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Research

Stomatal traits covary with leaf mycobiome diversity and composition

Austen Apigo[1,2]*, Sabrina Heitmann[1]*, Devin Leopold[1,3], Leander D. L. Anderegg[4] and Posy E. Busby[1]

[1]Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331, USA; [2]Department of Ecology and Evolutionary Biology, University of California Irvine, Irvine, CA 92697, USA; [3]Jonah Ventures, Boulder, CO 80301, USA; [4]Department of Ecology, Evolution, and Marine Biology, University of California Santa Barbara, Santa Barbara, CA 93106, USA

Author for correspondence:
Posy E. Busby
Email: posy.busby@oregonstate.edu

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Summary

- The scope of plant control over its microbiome is a central question in evolutionary biology and agriculture. Leaf traits are known to shape pathogen colonization and disease development, but their impact on the broader community of largely non-pathogenic fungi that colonize plant leaves remains an open question.
- We used reciprocal common gardens of the model tree, *Populus trichocarpa* (black cottonwood), to examine relationships between leaf traits and the leaf mycobiome in two strongly contrasting environments. We measured six leaf traits (stomatal length, stomatal density, carbon-to-nitrogen ratio, leaf thickness, leaf dry matter content, and specific leaf area) and used fungal marker gene sequencing to characterize leaf fungal communities for 57 tree genotypes replicated in one mesic and one xeric common garden (809 trees).
- Several leaf traits covaried with the leaf mycobiome, yet one relationship was paramount: plant genotypes with longer, sparser leaf stomata hosted a greater richness and diversity of more similar fungal species compared to plant genotypes with shorter, denser leaf stomata.
- These relationships, while modulated by the environment plants were sourced from and grown in, suggest that stomatal traits may be a general mechanism through which plants and the leaf mycobiome influence one another.

Introduction

Predicting how plant microbiomes assemble is an expanding research area, with growing interest in the extent to which plants influence their microbiomes through heritable traits (Wagner, 2021). Understanding these mechanisms is key to engineering crops that reliably recruit beneficial partners and to anticipating how plant–microbial interactions will respond to climate change. If microbiomes are influenced by genetically controlled plant traits, then plant genetics should offer predictive power over microbial community assembly. Indeed, many studies have shown that plant genotypic variation shapes the structure of microbial communities (Bálint *et al.*, 2015; Wagner *et al.*, 2016; Leopold & Busby, 2020; Morella *et al.*, 2020; VanWallendael *et al.*, 2022). However, in field settings, plant genotype typically explains a small portion of variation in the species composition of the plant microbiome (Wagner, 2021). This raises the question of whether considering plant genetic variation alone is sufficient to guide ongoing efforts to develop model plant systems capable of reliably controlling their microbiomes.

Trait-based approaches offer a promising framework to further understand how plant genetic variation contributes to microbiome assembly through measurable phenotypes. Increasing evidence has shown that physical and chemical properties of leaves, or leaf traits, can influence which microbes successfully colonize and establish (Kembel *et al.*, 2014; Christian *et al.*, 2020; Gonzá lez-Teuber *et al.*, 2020; Tellez *et al.*, 2022). However, existing studies often fall into two extremes: studies either compare genetic mutants in controlled laboratory settings, where trait and environmental variation are minimized, or survey different plant species in natural environments, where host effects are difficult to separate from environmental variation. Highly controlled gene knockouts are useful to establish the causal consequences of plant traits on microbiomes, and field surveys can reveal general ecological processes that operate across species and environments. Yet, there remains a critical need for approaches that assess leaf trait–microbiome relationships under realistic field conditions while also controlling for plant genetic background.

Although widely measured leaf traits associated with the leaf economics spectrum (Wright *et al.*, 2004) – such as leaf area (Wright *et al.*, 2017) or leaf nitrogen content (Reich & Oleksyn, 2004) – are useful indicators of plant strategies and performance, they often show weak or inconsistent relationships with

*These authors contributed equally to this work.

microbial community structure (Lajoie & Dariel, 2025). This may be because leaf traits that influence microscale conditions play a more direct role in structuring microbial communities. For example, bacteria on leaf surfaces tend to aggregate near leaf trichomes (Monier & Lindow, 2004), which buffer them from microclimatic stress, and proliferate in response to soluble carbohydrates leached onto leaf surfaces (Leveau & Lindow, 2001). In addition to surface-dwelling microbes, plant leaves host diverse communities of fungi, many of which form localized, asymptomatic infections inside leaves as endophytes (Arnold, 2007; Rodriguez *et al.*, 2009). These fungi face the additional challenge of gaining access to leaf interiors and thus may be sensitive to plant traits that control microbial entry. For example, tropical trees with thicker and tougher leaves hosted less abundant and less diverse leaf fungal communities (Tellez *et al.*, 2022), suggesting that leaf structural traits can limit fungal entry and establishment.

A potential route fungi can use to bypass the leaf surface is through natural openings such as stomata. Stomata are entry points for microbes into their hosts, with a long history of research recognizing their importance for fungal pathogens (Wu & Liu, 2022), which often modify stomatal function or traits during infection (Doehlemann *et al.*, 2017; Dutton *et al.*, 2019; Ye *et al.*, 2020). Non-pathogenic fungi can also enter leaves through stomata (Huang *et al.*, 2018), raising the possibility that variation in stomatal size, number, or function could govern how the broader community of leaf fungi assembles. Across many plant species and ecosystems, stomatal length and density are negatively correlated: plants with longer leaf stomata typically have lower stomatal densities (Hetherington & Woodward, 2003; Wang *et al.*, 2015; Liu *et al.*, 2023). This widespread trade-off not only reflects physiological constraints on gas exchange and water loss (Franks & Beerling, 2009; Xie *et al.*, 2022) but may also be a general axis of selection by which plants regulate microbial access to internal tissues (Beyschlag & Eckstein, 2001; Muir, 2020). While there is some evidence that stomatal traits are associated with the bacterial community in and on leaves (Damerum *et al.*, 2021; Smets *et al.*, 2023), the role of stomata in shaping diverse leaf fungal communities composed of largely non-pathogenic members has received less attention.

Assessing the generality of leaf trait–mycobiome relationships requires examining their variability across multiple environments, as environmental variation can simultaneously influence both plant trait expression and the pool of fungal species available for colonization. For example, even if leaf traits are under strong genetic control (Dunlap & Stettler, 2001; McKown *et al.*, 2014; Klein *et al.*, 2025), plant genotypes expressing similar leaf phenotypes across different environments could still assemble different fungal communities depending on which fungi are locally present. Leaf traits can also exhibit plasticity, such that environmental shifts in leaf trait expression potentially interact with differences in the local fungal species pool to influence fungal community structure. Trait expression may further be shaped by evolutionary processes such as local adaptation, which may explain why leaf trait variation is often linked to climate (Xing *et al.*, 2021; Li & Prentice, 2024; Klein *et al.*, 2025), and thus depends on the geographic origin of the host plant.

For these reasons, we used reciprocal common garden studies of *Populus trichocarpa*, a model tree for studying plant trait heritability (Evans *et al.*, 2014; McKown *et al.*, 2014) and plant–microbial interactions (Dove *et al.*, 2021; Van Nuland *et al.*, 2023), to examine the relationships between intraspecific leaf traits and the leaf mycobiome. We grew a diverse panel of *P. trichocarpa* genotypes, spanning broad climatic origins from the core of the tree's range, in two climatically contrasting common gardens: a cool, wet site and a hot, dry site, hereafter referred to as the mesic and xeric common gardens, respectively (Fig. 1). We measured a suite of leaf traits (stomatal length, stomatal density, carbon-to-nitrogen ratio, leaf dry matter content, leaf thickness, specific leaf area) and used marker gene sequencing to characterize fungal communities that naturally assembled in and on leaves. This reciprocal transplant design allowed us to ask how leaf traits varied among plant genotypes and the extent to which these traits exhibited plasticity across the gardens. We then asked whether these leaf traits predicted fungal richness, diversity, and composition within each garden, and whether these relationships depended on the home site from which plant genotypes were sourced or the common garden in which plants were grown.

