2-10-2018

Structural and Defensive Roles of Angiosperm Leaf Venation Network Reticulation Ecross an Andes-Amazon Elevation Gradient

Benjamin Blonder
University of Oxford

Norma Salinas
University of Oxford

Lisa Patrick Bentley
University of Oxford

Alexander Shenkin
University of Oxford

Percy Chambi-Porroa
Universidad Nacional San Antonio Abad del Cusco

See next page for additional authors

Recommended Citation

Blonder, B., Salinas, N., Patrick Bentley, L., Shenkin, A., Chambi Porroa, P. O., Valdez Tejeira, Y., Boza
Espinoza, T. E., Goldsmith, G. R., Enrico, L., Martin, R., Asner, G. P., Díaz, S., Enquist, B. J., & Malhi, Y. (2018)
Structural and defensive roles of angiosperm leaf venation network reticulation across an Andes-Amazon
elevation gradient. *Journal of Ecology* doi:10.1111/1365-2745.12945

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Authors

Benjamin Blonder, Norma Salinas, Lisa Patrick Bentley, Alexander Shenkin, Percy Chambi-Porroa, Yolvi Valdez Tejeira, Tatiana Erika Boza Espinoza, Gregory R. Goldsmith, Lucas Enrico, Roberta Martin, Gregory P. Asner, Sandra Diaz, Brian J. Enquist, and Yadvinder Malhi

DR. BENJAMIN BLONDER (Orcid ID : 0000-0002-5061-2385)

DR. ALEXANDER SHENKIN (Orcid ID : 0000-0003-2358-9367)

Title

Structural and defensive roles of angiosperm leaf venation network reticulation across an Andes-

Amazon elevation gradient

Running head

Leaf venation network reticulation

Authors

Benjamin Blonder [*1,2]

Norma Salinas [1,3]

Lisa Patrick Bentley [1,4]

Alexander Shenkin [1]

Percy Orlando Chambi Porroa [5]

Yolvi Valdez Tejeira [5]

Tatiana Erika Boza Espinoza [6]

Gregory R. Goldsmith [7]

Lucas Enrico [8]

Roberta Martin [9]

Gregory P. Asner [9]

Sandra Díaz [1,8]

Brian J. Enquist [10]

Yadvinder Malhi [1]

Affiliations

*: Corresponding author; bblonder@gmail.com

1: Environmental Change Institute, School of Geography and the Environment, University of Oxford, Oxford, OX1 3QY, United Kingdom

2: School of Life Sciences, Arizona State University, Tempe, Arizona, USA

3: Sección Química, Pontificia Universidad Católica del Perú, San Miguel, Lima, Peru

4: Department of Biology, Sonoma State University, 1801 E. Cotati Ave., Rohnert Park, CA 94928, United States

5: Universidad Nacional de San Antonio Abad del Cusco, Cusco, Peru

6: Department of Systematic and Evolutionary Botany, University of Zurich, Zürich 8088, Switzerland

7: Schmid College of Science and Technology, Chapman University, Orange, CA 92866 USA

8: Instituto Multidisciplinario de Biología Vegetal (IMBIV, CONICET-UNC), and FCEFyN,

Universidad Nacional de Córdoba, Argentina

9: Department of Global Ecology, Carnegie Institution for Science, Stanford, California, United States

10: Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, Arizona,

United States

Summary

1. The network of minor veins of angiosperm leaves may include loops (reticulation). Variation in network architecture has been hypothesized to have hydraulic and also structural and defensive functions.

2. We measured venation network trait space in eight dimensions for 136 biomass-dominant angiosperm tree species along a 3,300 m elevation gradient in southeastern Peru. We then examined the relative importance of multiple ecological, and evolutionary predictors of reticulation.

3. Variation in minor venation network reticulation was constrained to three axes. These axes described branching vs. reconnecting veins, elongated vs. compact areoles, and high vs. low density veins. Variation in the first two axes was predicted by traits related to mechanical strength and secondary compounds, and in the third axis by site temperature.

4. Synthesis. Defensive and structural factors primarily explain variation in multiple axes of reticulation, with a smaller role for climate-linked hydraulic factors. These results suggest that venation network reticulation may be determined more by species interactions than by hydraulic functions.

Resumen (español)

1. La red de venas menores de hojas de angiospermas puede incluir lazos (reticulación). Se ha planteado la hipótesis que la variaciones en la arquitectura de la red de venas puede cumplir además funciones estructurales, hidráulicas y de defensa.

2. Hemos medido las características de la red de venas foliares en ocho dimensiones para 136 especies de arboles angiospermos dominantes en un gradiente altitudinal de 3,300 metros en el sureste de Perú. Examinamos la importancia relativa de variables ecológicas y evolutivas capaces de predecir patrones de reticulación.

3. Variación en la reticulación de la red de venas menores estaba limitada a tres ejes. Estos ejes describen venas ramificadas versus reconectadas, areolas elongadas versus compactas, y venas de alta densidad versus las de baja densidad. La variación en los primeros dos ejes pudo predecirse por características relacionadas a fuerza mecánica y compuestos secundarios, y en el tercer eje por la temperatura del lugar de estudio.

4. Síntesis. Factores defensivos y estructurales explican la variación en múltiples ejes de los patrones de reticulación, con un papel menor para factores hidráulicos ligados al clima. Estos resultados

sugieren que la reticulación de la red de la venación puede ser determinada más por la interacciones

entre especies que por funciones exclusivamente hidráulicas.

