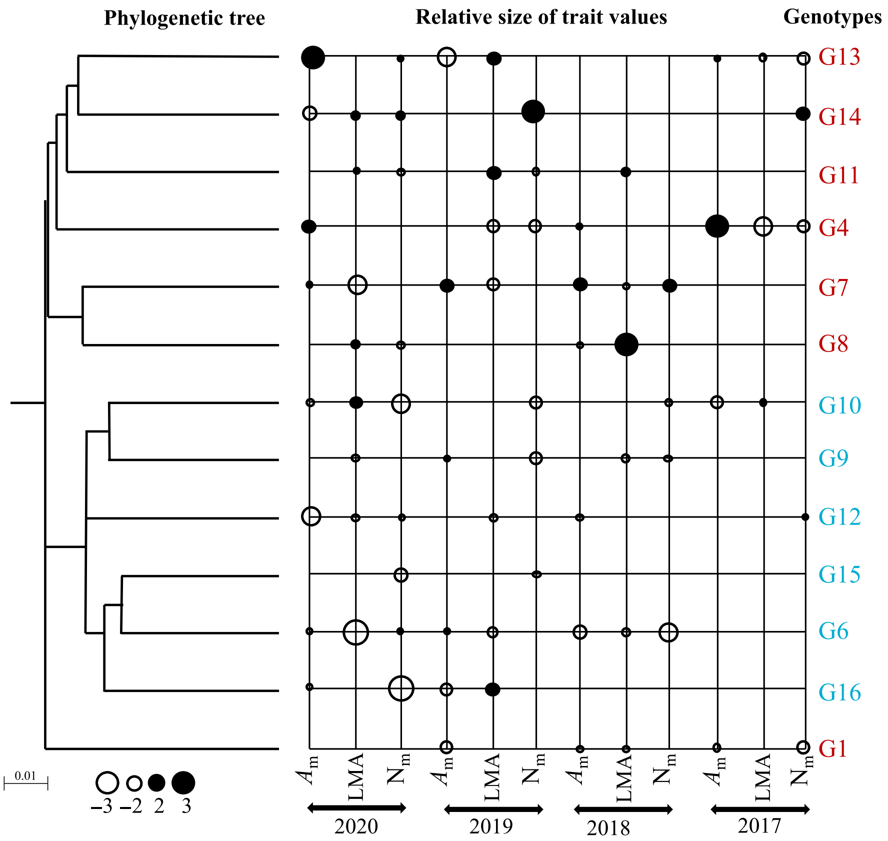

Fig. 1 Phylogenetic tree of the 13 wild blueberry genotypes out of 16 studied genotypes from *Vaccinium* species. Genotypes marked with red are *Vaccinium angustifolium* and genotypes marked with blue belong to *Vaccinium myrtilloides*. Two genotypes (G2 and G3) from *V. myrtilloides* species and one genotype (G5) from *V. angustifolium* species are missing from the phylogenetic analysis due to low-quality genome sequencing data. Relative size of the three key leaf economic spectrum traits (A_m, light-saturated photosynthetic rate, LMA, leaf mass per area; N_m, leaf nitrogen concentration per mass) values range from −3 to 3 for each year of study from 2017 to 2020. The size of the circle denotes the relative strength/size of trait values, with solid circles denoting higher positive size of trait values and open circles with four quadrants denoting lower negative size of trait values. The scale bar (0.01) of the phylogenetic tree represents the branch length and indicates the genetic distance between the genotypes.

within a genotype, less so across genotypes within a species, and the smallest between species (Fig. 2). However, the proportions varied substantially by trait (Fig. 2). Variation in A_a, A_m, LMA, LS was largest across years, g_s and Chl_m largest across stem within genotype, SH was largest across genotypes within a species, and SD variation was largest across species (Fig. 2).

Comparison of trait covariance (LES) among the genotypes of wild blueberry across years with those of Glopnet

The expected LES trait–trait relationships (covariance structure) from the global LES were found in some years in our data, but not consistently in all years (Figs 3a–f, S3). The among genotype trade-off between LMA and A_m was found in a vegetative year 2019 for both species, but in another vegetative year 2017, the same trait trade-off was only found among genotypes of *V. angustifolium* (Fig. 3c,d). The N_m and LMA relationship was also only found among genotypes of *V. myrtilloides* in crop year 2020 (Fig. 3f). When all genotypes from the two species were combined, LMA and A_m as well as A_m and N_m relationships were only found in vegetative years (2017 and 2019) and crop year 2018, respectively (Fig. S3a,b). Additionally, no significant relationships were found among LES traits when four-year averages were used for analyses (Fig. S4). Although our data did not find significant relationships between N_m and A_m in 2019 for *V. angustifolium*, between A_m and LMA in 2018, and between N_m and A_m in 2019 for *V. myrtilloides*, the null model distribution results suggested that these relationships should exist (Table S7; Figs S5, S6). Therefore, it could be because our sample size was not able to capture these trait–trait relationships. By contrast, the null model distribution results suggested that the other relationships (e.g. between N_m and LMA in all years for *V. angustifolium*, and in 2017, 2018, and 2019 for *V. myrtilloides*) did not exist (Table S7).

Coordination of leaf traits among the genotypes of wild blueberries across years

We did not consistently capture the trait–trait relationships between other structural and functional traits of leaves across years (Figs 4, 5, S7, S8). However, the trait–trait relationships among genotypes of two species differed by either vegetative year or crop year or by year even though they are both vegetative years or crop years. For example, there were significant relationships between A_m and C_m, and between Chl_m and N_m across the genotypes of *V. angustifolium* only in the crop year 2020 but in another crop year 2018, the relationship between A_m and C_m was only found across the genotypes of *V. myrtilloides* (Figs 4c,d, S8a). However, there were significant trait–trait relationships between A_m and C_m in both the crop years (2018 and 2020) across the genotypes of both species (Fig. S8a). Similarly, LMA was significantly related to A_N, A_p, and Chl_m across the genotypes of both species in both vegetative years (2017 and 2019; Fig. S8c–e).