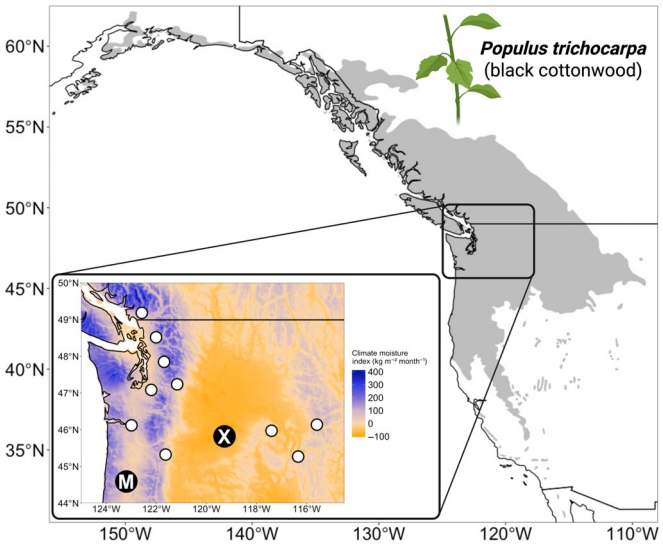

Fig. 1 *Populus trichocarpa* genotypes from the core of the tree's geographic range were transplanted into a mesic and xeric common garden to examine associations between leaf traits and the leaf mycobiome, and the extent to which these associations depended on environmental context. Plant genotypes (n = 57) sourced from 10 sites (small white circles) were transplanted and replicated 12 times in a mesic (large black circle labeled 'M') and xeric common garden (large black circle labeled 'X'). The map shows the geographic range of *P. trichocarpa*. The inset is overlaid with climate moisture index (kg m^{-2} $month^{-1}$), a measure of water availability (annual precipitation) relative to demand (potential evapotranspiration), with bluer hues corresponding to wetter environments (higher climate moisture index). This figure was created in BioRender (BioRender.com/85xzqou).

Materials and Methods

Common garden design

We established reciprocal common gardens using 59 *Populus trichocarpa* Torr. & A. Gray genotypes collected from 10 river valley sites spanning the core of the tree's native range across Oregon, USA, Washington, USA, and British Columbia, Canada (Fig. 1). Sites were selected to capture a broad climatic gradient: 30 plant genotypes originated from mild, mesic conditions west of the Cascade Mountain Range and 29 from xeric, seasonal environments to the east (Fig. 1). This regional contrast is known to drive differences in *P. trichocarpa* growth, phenology, and plant traits among populations (Dunlap & Stettler, 1996, 2001), providing a useful model system to identify leaf traits associated with the leaf mycobiome.

Cuttings from each *P. trichocarpa* genotype were grown in a clone bank in Corvallis, Oregon, for 2 yr. Each plant genotype was then transplanted in 2018 into two common gardens located at opposing ends of the climate gradient from which plant genotypes originated: a mesic garden in Corvallis, Oregon, and a xeric garden in Hermiston, Oregon. The gardens experience dramatically different climates, with the mesic garden receiving *c.* 110 cm of annual rainfall compared to 20 cm in the xeric garden and a mean summer temperature difference of *c.* 4°C. Each garden was planted using a randomized block design, each with 12 blocks that included one replicate of every *P. trichocarpa* genotype. To support plant growth and survival, the mesic garden was irrigated for the first two summers, and trees were surrounded by shade cloth in 2019 to limit weed competition, while the xeric common garden was irrigated annually during summer months. Tree survival was high at the time of leaf trait and leaf mycobiome sampling in the mesic (93% of trees survived) and xeric (87% of trees survived) common gardens.

Leaf trait measurements

In each common garden, we measured leaf traits on all trees in the four experimental blocks with the highest survival rate. Because each block contained one replicate of every *P. trichocarpa* genotype, four trees per genotype were considered for trait measurements. To standardize leaf age and collection location, we tagged 10 lower canopy, south-facing leaves per tree with a leaf plastochron index (Erickson & Michelini, 1957) of one, two, or three in spring 2020. In late summer 2020, we collected four of the 10 tagged leaves per tree for trait analysis. When tagged leaves were missing due to senescence or abscission, we substituted untagged leaves of similar age and canopy position. All leaves were placed in a ziplock bag with paper towels moistened with deionized water, kept on ice, and rehydrated for at least 12 h using a partial rehydration method (Pérez-Harguindeguy *et al.*, 2013).

We measured six leaf traits: stomatal length (pixels), stomatal density (stomata per microscope field), leaf dry matter content (mg g^{-1}), leaf thickness (μm), carbon-to-nitrogen ratio (%carbon/%nitrogen), and specific leaf area (mm^2 mg^{-1}). To measure stomatal length and density, a 2 cm^2 section of clear nail polish was applied to the abaxial (lower) side of one leaf per tree, to the right of the leaf midrib. Once dry, the stomatal peel was mounted on a microscope slide. At ×200 magnification, three microscope fields were haphazardly imaged while avoiding veins and damaged tissue. Using ImageJ (Schneider *et al.*, 2012), we measured stomatal length and density in each field and averaged values across the three fields per leaf. Leaf thickness, leaf dry matter content, and specific leaf area were measured on up to four leaves per tree. Leaf thickness was measured on either side of the midrib at the widest points a leaf blade expanded to with a Mitutoyo 7327 Micrometer Gauge (Mitutoyo, Takatsu-ku, Kawasaki, Japan). Rehydrated leaves were weighed, dried at 65°C for 72 h, then reweighed to determine dry mass. To measure leaf dry matter content, dry mass was divided by the wet mass. To measure specific leaf area, leaf area was calculated from digital images using ImageJ, then divided by dry mass. For each plant genotype in each common garden, one dry leaf per tree was combined into a single sample, ground for 1 min at 1000 strokes/minute with Geno/Grinder®, and analyzed for %C and %N with a Costech Elemental Analyzer (ECS 4010; Costech, Valencia, CA, USA).

Mycobiome sampling

To characterize the leaf mycobiome, we collected three leaves per tree across all 12 experimental blocks in each common garden during a 2-wk period in August and September 2019. Leaves with a healthy appearance were collected at a leaf plastochron index of five from opposing canopy directions. From each leaf, four leaf discs (*c.* 0.25 cm^2) were pooled into a single sterile 1.5 ml centrifuge tube using a hole punch that was cleaned with 70% ethanol between each plant, then placed on ice. Within 24 h, leaf discs were washed in 1 ml of molecular-grade sterile water with 0.1% Triton X-100 for 1 min (Brown *et al.*, 2018; Mahmoudi *et al.*, 2024) using a Geno/Grinder® 2010 (500 strokes min^{-1}). This procedure agitates leaf discs in the wash solution to remove loosely adhering dirt and microbial matter. After the leaf wash was removed, leaf discs were rinsed twice with molecular grade sterile water, then stored at −80°C.

Molecular and bioinformatic methods

Total genomic DNA was isolated from leaf discs using the 96 Well Synergy Plant DNA Extraction Kit (OPS Diagnostics, Lebanon, NJ, USA) following the manufacturer's instructions. Fungal communities were amplified using a two-stage PCR approach. The first stage used modified fungal-specific ITS3_KYO1 and ITS4 primers targeting the ITS2 region (Toju *et al.*, 2012) and included PNA clamp IP01 (PNA Bio, Thousand Oaks, CA, USA) to prevent plant ITS2 amplification (Cregger *et al.*, 2018). Stage-one PCR reactions (25 μl) included 12.5 μl MyFi™ Master Mix (Meridian Bioscience, Cincinnati, OH, USA), 2 μl template DNA, 1.25 μl of each 10 μM primer, 2.5 μl of the 10 μM PNA clamp, and molecular-grade water. Thermocycling conditions included an initial denaturation and enzyme activation cycle at 95°C (5 min), and 30 cycles of 95°C

(15 s), 55°C (1.5 min), and 72°C (1.5 min) followed by a final elongation cycle at 72°C (5 min; Nexus Gradient 6331, Eppendorf, Enfield, CT, USA). Temperature ramp rates were limited to 1°C to minimize chimera formation (Stevens *et al.*, 2013). For each 96-well plate, one DNA extraction control, one PCR control, and one control to evaluate sample 'index-hopping' or ' cross-talk' (van der Valk *et al.*, 2020) were included.

Stage-two PCR reactions (25 µl) included 12.5 µl Myfi™ Master Mix (Meridian Bioscience), 9 µl molecular grade water, 2.5 µl of each 10 µM primer, and 1 µl of stage-one PCR product. The stage-two primers were appended with 3–6 bp long staggered degenerate spacers to increase taxonomic coverage and overhang adapters to bind to the Illumina flow cell (Lundberg *et al.*, 2013). Thermocycling conditions included an initial cycle of 95°C (1 min), and 8 cycles of 95°C (20 s), 55°C (20 s), and 72°C (30 s) followed by a final elongation cycle at 72°C (5 min). Stage-two PCR products were cleaned and normalized using Just-a-Plate™ 96-well normalization and purification plates (Charm Biotech, Cape Girardeau, MO, USA). Equal volumes of the cleaned PCR products were pooled and sequenced using paired-end 250-bp Illumina MiSeq at the Center for Quantitative Life Sciences at Oregon State University.

Sequences were demultiplexed with PHENIQS v.2.0.4 (Galanti *et al.*, 2021) with a confidence threshold of 0.995. Adapters and primers with degenerate spaces were removed with CUTADAPT v.1.18 (Martin, 2011). Reads were trimmed of read-through adapter contamination from the 3' ends with SeqPurge (Sturm *et al.*, 2016). Quality filtering, denoising, and chimera removal were performed with DADA2 (Callahan *et al.*, 2016). ITS2 regions were extracted with ITSx (Bengtsson-Palme *et al.*, 2013). Plant contamination was removed by aligning the *P. trichocarpa* v.3.0 genome using BOWTIE2 (Langmead & Salzberg, 2012). Sequences were clustered into Operational Taxonomic Units (OTUs) at 99% similarity using DECIPHER (Wright, 2024). Taxonomy was assigned with DADA2 using the UNITE fungal database (Nilsson *et al.*, 2019; Wright, 2024).

Statistical analysis

We structured our statistical analyses around three aims: two corresponding to our proposed questions and one addressing an emergent pattern observed during analysis. First, we assessed leaf trait variation within each garden with principal component analysis and trait plasticity across gardens with correlation analysis and linear mixed models. Second, we tested whether leaf traits, common garden, and home site predicted fungal richness, diversity, or composition using linear mixed models and generalized linear latent variable models. Third, we discovered a correlation between stomatal traits and the relative abundance of *Melampsora*, a genus of common rust pathogens on *P. trichocarpa* (Dunlap & Stettler, 1996). We incorporated an additional analysis to address the possibility that rust abundance modulated the effect of stomatal traits on fungal community structure. All statistical analyses and data visualization used R v4.4.2 (R Core Team 2020, Vienna, Austria).

Analysis of leaf traits We averaged each leaf trait (stomatal length, stomatal density, leaf dry matter content, carbon-to-nitrogen ratio, leaf thickness, specific leaf area) across individuals of the same plant genotype. We removed two plant genotypes from the mesic garden and five from the xeric garden that did not have a full complement of trait data, for a total of 57 genotypes across both gardens.

We explored how leaf traits varied among plant genotypes within each garden in multivariate trait space. After separating the leaf trait data by the mesic garden (n = 57 plant genotypes) and the xeric garden (n = 54 plant genotypes), averaged trait values were scaled using the 'scale' function in base R, then visualized along their first two principal components using the 'prcomp' function in the stats package. To identify traits that strongly contributed to phenotypic differentiation among plant genotypes, we compared their squared loadings with respect to the first principal component, which quantifies the proportion of each trait's variance captured along that axis.

We examined how variable trait expression was across the two gardens. After combining the leaf trait data across both gardens (n = 57 plant genotypes in the mesic garden; n = 54 plant genotypes in the xeric garden), we correlated leaf trait values between plant genotypes in the mesic and xeric gardens using Pearson's product–moment correlation coefficient. We fit linear mixed models (leaf trait ~ garden × climate moisture index + 1|home site + 1|plant genotype) to test the effects of common garden, home site climate moisture index (CMI), and their interaction on each leaf trait. Home site and plant genotype were included as random intercepts to account for the sites plant genotypes originated from and repeated plant genotypes across gardens. CMI, extracted from the CHELSA dataset (Karger *et al.*, 2017), was used as an index of a plant genotype's climatic origin. It is the difference between annual precipitation and potential evapotranspiration (calculated using the Penman-Monteith equation) and represents the long-term balance between water supply and demand.

Analysis of fungal community structure We combined leaf trait and mycobiome data across both gardens to test the effects of leaf traits, home site CMI, and common garden on fungal richness and diversity using linear mixed models (fungal richness or diversity ~ stomatal length (SL) + stomatal density (SD) + carbon-to-nitrogen ratio (CN) + leaf dry matter content (LDMC) + leaf thickness (LT) + specific leaf area (SLA) + CMI + garden + 1|home site + 1|plant genotype). The fungal community sequenced from each plant individual was rarefied to a sequencing depth of 2007 reads. For the fungal community associated with each plant individual, fungal richness was measured as the number of OTUs and fungal diversity was measured as the Shannon diversity index (Shannon & Weaver, 1949) with the 'specnumber' and 'diversity' functions, respectively, in the VEGAN package (Oksanen *et al.*, 2024). Fungal richness and diversity values were then averaged across individuals of the same plant genotype. Leaf traits and CMI were scaled using the scale() function. Multicollinearity among leaf traits, CMI, and garden was assessed using the 'check_collinearity' function in the

PERFORMANCE package (Lüdecke *et al.*, 2021). Because stomatal length depended on CMI ($\beta = 0.056$, $t_8 = 4.74$, $P = 0.0015$) and garden ($\beta = -0.064$, $t_{52} = -11.60$, $P < 0.001$), and was the only variable associated with fungal richness and diversity in both common gardens, we tested whether CMI or garden modified those relationships. We compared the aforementioned model to two candidate models with AICc, each with one of the following interaction terms: SL × CMI or SL × garden. We used partial regression plots to visualize the relationship between stomatal length and fungal richness or diversity while controlling for other leaf traits, CMI, and garden (Moya-Laraño & Corcobado, 2008). Semi-partial coefficients of determination (R^2) were calculated for each predictor variable in each linear mixed model with the 'r2beta' function in the R2GLMM package (Edwards *et al.*, 2008).

We tested the effects of leaf traits and home site CMI on fungal composition using generalized linear latent variable models (GLLVM). Models were fit with a negative binomial distribution with home site as a random effect using the 'gllvm' function in the GLLVM package (Niku *et al.*, 2019). We modeled the mesic and xeric garden separately because their species compositions were distinct (PERMANOVA: $F_{1,102} = 40.66$, $R^2 = 0.16$, $P = 0.001$; Supporting Information Fig. S1). Within each garden, read counts were summed across plant individuals of the same genotype, natural log transformed, and included as a row offset to account for differences in sequencing depth. OTUs with a relative abundance < 0.001% or occurring in fewer than five plant genotypes per garden were excluded. For the mesic garden analysis, we excluded stomatal density as a covariate due to its strong correlation with stomatal length (Pearson's $r = -0.87$, $P < 0.001$). Then, leaf traits and CMI were scaled using the scale() function. We used AIC to compare candidate models with 0–2 unconstrained latent variables, 1–2 constrained latent variables, and combinations of both, with either shared or unshared factor loadings. For both common gardens, the best-fitting models were concurrent ordinations (van der Veen *et al.*, 2023) with two unconstrained latent variables and two constrained latent variables sharing the same factor loadings. From these models, we identified leaf traits that significantly explained compositional variation along one or both constrained latent variables.

Because stomatal length was the only variable associated with fungal composition in both gardens, we examined whether plant genotypes with greater stomatal length harbored more similar or more dissimilar fungal communities. We separated the dataset by garden, then randomly subsampled 10 plant genotypes and their fungal communities 1000 times. For each subsample, we calculated the mean stomatal length and the mean pairwise Bray–Curtis dissimilarity using the 'vegdist' function in the VEGAN package. Analyses repeated with 5 or 15 plant genotypes per subsample generated similar results.