The relationships between A_P and Chl_m with LMA were only present across the genotypes of *V. angustifolium* in the vegetative

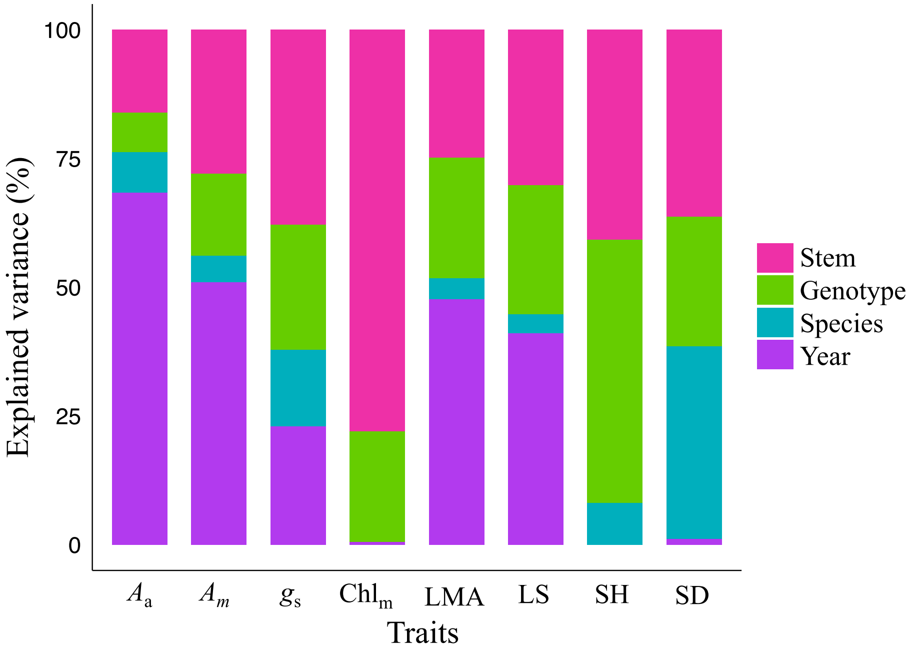

Fig. 2 Source of variation for different traits of *Vaccinium angustifolium* and *Vaccinium myrtilloides* studied across three (2018, 2019, 2020) years. Variance structure at the stem (red), genotype (green), species (blue), and year (purple) levels for light-saturated photosynthetic rate per area (A_a), light-saturated photosynthetic rate per mass (A_m), stomatal conductance per leaf area (g_s), Chl concentration per mass ($\mathrm{Chl_m}$), leaf mass per area (LMA), leaf size (LS), stem height (SH), and stem diameter (SD). Explained variance is obtained from four-level (stem, genotypes species, year) nested variance partition where year is used as a nested term. For 2017, A_m data of only four genotypes of *V. myrtilloides* were available, while LS, SH and SD data were missing due to weather and logistic issues. Hence, 2017 is excluded from the analysis. Overall, n = 6–8 genotypes per species, n = 6 stems per genotype.

year 2017 and across the genotypes of *V. myrtilloides* in another vegetative year 2019 (Fig. 5c–f). Similarly, the relationships between A_m and $\mathrm{Chl_m}$ as well as between $\mathrm{Chl_m}$ and $\mathrm{N_m}$ were only found in vegetative year 2019 across the genotypes of both species (Fig. S5b,f). The relationship between A_N and LMA was present across the genotypes of *V. angustifolium* in both vegetative years (2017 and 2019; Fig. 5a).

Relationships between the same leaf trait of different years

The same leaf traits of different years showed inconsistent relationships across the genotypes of two species. The LMA of 2017 and 2018, as well as that of 2017 and 2019, were not related (Fig. 6a,c), while that of 2019 and 2020, as well as 2018 and 2020, were related across the genotypes of two species (Fig. 6b,d). The LS of two crop years (2018 and 2020) and one crop cycle (2019 and 2020) were not related across the genotypes of two species (Fig. 6e,f).

Overall trait variation and trait network across years and the influence of environmental factors

The multivariate space of leaf functional, structural, and nutrient traits, as shown in the PCA results (Figs 7, S9), indicate trait shifts over the years driven by changing environmental conditions (Fig. 7a,b). Wild blueberries in crop years (2018 and 2020) occupied the same negative side of the PCA along the PC1, while wild blueberries in 2018 occupied the third quadrant, and that of 2020 second quadrant (Fig. 7a). Wild blueberries in the vegetative year 2019 occupied the positive side of the PCA, that is first and fourth quadrants (Fig. 7a). This temporal differentiation in traits of wild blueberries seemed to be driven by changing environmental conditions as temperature was positively related to leaf carbon traits ($\mathrm{C_m}$, C : N) in crop years, and rainfall was associated and positively related to leaf photosynthetic traits (A_a, A_m, A_P, A_N, g_s), leaf nutrient traits ($\mathrm{N_m}$, $\mathrm{Chl_m}$, $\mathrm{B_m}$, $\mathrm{Mn_m}$, $\mathrm{K_m}$, $\mathrm{Cu_m}$), leaf structural traits (LMA, LS), and water use efficiency (Alg) in a vegetative year (2019; Fig. 7a). Overall, *V. angustifolium* genotypes were located more toward the center, and *V. myrtilloides* genotypes were located away from the center in crop years. However, in the vegetative year 2019, the genotypes of the two species showed general overlap in the PCA (Fig. 7b). The PCAs with leaf nutrient traits and leaf structural traits separated generally agree with the PCA with all traits, with the temporal separation mostly correlated with changing environmental conditions over the years (Fig. S9).

Our trait network analysis revealed high year-year variations in the overall pattern (Fig. 8). The presence or absence of trait–trait relationships between A_m, LMA, and $\mathrm{N_m}$ as shown in Fig. 3 was consistent with the correlation network observed here in Fig. 8 across all years. Moreover, the traits were more strongly correlated in dry years (2018 and 2020) than that of the wet year, 2019 (Figs 8, S10).

Discussion

Our study reveals high variation in leaf structure and physiology among individual stems, among genotypes, and across years in wild blueberries, which have previously been shown to have high intergenotype variations in plant height, leaf and berry color, phenology, and yield (Kloet, 1978; Smagula *et al.*, 1997; Barai *et al.*, 2022). The large variation found in leaf structure, physiology, and nutrient concentrations across the genotypes in a common garden (farm) suggests high genetic diversity, while the high variation within a genotype across years suggests high plasticity. Interestingly, the global LES trade-offs were not always/consistently found in all years. Thus, global LES

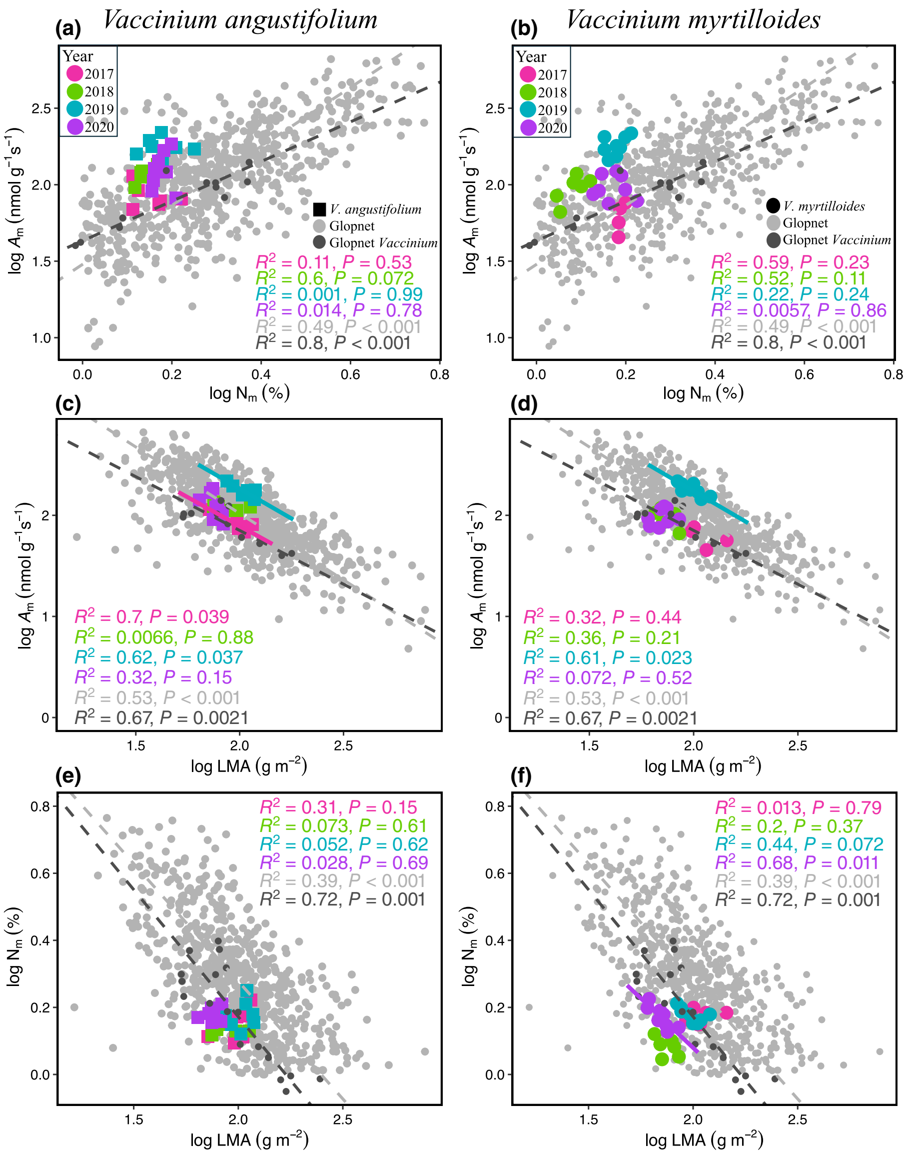

Fig. 3 Mass-based photosynthetic rate (A_m) in relation to leaf nitrogen concentration (N_m; a, b), and leaf mass per area (LMA; c, d), and leaf nitrogen concentration (N_m) in relation to leaf mass per area (LMA; e, f) for the genotypes of *Vaccinium angustifolium* (squares) and *Vaccinium myrtilloides* (circle) in 2017 (red), 2018 (green), 2019 (blue), and 2020 (purple) study year. The points on the background represent the global dataset (Glopnet; light gray circles) and *Vaccinium* species in the glopnet (Glopnet *Vaccinium*; dark gray circles). The gray points are from a global dataset of Wright *et al*. (2004). All variables were log-transformed. Break lines are standardized major axis (SMA) lines fitted to the global dataset (light gray line), and Glopnet *Vaccinium* (dark gray line), whereas solid lines are SMA lines fitted across the genotypes of *V. angustifolium* and *V. myrtilloides* according to their corresponding year (red line for 2017, green line for 2018, blue line for 2019, purple line for 2020). P < 0.05 are significant and are marked with a corresponding line color as described above. P values > 0.05 are non-significant and are not marked with the corresponding line color. For 2017, A_m data of only four genotypes of *V. myrtilloides* were available due to weather and logistic issues. Overall, n = 6–8 genotypes per species.

relationships might be absent at small scales such as in a single field site, suggesting temporal instability and/or limited trait variation at small scales. This is supported by the null model distribution results showing that the absence of some relationships could be due to limited sample size, but not for most relationships. This finding is in agreement with Messier *et al.* (2017), that at local scales, global LES trait-trait relationships might be absent or appear inverted (also see Albert *et al.*, 2010b; Asner *et al.*, 2014). Here, we added a new explanation for the absence of the LES relationships from the perspective of temporal instability and the influence of temporal changes in environmental conditions. Indeed, we found that the trait syndrome was shifted by changing environmental conditions over the years, as suggested by the PCA and trait network analysis results. Interestingly, the trait syndromes of two species were separated in 1 yr, but overlapped in the other 2 yr, suggesting a temporal dimension in niche overlap and separation. This new temporal dimension also suggests that caution is needed in the interpretation and extrapolation of results from one year or single time point studies. This is particularly true in environments with high year-to-year variability.