Analysis of *Melampsora* relative abundance We tested whether *Melampsora* rust modified the relationships between leaf traits and fungal richness or diversity. For each plant genotype, rust relative abundance was calculated as the sum of read counts for 21 OTUs identified to the genus *Melampsora* divided by the rarefied sequencing depth per genotype (2007 reads). Using the combined data across both gardens, we fit a linear mixed model to test the effects of rust, leaf traits, home site CMI, and common garden on fungal richness and diversity, with stomatal density excluded as a covariate due to collinearity (fungal richness or diversity ~ rust relative abundance (RRA) + SL + CN + LDMC + LT + SLA + CMI + garden + 1|home site + 1|plant genotype). We compared the aforementioned model to five candidate models using AICc, each with one of the following interaction terms: SL × RRA, SL × garden, SL × CMI, RRA × garden, and RRA × CMI. The first three candidate models allowed us to test whether the relationship between stomatal length and fungal richness or diversity depended on rust, common garden, or home site climate. Because rust itself depended on garden ($\beta = -0.068$, $t_{52} = -2.89$, $P = 0.0056$) and home site CMI ($\beta = -0.085$, $t_8 = -2.73$, $P = 0.026$), we included the latter two candidate models to test whether environmental context modified the effect of rust. For fungal composition, we re-ran the GLLVM associated with each garden without *Melampsora* OTUs to assess whether the association between stomatal length and fungal composition was dependent on plant genotypes with shorter stomata harboring a greater relative abundance of rust.

Results

Longer leaf stomata are more sparsely distributed across leaf surfaces

Leaf trait variation among plant genotypes reflected their home sites, with stomatal length and density emerging as a consistent axis of phenotypic differentiation in both common gardens (Figs 2a,b, S2). When considering all measured leaf traits, much of the variation in stomatal length and density was explained by the first principal component in both the mesic (58.04%) and xeric (66.54%) gardens (Figs 2a,b, S2). Stomatal length and density were negatively correlated in both the mesic (Pearson's $r = -0.87$, $P < 0.001$) and xeric common gardens (Pearson's $r = -0.76$, $P < 0.001$; Fig. 2c,d). Thus, longer leaf stomata were more sparsely distributed across leaf surfaces, while shorter leaf stomata were more densely clustered (Fig. 2). For example, in the mesic garden, as stomatal length nearly doubled from 0.38 to 0.69 pixel lengths, stomatal density decreased more than threefold from 73.55 to 22.58 stomata per unit area (Fig. 3).

When comparing leaf trait expression across common gardens, plant genotypes expressed stomatal traits more consistently than any other measured trait (Pearson's $r = 0.76$, $P < 0.001$; Fig. S3). In both gardens, plant genotypes from wetter home sites (i.e. higher home site CMI) expressed longer and sparser stomata, whereas those from drier sites expressed denser and shorter stomata (Figs 2, S3a,b, S4a,b). However, the effect of home site climate on stomatal traits was weaker in the xeric garden for both stomatal length (garden × CMI interaction: $\beta = -0.031$, $t_{52} = -5.57$, $P < 0.001$; Fig. S4a) and density (garden × CMI interaction: $\beta = 3.51$, $t_{52} = 3.32$, $P = 0.0017$; Fig. S4b), where stomatal trait values were also more constrained in their expression (Fig. 2e,f).

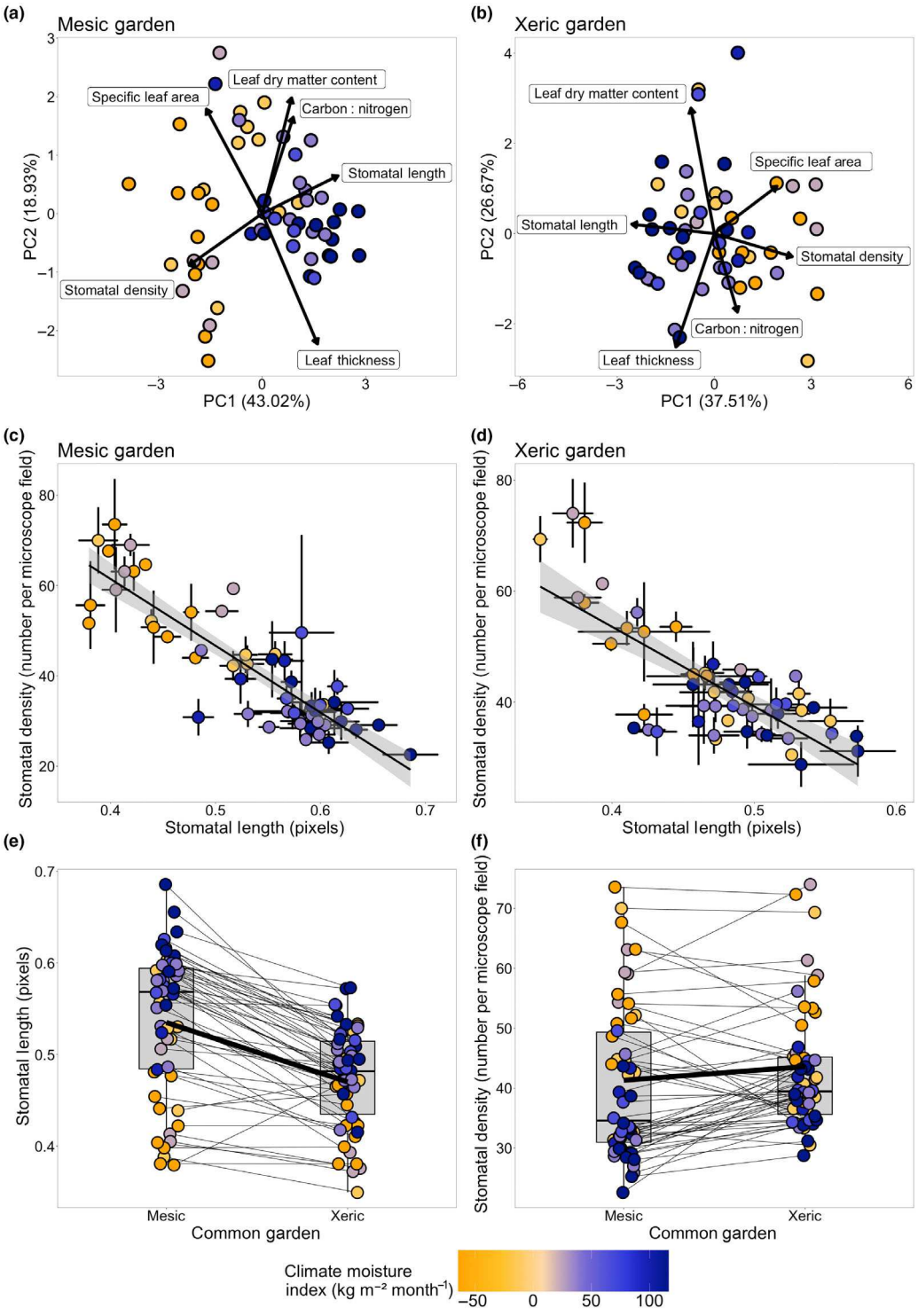

Fig. 2 *Populus trichocarpa* genotypes from wetter environments express longer and sparser stomata in both the mesic and xeric common gardens. (a, b) Leaf trait variation among plant genotypes reflects their home sites, with stomatal length and density distinguishing plant genotypes along the first principal component. Each point corresponds to a single plant genotype, and its position in ordination space is based on averaged trait values among replicate plants of the same plant genotype. (c, d) Plant genotypes from wetter home sites express longer and sparser leaf stomata, while those from drier home sites express shorter and denser stomata (mesic garden: Pearson's $r = -0.87$, $P < 0.001$; xeric garden: Pearson's $r = -0.76$, $P < 0.001$). Each point is the mean trait value of replicate plants of the same plant genotype with SE bars. Grey shading refers to 95% confidence intervals. (e, f) Stomata are longer in the mesic garden (linear mixed model: $P < 0.001$), while stomatal density is similar across gardens (linear mixed model: $P = 0.052$). Each point is the mean trait value of replicate plants of the same plant genotype. Thin lines connect the same plant genotypes in each garden. The thick line depicts the change in mean stomatal length or density across the gardens after accounting for home site and plant genotype. Horizontal lines within each boxplot refer to median values and whiskers extend to 1.5 times the interquartile range. In all panels, points are color coded by the climate moisture index from which plant genotypes were sourced.