Large temporal variation in leaf functional traits across genotypes

Our results suggest the importance of the temporal dimension of trait variation, which can sometimes contribute more to overall variation compared with spatial variations (across and intra-genotype variations). This is supported by the fact that temporal variation (year) was the largest source of variation for many important traits including photosynthetic rates (A_m), LMA, and LS. Some traits, especially those known to shift with changes in environmental conditions such as LMA, A_m, and N_m (Poorter *et al.*, 2009; Becklin *et al.*, 2016; Ghimire *et al.*, 2017; Dusenge

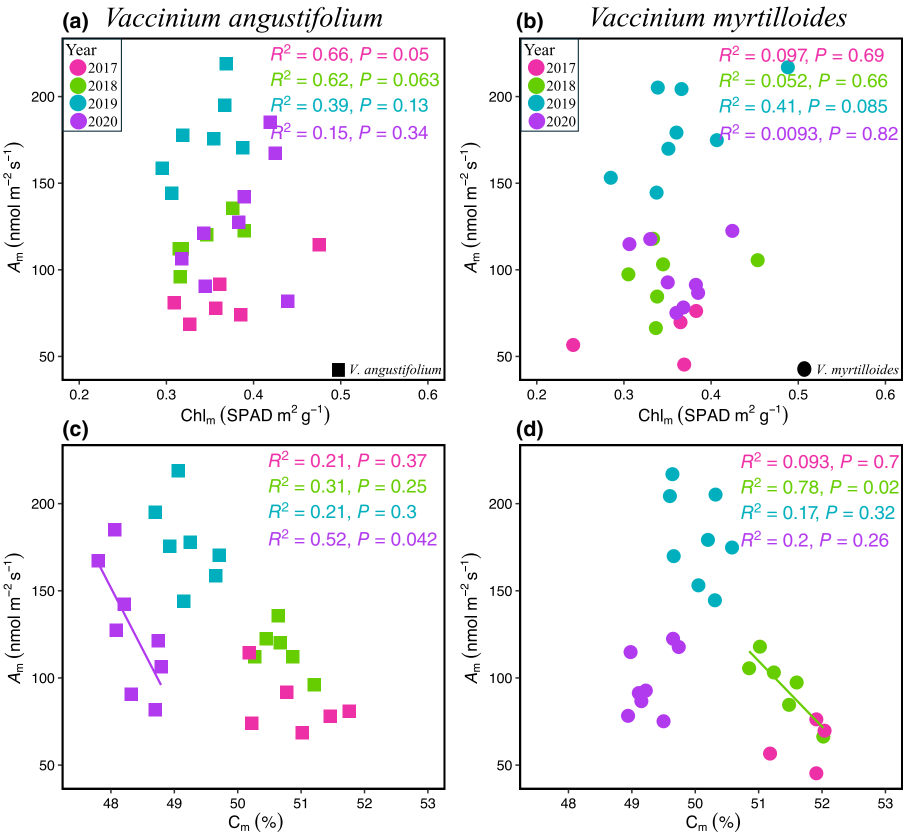

Fig. 4 Mass-based photosynthetic rate (A_m) in relation to Chl concentration per mass (Chl_m; a, b) and carbon concentration per mass (C_m; c, d) across the genotypes of *Vaccinium angustifolium* (squares) and *Vaccinium myrtilloides* (circle) in 2017 (red), 2018 (green), 2019 (blue), and 2020 (purple) study year. Points are means. Linear (c, d) regressions were fitted across the genotypes of *V. angustifolium* and *V. myrtilloides* according to their corresponding year (red line for 2017, green line for 2018, blue line for 2019, and purple line for 2020). $P < 0.05$ are significant and are marked with a corresponding line color as described above. P values > 0.05 are nonsignificant and are not marked with the corresponding line color. For 2017, A_m data of only four genotypes of *V. myrtilloides* were available due to weather and logistic issues. Overall, n = 6–8 genotypes per species.

et al., 2019), varied strongly across years, suggesting high temporal trait plasticity (Lázaro-Nogal *et al.*, 2015). This could be attributed to the strong variation in environmental conditions observed over the years; that is, 2019 was an extremely wet year, while 2018 and 2020 were dry and high temperature years. However, leaf phosphorus (P_m) and Chl concentration (Chl_m) did not vary across years, although they are known to change with environmental conditions such as mean annual temperature and precipitation (Gong *et al.*, 2020; Zhang *et al.*, 2021). Therefore, P_m and Chl_m are not always good indicators of plant responses to environmental change.

Significantly higher values of photosynthetic traits (A_a, A_m, A_P), LMA, N_m, and lower mean values of leaf size (LS), carbon (C_m), iron (Fe_m), and carbon nitrogen ratio (C : N) of *V. angustifolium* than *V. myrtilloides* in some years (Tabel S4; Notes S1) likely suggests that *V. angustifolium* invests more in photosynthetic traits than in structural and stress tolerance traits. This strategy of larger investment in photosynthetic machinery can result in a faster growth rate (Mathan *et al.*, 2016), greater aboveground biomass (Thompson *et al.*, 2017), sugar accumulation and yield increase (Ainsworth & Long, 2005; van der Kooi *et al.*, 2016), and increased abundance (R. Li *et al.*, 2015; X. Li *et al.*, 2015). This could explain the higher yield and higher dominance of *V. angustifolium* species in the field (Strik & Yarborough, 2005; Jones *et al.*, 2014; Drummond, 2019; Drummond & Rowland, 2020). By contrast, significantly higher values of leaf size, C_m, C : N, and Fe_m (traits related to toughness and construction) and lower mean values of photosynthetic traits of *V. myrtilloides* than *V. angustifolium* suggests that this species favors a more conservative strategy and makes greater investments in structural and stress tolerance traits. This pattern suggests that the differentiated resource use of the two species may reduce competition to some extent and facilitate their coexistence. This is in line with Pastore *et al.* (2021), which suggests that species with differentiated resource use may coexist.

Global LES trait relationships do not always hold among the genotypes

The LES relationship or trade-offs previously reported on a global scale (Field & Mooney, 1986; Reich *et al.*, 1997; Wright *et al.*, 2004) were not always captured among genotypes within and across two species of wild blueberries. Although the absence of some relationships in some years could be attributed to the limited sample size in this study, as suggested by the null model distribution results, the nonexistence of most relationships in most years was confirmed by the null model analysis. Another study on trees by Messier *et al.* (2017) suggested that LES relationships observed at a global scale might not be extrapolatable to smaller scales (site, species, and individual stems) due to a shift in the main driving factors of phenotypic integration (biophysical constraints, genetic constraints, and environmental filtering). Here, our results suggest the temporal instability of these trait–trait relationships, which could at least partly be explained by the effect of changing environmental conditions (Cui *et al.*, 2020). This is also supported by the trait network

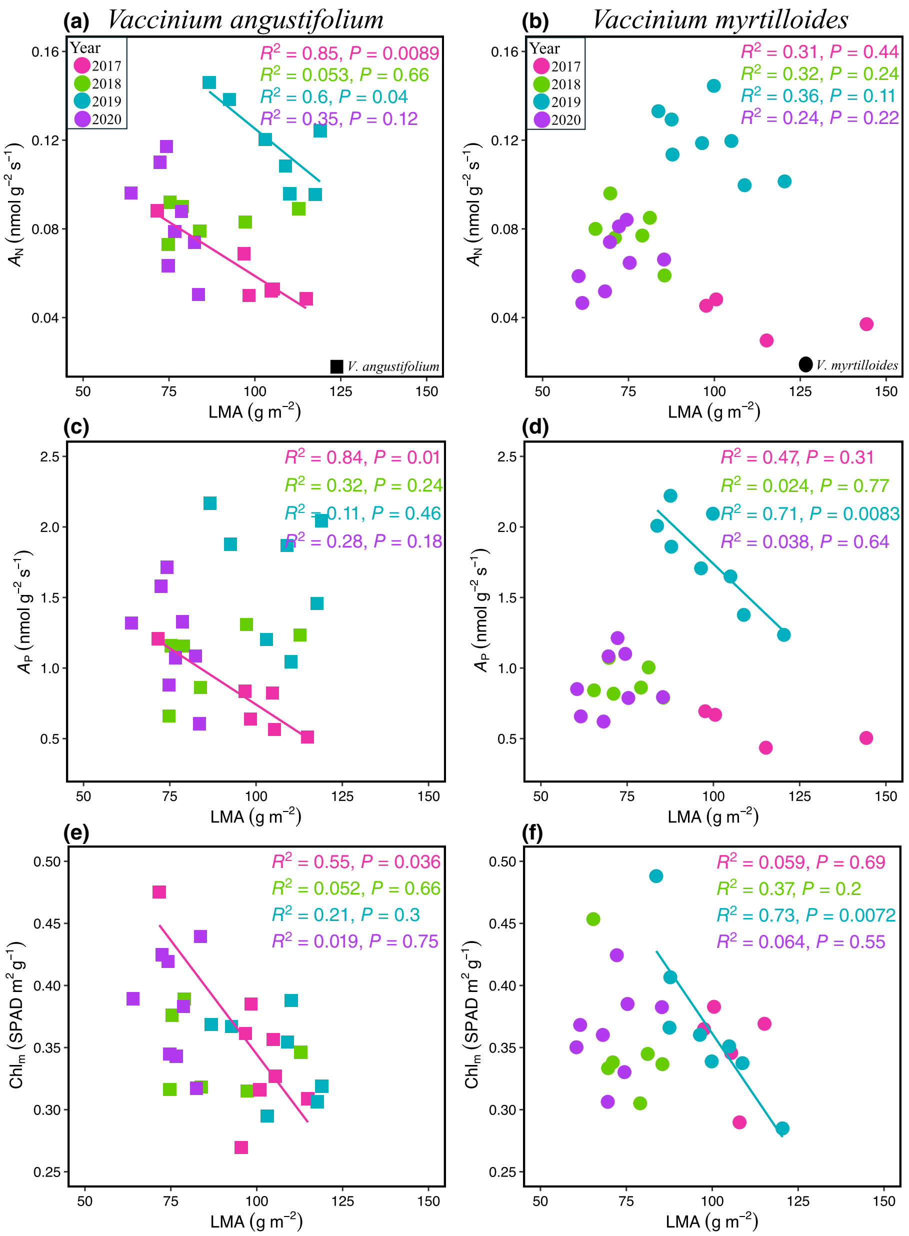

Fig. 5 Photosynthetic nitrogen (N) use efficiency (A_N; a, b), photosynthetic phosphorus (P) use efficiency (A_P; c, d), and Chl concentration per mass (Chl_m; e, f) in relation to leaf mass per area (LMA) across the genotypes of *Vaccinium angustifolium* (squares) and *Vaccinium myrtilloides* (circle) in 2017 (red), 2018 (green), 2019 (blue), and 2020 (purple) study year. Points are means. Linear (c–f) regressions were fitted across the genotypes of *V. angustifolium* and *V. myrtilloides* according to their corresponding year (red line for 2017, green line for 2018, blue line for 2019, and purple line for 2020). $P < 0.05$ are significant and are marked with a corresponding line color as described above. P values > 0.05 are nonsignificant and are not marked with the corresponding line color. For 2017, A_m data of only four genotypes of *V. myrtilloides* were available due to weather and logistic issues. Overall, n = 6–8 genotypes per species.

analyses (Fig. 8) showing stronger overall trait–trait correlations in drier years than in wet years. Additionally, the presence of LES trait–trait relationships in some years but not in all years highlights the importance of studying the temporal dynamics of these relationships, especially for local scale studies that seek to test these relationships. The absence or presence of the trait–trait relationships based on data from a single year could not tell the whole story of functional trade-offs constraining trait development.