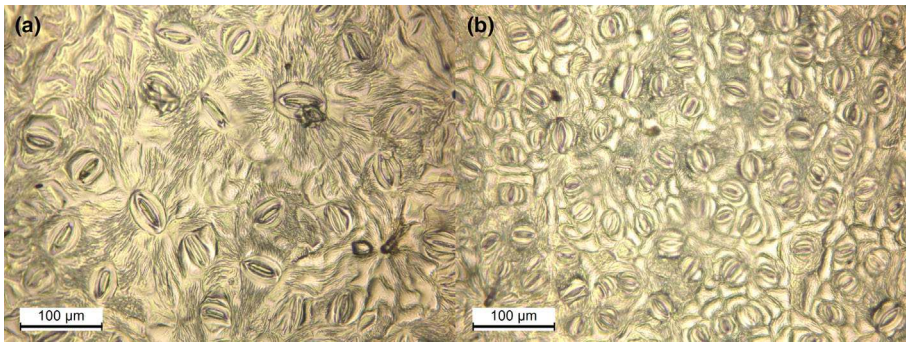

Fig. 3 Examples of variation in abaxial stomatal length and density in *Populus trichocarpa*. (a) Representative example of long and sparse stomata (*P. trichocarpa* genotype HRSP-27-5). (b) Representative example of short and dense stomata (*P. trichocarpa* genotype BESC-1243). These plant genotypes were grown in the mesic common garden.

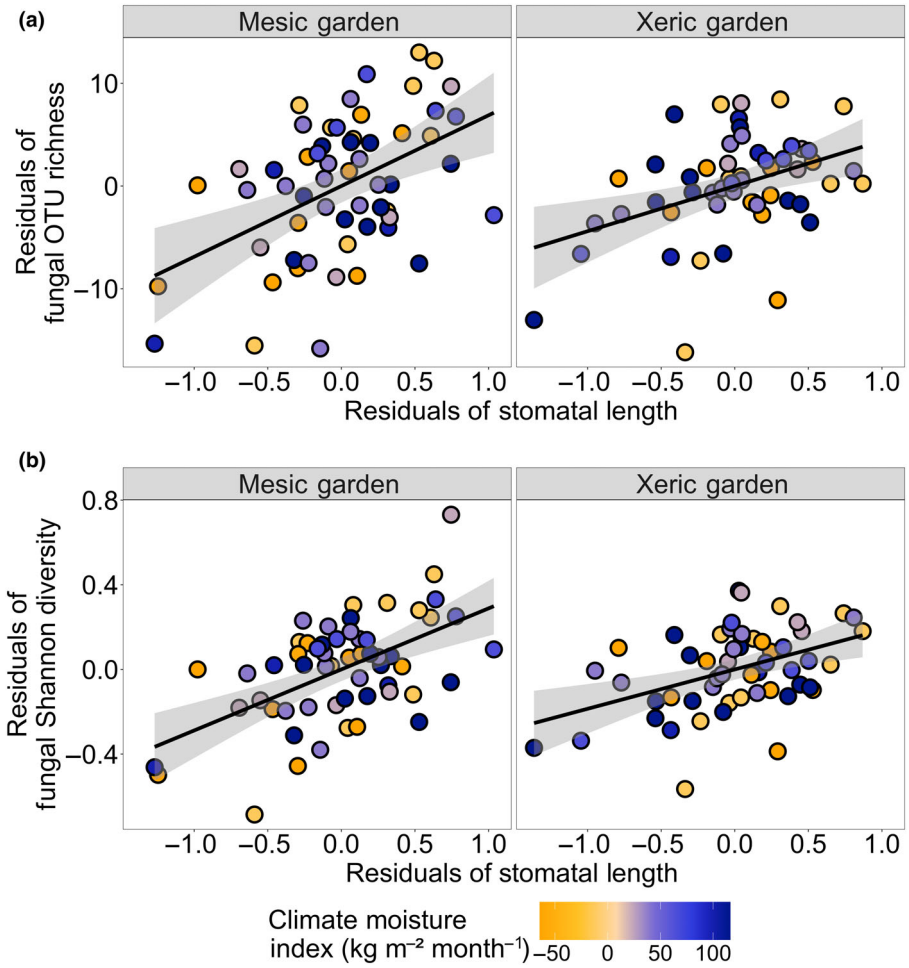

Fig. 4 *Populus trichocarpa* genotypes with longer and sparser leaf stomata host a greater richness and diversity of leaf fungi. (a, b) Longer and sparser leaf stomata are associated with greater fungal richness (semi-partial R^2 = 0.16) and diversity (semi-partial R^2 = 0.23) in both the mesic and xeric common gardens. Partial regression plots illustrate the relationship between stomatal length and leaf fungal richness or diversity after accounting for other leaf traits, common garden, and home site climate moisture index. Each point is based on the mean trait or community structure value among replicate plants of the same plant genotype. Points are color coded by the climate moisture index from which plant genotypes were sourced. Grey shading refers to 95% confidence intervals. OTU, Operational Taxonomic Units.

Longer stomata support greater leaf fungal richness and diversity

Leaf fungal richness ($\beta = 5.09$, $t_{46} = 4.50$, $P < 0.001$; semi-partial $R^2 = 0.16$) and diversity ($\beta = 0.23$, $t_{46} = 5.68$, $P < 0.001$; semi-partial $R^2 = 0.23$) were greater in plant genotypes with longer stomata (Figs 4, S5; Table S1). The effect of stomatal length on fungal richness and diversity depended on the CMI of a plant genotype's home site. Specifically, stomatal length had stronger positive associations with fungal richness ($\beta = -1.31$, $t_{46} = -2.06$, $P = 0.045$) and diversity ($\beta = -0.059$, $t_{46} = -2.66$, $P = 0.011$) among plant genotypes from drier home sites (Fig. S6; Table S1). However, home site climate alone was not associated with fungal richness ($P = 0.29$) or diversity ($P = 0.76$) as a standalone fixed effect.

Although stomatal length and density were negatively correlated in both gardens, stomatal density explained minimal variation in fungal richness ($P = 0.16$; semi-partial $R^2 = 0.019$) and diversity ($\beta = 0.078$, $t_{46} = 2.25$, $P = 0.030$; semi-partial $R^2 = 0.046$). Leaves with lower nitrogen content hosted greater fungal richness ($\beta = 0.97$, $t_{46} = 1.70$, $P = 0.097$) and diversity ($\beta = 0.048$, $t_{46} = 2.38$, $P = 0.021$). However, carbon-to-