Notably, the N_m and LMA relationship among genotypes within *V. myrtilloides* was only captured in crop year 2020 but was entirely absent among genotypes within *V. angustifolium.* The presence of this relationship is in agreement with the global trends (Wright *et al.*, 2004), which could be explained by the dilution of nitrogen by the mass of thick and sclerified cell walls in high LMA leaves. The absence of the N_m and LMA relationship could have arisen from the narrow range of trait space covered in our study. This finding is similar to Reich *et al.* (1999), whose six-site dataset revealed an absence of within-species N_m and LMA trait relationships. The A_m and N_m relationship was also absent among genotypes within both species, but this relationship was present among genotypes across two species in the crop year 2018. This could be due to significantly lower nitrogen values of wild blueberry species than the Glopnet species mean values (Table S4), resulting in a narrow range of N_m. However, combining two species resulted in a wider trait space, allowing the causal relationship between A_m and N_m (Field & Mooney, 1986) to be detectable. This indicates the effect of narrow trait ranges at local scales in detecting trait–trait relationships (Wright & Sutton-Grier, 2012).

Interestingly, the LMA and A_m trade-off could be captured in one vegetative year 2019, but the same trade-off was only found

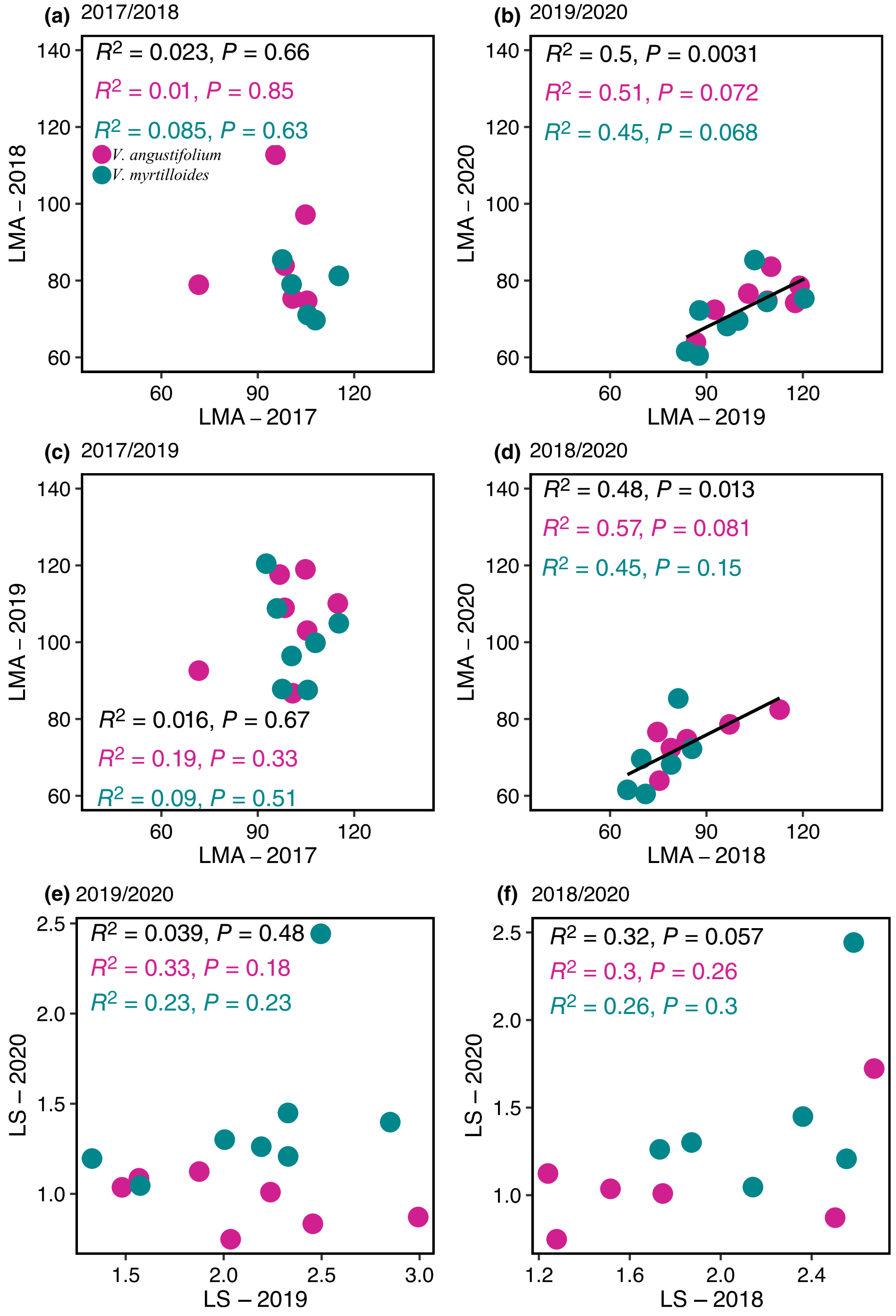

Fig. 6 Relationship of leaf mass per area (LMA) of 2017 with LMA of 2018 (a), LMA of 2019 with LMA of 2020 (b), LMA of 2017 with LMA of 2019 (c) LMA of 2018 with LMA of 2020 (d), Leaf size (LS) of 2018 with LS of 2020 (e), and LS of 2019 with LS of 2020 (f) across and within *Vaccinium angustifolium* (red circles) and *Vaccinium myrtilloides* (blue circles). Points are means. Linear (b, d, e) regressions were fitted to the data across (black solid line) and within (red solid line, *V. angustifolium* and green solid line, *V. myrtilloides*) blueberry species. $P < 0.05$ are significant and are marked with a corresponding line color as described above. P values > 0.05 are nonsignificant and are not marked with the corresponding line color. Overall, $n = 6$–8 genotypes per species.

among genotypes within *V. angustifolium* in another vegetative year 2017. Similarly, we were also able to capture the A_N and LMA as well as A_P and LMA relationships in vegetative years. This could be due to the higher and broader range of LMA covered, and the higher energy allocation of blueberries in the vegetative growth phase. This is supported by the result that both *V. angustifolium* and *V. myrtilloides* had higher LMA in the vegetative than in crop years. By contrast, the absence of the A_m and LMA relationship in crop years could be due to the narrower range of LMA in those years. In addition to the inconsistent relationships between LES traits (LMA, A_m, and N_m), the relationships between other leaf functional and structural traits such as A_m with C_m, Chl_m, and C : N, and LMA with Chl_m, Fe_m, and photosynthetic nutrient use efficiency (A_N and A_P) among genotypes within and across two species were also unstable across years. Therefore, the temporal instability of trait–trait relationships applies to a wide range of leaf functional traits.

Changing environmental conditions across years may facilitate the coexistence of diverse genotypes and species

Temporal changes in environmental conditions including temperature and rainfall across years, high plasticity of the genotypes, and differential responses of species and genotypes to changing

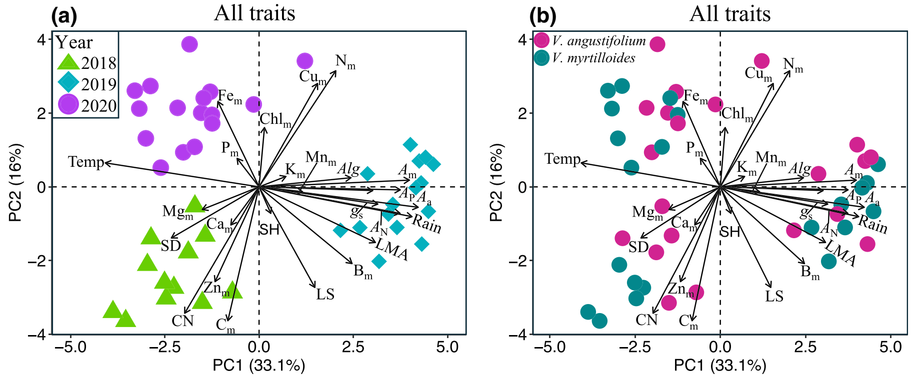

Fig. 7 Principal component analysis (PCA) of mean values of the combination of leaf functional traits, leaf structural traits, and leaf nutrient traits combined for 2018, 2019, 2020 with years loading on the background (a) and with species loadings on the background (b). Traits included in the analysis are area-based light-saturated photosynthetic rate (A_a), stomatal conductance (g_s), water use efficiency (Alg), mass-based photosynthetic rate (A_m), photosynthetic nitrogen (N) use efficiency (A_N), photosynthetic phosphorus (N) use efficiency (A_P), Chl concentration per mass (Chl_m), leaf mass per area (LMA), leaf size (LS), plant height (PH), stem diameter (SD), nitrogen concentration per mass (N_m), carbon concentration per mass (C_m), carbon nitrogen ratio (CN), calcium concentration per mass (Ca_m), potassium concentration per mass (K_m), magnesium concentration per mass (Mg_m), phosphorus concentration per mass (P_m), aluminum concentration per mass (Al_m), boron concentration per mass (B_m), copper concentration per mass (Cu_m), iron concentration per mass (Fe_m), manganese concentration per mass (Mn_m), and zinc concentration per mass (Zn_m). Green triangles represent 2018, blue squares represent 2019, and purple circles represent 2020 on the background of the PCA (a). Red circles represent genotypes of *Vaccinium angustifolium*, and blue circles represent genotypes of *Vaccinium myrtilloides* (b). Note that 2017 data were not included in analyses because not all traits were measured.

environmental conditions may all facilitate the coexistence of diverse genotypes and species. Different environmental conditions in different years favor different trait suites (combination of traits), and thus provide unique temporal windows for different species to perform relatively better compared to other coexisting species (Gendron, 1987; Chesson, 2000; Adler *et al*., 2006; Angert *et al*., 2009). Here in the wild blueberry system, different genotypes show higher physiological performances in different years, compared with other genotypes. This suggests a differentiation in temporal niches among different genotypes, facilitating their coexistence. According to modern coexistence theory, species coexistence in the same environment may occur when (1) two species have differentiated resource use (niche differentiation), (2) they have equivalent competitive capacities despite partially overlapping resource use (Pastore *et al*., 2021), or (3) they have equal or close performance and competitive capacities despite sharing the niche (Hubbell, 2001). Two studied species exhibited significant differences in most of the leaf structural, physiological, and nutrient content traits, and there was little to no overlap in the traits in both 2018 and 2020, which suggests differentiated traits and resource use. However, there was a clear overlap between the traits and genotypes during the high rainfall year 2019 (Fig. 7b). The wet conditions of 2019 allowed for the alleviation of water stress, which might have weakened the advantages of *V. angustifolium* leading to comparable performances of both species. This temporal dynamics of environmental variation may play an important role in shaping the coexistence of blueberry species and different genotypes. This finding strengthens our understanding of the coexistence mechanisms of wild blueberries and the role of environmental variability in driving trait variation. Therefore, fluctuating environmental conditions could play a significant role in shaping the species composition of an ecosystem.

Limitations of this study and future work

While the sample size we used (6–8 genotypes per species) was able to capture the high variation among genotypes of wild blueberries, this limited sample size may potentially make it hard to capture trait–trait relationships. However, the null model analysis suggests that the absence of only a few relationships was due to a limited sample size, and in most cases, the relationships did not exist. To confirm the null model analysis results, a field study including most genotypes (i.e. *c*. 200) in a common farm could be conducted to reveal the sample size needed to capture trait–trait relationships. Moreover, while our approach of monitoring the same individuals in the same ecosystem over the years can unfold natural temporal dynamics of traits and trait–trait relationships, and avoid confounding effects of using different individuals, the changing environmental conditions are a combined effect of different environmental factors. A future study could combine temporal monitoring in the natural field with control experiments manipulating different environmental conditions to reveal the mechanisms regulating the temporal instability of the trait–trait relationships.