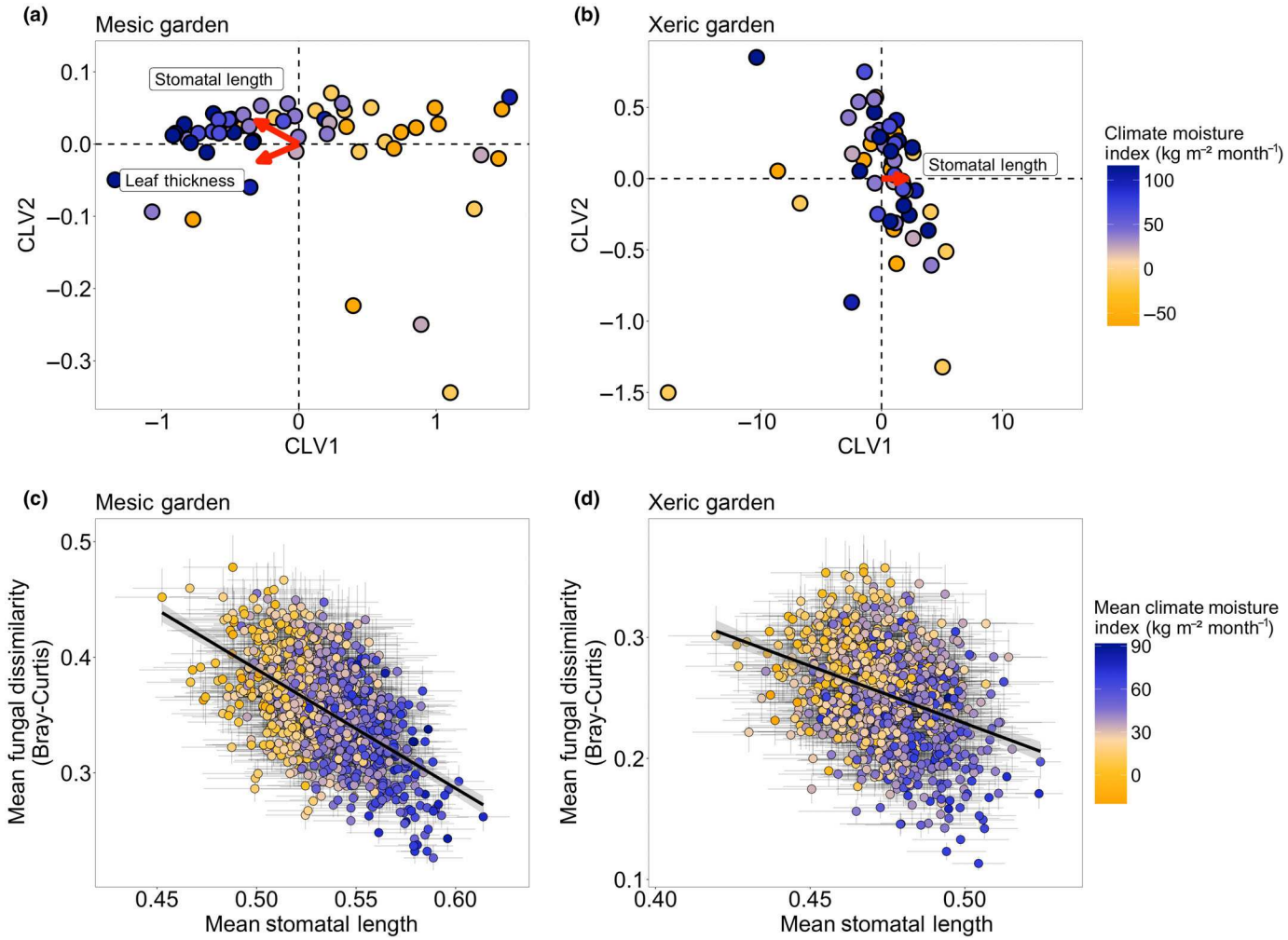

Fig. 5 *Populus trichocarpa* genotypes with longer and sparser leaf stomata host more compositionally similar communities of leaf fungi. (a) In the mesic garden, stomatal length and leaf thickness are associated with fungal composition. (b) In the xeric garden, stomatal length is associated with fungal composition. Red vectors represent plant traits that significantly contribute to variation in fungal community composition along one or both constrained latent variables in a generalized linear latent variable model ($P < 0.05$). Fungal Operational Taxonomic Units with a relative abundance < 0.001% or occurring in fewer than five plant genotypes were excluded. (c, d) Fungal communities are more compositionally similar among plant genotypes with longer stomata in both the mesic (Pearson's $r = -0.56$, $P < 0.001$) and xeric (Pearson's $r = -0.35$, $P < 0.001$) common gardens. Ten plant genotypes and their associated fungal communities were subsampled with replacement 1000 times per garden to assess how pairwise fungal dissimilarities (Bray–Curtis) varied with stomatal length. Each point is the mean stomatal length or mean dissimilarity across plant genotypes in a subsampling event with SE bars. Points are color coded by the climate moisture index from which plant genotypes were sourced. CLV, constrained latent variables.

nitrogen ratio (semi-partial $R^2 = 0.026$–0.050), along with leaf dry matter content (semi-partial $R^2 = 0.001$–0.003), leaf thickness (semi-partial $R^2 = 0.004$–0.008), and specific leaf area (semi-partial $R^2 = 0.005$–0.006), minimally explained fungal richness and diversity.

In addition to stomatal traits and leaf chemistry, plants grown in the xeric garden had lower fungal richness ($\beta = -8.15$, $t_{46} = -4.29$, $P = 0.001$) and diversity ($\beta = -0.33$, $t_{46} = -4.92$, $P < 0.001$) than those in the mesic garden. Despite these garden-level differences, stomatal length maintained a consistent positive relationship with fungal richness and diversity, regardless of common garden environment (fungal richness model with SL × garden interaction: $P = 0.53$; fungal diversity model with SL × garden interaction: $P = 0.32$).

Longer stomata support more similar leaf fungal communities

Leaf fungal communities were more similar in community composition among plant genotypes with longer leaf stomata in both common gardens (Fig. 5). Stomatal length was significantly associated with variation in fungal community composition along one or two constrained latent variables (CLV) in the mesic (CLV1: estimate = 0.31, $z = 2.73$, $P = 0.0064$; CLV2: estimate = −0.027, $z = -3.37$, $P < 0.001$) and xeric (CLV1: estimate = 2.04, z = 2.07, $P = 0.038$) gardens (Fig. 5a,b). Plant genotypes with longer stomata were more clustered in concurrent ordination space (Fig. 5a,b), indicating that greater stomatal length was associated with greater fungal community similarity

(Fig. 5c,d). This pattern was confirmed through repeated subsampling of plant genotypes and fungal communities, which showed that plant genotypes with longer stomata hosted more compositionally similar fungal communities in both the mesic (Pearson's $r = -0.56$, $P < 0.001$) and xeric (Pearson's $r = -0.35$, $P < 0.001$) common gardens (Fig. 5c,d).

The other measured leaf traits had inconsistent or no relationships with fungal composition. Leaf thickness (CLV1: estimate = 0.29, $z = 3.22$, $P = 0.0013$) was associated with fungal composition in the mesic garden but not in the xeric garden (Fig. 5a,b; Table S2). Stomatal density, carbon-to-nitrogen ratio, specific leaf area, and leaf dry matter content were not associated with fungal composition (Table S2).

Longer stomata are associated with lower *Melampsora* rust

Melampsora species, common rust pathogens on *P. trichocarpa*, were represented by 21 OTUs that were greater in relative abundance in the mesic garden (22.30% ± 19.20% SD) than in the xeric garden (15.19% ± 16.64%; $\beta = -0.064$, $t_{53} = -2.73$, $P = 0.0086$; Figs S7, S8). Rust relative abundance was negatively correlated with stomatal length in both the mesic (Pearson's $r = -0.69$, $P < 0.001$) and xeric (Pearson's $r = -0.44$, $P < 0.001$) gardens (Figs 6a, S8). Although greater rust relative abundance was associated with lower fungal richness ($\beta = -4.25$, $t_{47} = -7.47$, $P < 0.001$) and diversity ($\beta = -0.20$, $t_{46} = -8.09$, $P < 0.001$; Fig. 6b), longer stomata were still associated with greater fungal richness ($\beta = 1.99$, $t_{47} = 2.87$, $P = 0.0061$) and diversity ($\beta = 0.066$, $t_{46} = 2.67$, $P = 0.011$; Fig. 6c; Table S3). Stomatal length was still associated with fungal composition after removing rust OTUs in both the mesic (CLV2: estimate = −0.29, $z = -2.27$, $P = 0.023$) and xeric gardens (CLV2: estimate = −0.40, $z = -2.29$, $P = 0.022$).

Discussion

Our findings reveal that stomatal traits may be a general means through which plants and the leaf mycobiome influence one another. Using reciprocal common gardens planted with a diverse panel of *P. trichocarpa* genotypes, we found that plant genotypes with longer and sparser stomata supported greater leaf fungal richness, diversity, and community similarity than those with shorter and denser stomata (Figs 4, 5). These associations persisted across two strongly contrasting environments when accounting for covarying leaf traits, plant home site, and a common leaf pathogen, demonstrating the predictive value of stomatal traits on the leaf mycobiome under field conditions. Stomatal length alone explained 16–23% of the variation in fungal richness and diversity, greatly exceeding the explanatory power of commonly measured leaf traits used to infer plant ecological strategies, such as specific leaf area (< 1%) and carbon-to-nitrogen ratio (3–5%).

Stomatal traits are a consistent axis of leaf trait variation

Stomatal length and density varied dramatically among plant genotypes within each common garden, with the longest stomata occurring at densities 61.15% to 69.30% lower than the shortest stomata in the xeric and mesic gardens, respectively (Figs 2c,f, 3). Despite variation in leaf trait expression in each garden, stomatal traits accounted for much of the trait variation differentiating plant genotypes (Fig. S2) and were more consistent in their expression than any other measured leaf trait (Fig. S3). Plant genotypes from cooler, wetter home sites expressed longer and sparser stomata than those from hotter, drier home sites (Figs 2, S3a,b, S4a,b), aligning with work linking stomatal trait variation to climate adaptation (Dunlap & Stettler, 2001; McKown *et al.*, 2014; Klein *et al.*, 2025). Although the stomatal size-density relationship is widespread among vascular plants (Hetherington & Woodward, 2003; McKown *et al.*, 2014; Wang *et al.*, 2015; Liu *et al.*, 2023) and readily quantifiable, stomatal traits are an underrecognized axis of leaf phenotypic variation that could influence leaf microbiome structure (Lajoie & Dariel, 2025).