Conclusion

In conclusion, we introduced a novel temporal dimension in studying LES traits and trait–trait relationships. We found high temporal variation in functional traits and temporal instability of the LES trait–trait relationships in wild blueberries across diverse genotypes, which could not be solely explained by the limited sample size. The high temporal variation may be attributed to shifting environmental conditions across temporal scales, highlighting the vital role of changing environmental conditions in shaping functional traits, species coexistence, and

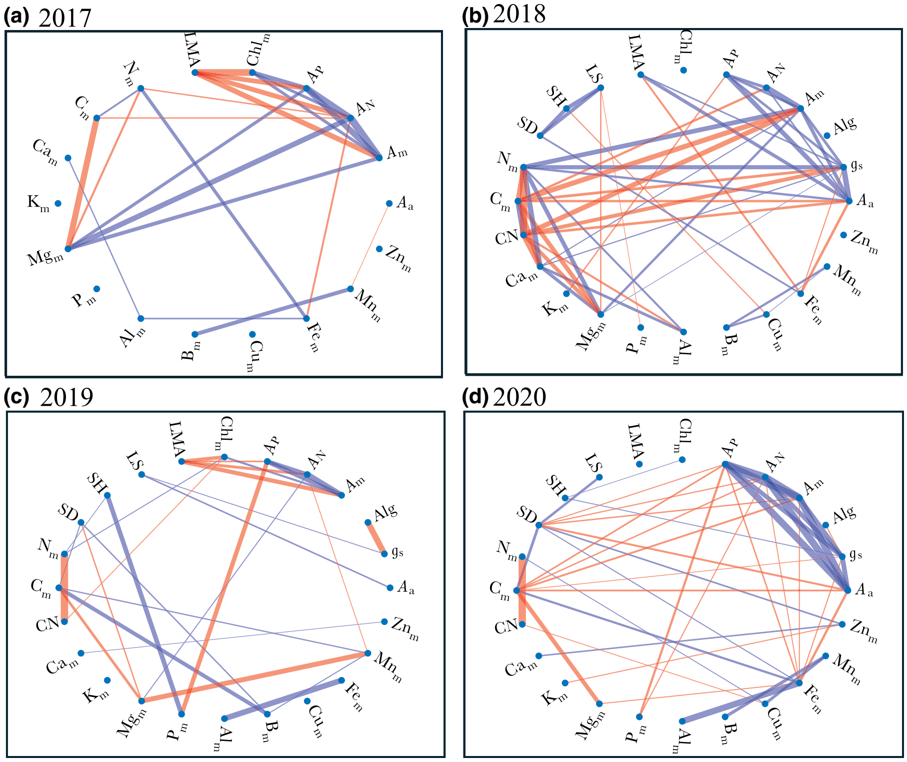

Fig. 8 Trait–trait correlation network showing correlation pattern among different leaf functional, structural, and nutrient traits for year 2017 (a), 2018 (b), 2019 (d), and 2020 (d) across *Vaccinium angustifolium* and *Vaccinium myrtilloides*. Only significant correlations at the P value ≤ 0.05 level are shown. The locations of the traits were optimized using network analysis tools (IGRAPH package, MATLAB), and reflect correlation strength. Correlation strength is indicated by the thickness of edge lines and distance among traits. Positive correlations are shown as blue, and negative correlations are shown as red. Traits included in the trait correlation network are area-based light-saturated photosynthetic rate (A_{a}), stomatal conductance (g_{s}), water use efficiency (Alg), mass-based light-saturated photosynthetic rate (A_{m}), photosynthetic nitrogen (N) use efficiency (A_{N}), photosynthetic phosphorus (N) use efficiency (A_{P}), Chl concentration per mass (Chl$_{\mathrm{m}}$), leaf mass per area (LMA), leaf size (LS), stem height (SH), stem diameter (SD), nitrogen concentration per mass (N$_{\mathrm{m}}$), carbon concentration per mass (C$_{\mathrm{m}}$), carbon nitrogen ratio (CN), calcium concentration per mass (Ca$_{\mathrm{m}}$), potassium concentration per mass (K$_{\mathrm{m}}$), magnesium concentration per mass (Mg$_{\mathrm{m}}$), phosphorus concentration per mass (P$_{\mathrm{m}}$), aluminum concentration per mass (Al$_{\mathrm{m}}$), boron concentration per mass (B$_{\mathrm{m}}$), copper concentration per mass (Cu$_{\mathrm{m}}$), iron concentration per mass (Fe$_{\mathrm{m}}$), manganese concentration per mass (Mn$_{\mathrm{m}}$), and zinc concentration per mass (Zn$_{\mathrm{m}}$). Note that data for six traits in 2017 were not collected, and hence, they were not included in the analysis.

community composition. This leads to the conclusion that caution must be taken when interpreting the results of studies limited by a single year or single time point to understand the functional trade-offs constraining trait development, as well as the influence of traits in individual performance through time. Future studies should aim to uncover the role of changing environmental conditions in shaping trait variation across temporal scales. These findings expand our understanding of the relationships shown for leaf traits of Glopnet species with a new temporal dimension, highlighting the importance of variation at temporal scales in reframing modern understanding of leaf design, general constraints in trait development, niche differentiation, and species coexistence.

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Competing interests

None declared.

Author contributions

Y-JZ and PP designed the study and experiment. PP and Y-JZ collected and analyzed the data. PP wrote the manuscript with contributions from all authors including Y-JZ, SA, JW, and BM.

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Data availability

The data that support the findings of this study are available in Tables S4–S6 and Dataset S1.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Dataset S1 Dataset of the present study.

Fig. S1 Map showing the location of different genotypes.

Fig. S2 Diversity in leaf form, color, size and morphology.

Fig. S3 Leaf economic spectrum trait–trait relationships with two species combined.

Fig. S4 Leaf economic spectrum trait–trait relationships of four-year average values.

Fig. S5 Null model distribution of leaf economic spectrum trait–trait relationships of *Vaccinium angustifolium*.

Fig. S6 Null model distribution of leaf economic spectrum trait–trait relationships of *Vaccinium myrtilloides*.

Fig. S7 Additional trait–trait relationships for different species.

Fig. S8 Additional trait–trait relationships across two species.

Fig. S9 Principal component analysis of mean values of leaf nutrients and leaf functional traits.

Fig. S10 Corrplot correlogram of traits included in the trait network analysis.

Notes S1 Supporting results.

Table S1 Nomenclature, distribution, and native habitats of studied wild blueberry species.

Table S2 Average of mean temperature, maximum relative humidity, and total precipitation of the study site.

Table S3 Traits, symbols, and units.

Table S4 Mean values of traits of studied species in different years.

Table S5 Comparison of trait values between two species.

Table S6 Means and SE for each trait by species.

Table S7 Null model distribution results of leaf economic spectrum trait–trait relationships.