Potential drivers of stomatal–mycobiome associations

Our findings suggest that fewer stomata of larger size may have selected for a greater richness and diversity of more similar fungal taxa (Figs 4, 5). Relative to an arrangement of shorter, denser stomata, larger, sparser stomatal openings increase the distance fungal hyphae must travel on the leaf surface to reach a pore. Thus, large stomata, through their sparsity, may decrease the rate at which fungi colonize the leaf interior (P. E. Busby *et al.*, unpublished). Such sparsity may impose strong host filtering, as fewer colonization events could provide more opportunity for stomatal and other leaf traits to shape which fungi successfully establish. Consistent with this, longer and sparser stomata homogenized fungal composition to a more predictable community of fungal taxa (Fig. 5), which may be indicative of greater host control over mycobiome assembly. Moreover, if fewer stomatal entry points decrease fungal colonization of the leaf interior, such leaves may be expected to host a lower richness of fungi because fewer fungal species arrive to establish (MacArthur & Wilson, 1967). However, we observed the opposite pattern: leaves with fewer, longer stomata hosted greater fungal richness and diversity. Strong host filtering may prevent any single fungus from dominating the leaf interior, promoting coexistence among a broader suite of fungal taxa. Additionally, if the rate at which fungi turn over or go locally extinct is lower in leaves with longer and sparser stomata, fungal richness may accumulate despite reduced fungal immigration.

By contrast, denser stomata of smaller size were associated with a lower richness and diversity of more dissimilar fungal taxa (Figs 4, 5). Smaller but denser stomatal entry points should reduce the distance fungal hyphae must travel on the leaf surface to reach a pore (P. E. Busby *et al.*, unpublished), increasing the likelihood of fungal colonization. If greater opportunities for fungal colonization in plants with shorter, denser stomata weaken host filtering on the mycobiome, then greater variability in fungal composition may reflect greater stochasticity as the mycobiome assembles. Stochastic processes such as priority effects, where early arriving fungi may preempt colonization by other fungi (Vannette & Fukami, 2014; Fukami, 2015), could explain why fungal richness remains low

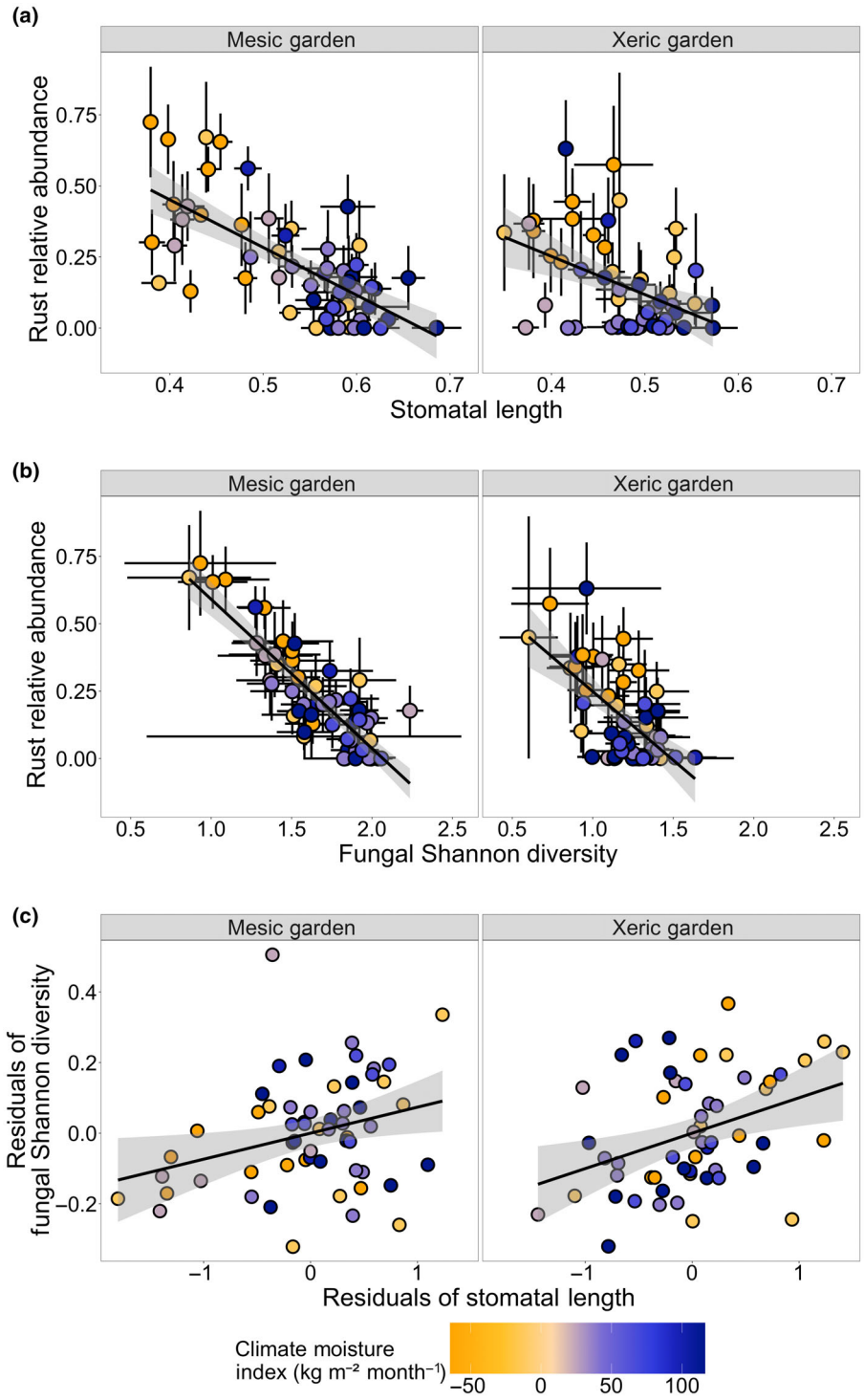

Fig. 6 Relationships between stomatal length and fungal richness or diversity are not solely driven by the relative abundance of *Melampsora*, a genus of common rust pathogens on *Populus trichocarpa*. (a) Rust relative abundance is negatively correlated with stomatal length in both the mesic (Pearson's $r = -0.69$, $P < 0.001$) and xeric (Pearson's $r = -0.44$, $P < 0.001$) common gardens. (b) Rust relative abundance is negatively correlated with fungal diversity in both the mesic (Pearson's $r = -0.87$, $P < 0.001$) and xeric (Pearson's $r = -0.62$, $P < 0.001$) common gardens. (c) Stomatal length remains positively associated with fungal diversity (linear mixed model: $P = 0.011$), after accounting for rust relative abundance, common garden, home site climate moisture index, and other leaf traits. In all panels, each point represents a single plant genotype. Error bars refer to SE. Colors refer to the climate moisture index from which plant genotypes were sourced. Grey shading refers to 95% confidence intervals.

even when the potential for fungal immigration is high. For example, a fast-growing or abundant fungus in the local species pool that colonizes many stomatal entry points could limit opportunities for later colonizers and reduce the potential for fungal coexistence. Thus, strong competitive priority effects, by increasing the likelihood that later arriving fungi cannot establish, may explain why shorter, denser leaves had lower fungal richness. Priority effects in leaf mycobiome assembly have been shown to be important in this system (Leopold & Busby, 2020). However, additional work integrating measures of stomatal density, fungal load inside of leaves, and fungal species colonization and extinction through community assembly is needed to test whether more stomatal entry points increase fungal immigration and to what extent stomatal density amplifies host filtering or stochastic processes.

While stomatal traits may regulate colonization of the mycobiome, the expression of stomatal traits may also depend on the

leaf mycobiome. Inoculation-based studies have shown that stomatal density was reduced in plants colonized by non-pathogenic bacterial and yeast endophytes (Rho *et al.*, 2018) or a bacterial pathogen (Dutton *et al.*, 2019). Microbe-driven changes in stomatal development could arise when infection alters phytohormone levels or activates immune receptors in mature leaves, which in turn influence epidermal differentiation in newly developing leaves (Dutton *et al.*, 2019). At the same time, stomatal trait expression is also bounded by genomic constraints, with the loci associated with stomatal size having been identified in *P. trichocarpa* and linked to climate adaptation (Klein *et al.*, 2025). Understanding how fungal-driven variation in stomatal traits operates within the genetic and developmental limits of the host plant will be key to understanding how the local fungal species pool could mediate stomatal trait–mycobiome associations.

It is also possible that unmeasured leaf properties that covary with stomatal length and are relevant to fungal colonization explain the associations between stomatal length and the leaf mycobiome. For example, the same plant gene family – 3-ketoacyl-CoA synthase genes – is associated with both stomatal traits (Klein *et al.*, 2025) and cuticular wax variation (Gonzales-Vigil *et al.*, 2017), two traits that likely impact leaf microbiome assembly in distinct ways. Longer stomata may correlate with shifts in the abundance and topography of other leaf anatomical features that influence fungal establishment, such as trichome or vein density, which could impact the availability of soluble carbon sources or the aggregation of moisture on leaf surfaces (Leveau & Lindow, 2001). Larger pore sizes could further shape the leaf mycobiome by facilitating the entry of a common endophytic mite (*Schizoempodium mesophyllincola*) in *P. trichocarpa*, which feeds on spongy mesophyll cells after stomatal entry (Oldfield *et al.*, 1998) and can negatively impact co-occurring biotrophic fungi (Busby *et al.*, 2019). Finally, stomatal length may be associated with the duration and rate at which stomata remain open or closed (Fanourakis *et al.*, 2015). A previous common garden study found no relationship between stomatal conductance, a measure of gas exchange dependent upon stomatal opening, and abaxial stomatal length or density among *P. trichocarpa* genotypes from mesic environments (McKown *et al.*, 2014). However, further exploration of this trend including plant genotypes from xeric environments is still needed.

The mechanisms we propose underlying stomatal length–mycobiome associations are largely interpreted through the lens of fungi colonizing the leaf interior through stomata. Although washing leaf discs with a surfactant is expected to dislodge fungal spores or hyphal fragments on the leaf surface (Brown *et al.*, 2018; Mahmoudi *et al.*, 2024) and may bias sequencing toward fungi inside leaves, our leaf washing procedure likely captured fungi living on leaf surfaces. Strictly epiphytic fungi and those that subsequently colonize the leaf interior may have different responses to stomatal traits. For example, surface-dwelling fungi may have stronger associations with epidermal traits correlated with stomatal area, such as pavement cell area, while endophytic fungi may depend more on the traits of stomata themselves (size, density, depth, closure). Additional work that isolates the epiphytic from the endophytic fungal community will help to elucidate whether stomatal traits can influence leaf surface colonization, leaf interior colonization, or both.

Environmental context mediates stomatal–mycobiome associations

Although stomatal traits covaried with mycobiome structure in both common gardens, the strength of this relationship was reduced in the xeric garden (Figs 4, 5). The trend where plant genotypes from wetter home sites expressed longer, sparser stomata and those from drier home sites expressed shorter, denser stomata was weaker in the xeric garden (Fig. S4a,b). This narrower range of trait variation in the xeric garden may have contributed to weaker relationships between stomatal traits and the leaf mycobiome (Fig. S5). Conditions unique to the xeric garden, such as greater desiccation on the leaf surface, may have preempted host selection altogether by suppressing fungal survival on the leaf surface, such that leaf traits exerted weaker control over their mycobiomes simply because there were fewer fungal taxa their traits could influence. In addition, drier conditions can promote longer durations of stomatal closure, further reducing access to leaf interiors (McAdam *et al.*, 2015), which could in turn homogenize fungal communities to the same drought-tolerant taxa (Fig. S1). The plant trait variation expressed in our common gardens does not reflect intraspecific variation that would be found in a single wild population of *P. trichocarpa*. Yet, by generating significant leaf trait variation and controlling for host genetic identity, our findings signal that environmental context may limit the ability of a plant and its mycobiome to influence one another.

Stomatal-mycobiome associations amid *Melampsora* rust pressure

Our leaf sampling for mycobiome characterization coincided with the emergence of *Melampsora*, a genus of rust pathogens that commonly infects *P. trichocarpa* late in the growing season (Dunlap & Stettler, 1996). We found that plants with greater rust relative abundance hosted fewer fungal taxa (Fig. 6b), consistent with other plant systems showing that pathogen infection is often accompanied by shifts in the diversity and composition of the non-pathogenic microbiome (Bulgari *et al.*, 2014; Li *et al.*, 2022; Zhang *et al.*, 2023). The persistence of the associations with stomatal length and mycobiome structure (Fig. 6c) after accounting for rust relative abundance suggests that stomatal length contributes to fungal community assembly even under pathogen pressure.

Resistance to rust tends to be greater in plant genotypes from cooler, wetter environments (Dunlap & Stettler, 1996; Leopold & Busby, 2020), which also tend to have longer, sparser stomata and experience greater rust pressure (Dunlap & Stettler, 1996). Consistent with this, plants from wetter climatic origins in our study had lower rust relative abundance (Fig. 6a). While this pattern is thought to manifest through resistance genes (Yin *et al.*, 2004; La Mantia *et al.*, 2013) or reduced infection probability through fewer stomatal entry points (Muir, 2020), longer and sparser stomata may confer

indirect resistance by promoting the assembly of diverse mycobiomes that suppress rust pathogens emerging late in the growing season (McLaren & Callahan, 2020). The *Melampsora* OTUs detected in our study likely represent multiple rust strains, which together were negatively correlated in their relative abundance with fungal richness and diversity in both gardens (Fig. 6b). Stomatal-mediated assembly of a protective mycobiome may be a broad-spectrum means of rust resistance effective across different *Melampsora* lineages.

Conclusions

Plant biologists have long focused on plant traits to understand plant strategies and performance. Microbial ecologists are increasingly asking whether these same plant traits influence the assembly of leaf microbial communities. Here, we show that plants with longer and sparser stomata hosted a greater richness and diversity of similar fungal species. Our findings signal that stomatal traits may be a general means through which plants and the leaf mycobiome influence one another. Further work testing this hypothesis and clarifying how stomatal length mediates microbial assembly independent of stomatal density and other leaf traits that likely influence microbial colonization (e.g. cuticular waxes and leaf exudates) will be key to strategies aimed at improving plant health and forecasting how microbiomes respond to novel plant phenotypes.

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Competing interests

None declared.

Author contributions

PEB and DL designed and supervised the research. SH collected the data and contributed to data analysis and interpretation. AA analyzed the data and wrote the manuscript. LDLA assisted with data collection, analysis, and interpretation. All authors contributed feedback on the manuscript. AA and SH contributed equally to this work.

ORCID

Leander D. L. Anderegg https://orcid.org/0000-0002-5144-7254
Austen Apigo https://orcid.org/0000-0002-2525-0394
Posy E. Busby https://orcid.org/0000-0002-2837-9820
Sabrina Heitmann https://orcid.org/0000-0002-3222-5648
Devin Leopold https://orcid.org/0000-0003-2124-2116

Data availability

Data and code associated with this study are archived in Figshare (doi: 10.6084/m9.figshare.30338440). Sequencing data are archived in the National Center for Biotechnology Information Sequence Read Archive database (BioProject accession number: PRJNA896565).

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Supporting Information

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

Fig. S1 Leaf fungal communities differ in community composition between the two common gardens.

Fig. S2 Stomatal length and density show the strongest associations with the first principal component in multivariate trait space.

Fig. S3 Stomatal length and density show the most consistent trait expression across the mesic and xeric common gardens.

Fig. S4 Relationships between leaf traits and the climate moisture index from which plant genotypes were sourced.

Fig. S5 Longer stomata are associated with greater fungal richness and diversity.

Fig. S6 Plant genotypes from drier home sites exhibit stronger associations between stomatal length and fungal richness or diversity.

Fig. S7 Plant genotypes in the mesic garden have greater relative abundances of *Melampsora*.

Fig. S8 Plant genotypes with longer and sparser stomata are dominated by fungi in the Dothideomycetes, while those with shorter and denser stomata have greater relative abundances of rust pathogens in the Pucciniomycetes.

Table S1 Results from linear mixed models testing the effect of leaf traits, common garden, and home site climate moisture index on fungal richness or diversity.

Table S2 Results from generalized linear latent variables models testing the effect of leaf traits, and home site climate moisture index on fungal composition.

Table S3 Results from linear mixed models testing the effect of rust relative abundance, leaf traits, common garden, and home site climate moisture index on fungal richness or diversity.

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