Key words

reticulation, loop, venation network, trait space, tropical forest, redundancy, damage resilience,

damage resistance, leaf performance

Introduction

Leaf function is important for determining plants fitness across biotic and abiotic contexts. Venation

mediates water and carbon fluxes (Brodribb *et al.*, 2007; Brodribb *et al.*, 2010), may provide

structural or defensive functions (Givnish, 1979; Méndez-Alonzo *et al.*, 2013), and may influence

whole-plant carbon construction cost (Sack and Scoffoni, 2013). The costs and the benefits of a leaf

may be constrained by the architecture of the venation network (Sack *et al.*, 2004; Brodribb *et al.*,

2007; Blonder *et al.*, 2011; Sack and Scoffoni, 2013; Buckley *et al.*, 2015), which in turn maybe

constrained by the evolution of novel venation phenotypes across clades (Trivett and Pigg, 1996;

Brodribb *et al.*, 2010). Because multiple functions map differently onto the same structure, not all

function / cost axes can simultaneously be optimized by a network constrained to a planar geometry,

For example, a network cannot simultaneously maximize efficiency and redundancy (Katifori *et al.*,

2010). As a result, tradeoffs may be necessary (Katifori *et al.*, 2010; Blonder *et al.*, 2011).

Describing reticulation in minor venation networks

Venation networks vary widely in their geometry, with some that form extensive loops (closed

interconnections of veins, or anastomoses (Roth-Nebelsick *et al.*, 2001)) that enclose areoles, and

others that do not. This variation is reflected in the general term, 'reticulation' (Trivett and Pigg,

1996; Roth-Nebelsick *et al.*, 2001). Because vein orders often smoothly transition into each other, we use 'minor' to refer to the patterns characteristic of the ultimate veins, though these patterns may include features created by higher-order veins. Some leaves have areoles in their minor veins that are simple polygons, while others have freely ending veinlets that do not connect to other veins (von Ettingshausen, 1861; Ellis *et al.*, 2009). Reticulation patterns may be described by scale-dependent (with units) or scale-independent (dimensionless) statistics.

Scale-dependent reticulation patterns have been described by vein density, the length of veins per unit leaf area, and by loopiness, the number of areoles per unit leaf area. Scale-independent reticulation patterns describe the shape of loops and other structures in the network (e.g. freely ending veinlets). Rapid evolution of minor venation across clades is also thought to be possible, consistent with functional roles of reticulation patterns (Givnish *et al.*, 2005; Boyce *et al.*, 2009b; Horn *et al.*, 2009; Blonder *et al.*, 2016).

Scale-dependent patterns in vein density have been shown to be predictive of transpiration and photosynthesis rates (Brodribb *et al.*, 2007), environmental change (de Boer *et al.*, 2012) and climate niches (Manze, 1967; Uhl and Mosbrugger, 1999; Blonder and Enquist, 2014). However, much less is known about the ecological significance of variation in scale-independent patterns of reticulation, about the drivers of this variation, or about the evolutionary patterns and constraints underlying this variation. This is surprising, given the differences in reticulation readily observed across taxa (e.g. between *Ginkgo biloba* [maidenhair tree], with no looping, and *Malus pumila* [orchard apple] with extensive looping).

Functional hypotheses for reticulation

A wide body of empirical evidence has shown that minor venation patterns reflect hydraulic functioning across environments (Sack and Scoffoni, 2013; Blonder and Enquist, 2014; Blonder *et al.*, 2017). Higher vein density may occur in with warmer and drier abiotic conditions (Sack and Scoffoni, 2013). Sites with higher potential evapotranspiration or temperatures should select for species that

achieve high carbon assimilation rates by transpiring either a high amount of water when water is temporarily abundant or that use the same amount of water more efficiently when water is not (Blonder *et al.*, 2014). Thus, higher vein density or water use efficiency should be associated with warmer environments (Uhl and Mosbrugger, 1999; Sack and Scoffoni, 2013; Blonder *et al.*, 2014; Blonder *et al.*, 2016; Blonder *et al.*, 2017). Because many scale-dependent reticulation metrics are necessarily correlated based on geometrical scaling considerations (Sack *et al.*, 2012; Blonder *et al.*, 2013; Carins Murphy *et al.*, 2016), warmer and less wet environments are expected to also have higher reticulation due to their direct impacts on vein density.

Reticulation may also mediate efficiency vs. redundancy tradeoffs for leaf hydraulics. Theoretical models suggest that networks that only branch (no loops) provide the most efficient transport of such resources from a central point under constant conditions (West *et al.*, 1997; Dodds, 2010; McKown *et al.*, 2010; Price *et al.*, 2010), while networks that branch and also have loops provide alternate flow pathways that provide more resilience to fluctuating resource loads (Nardini *et al.*, 2001; Sack *et al.*, 2008; Corson, 2010; Katifori *et al.*, 2010; Price and Weitz, 2014). Such fluctuating loads and damage could arise from the occurrence of sunflecks in shaded conditions (Givnish, 1979; Zwieniecki *et al.*, 2002; Givnish *et al.*, 2005), drought stress and xylem cavitation (Brodribb *et al.*, 2016). Thus, reticulation could be associated with traits related to photosynthetic capacity and light environment.

Reticulation may also have multiple structural and defensive functions beyond these direct and indirect hydraulic functions (Sack and Scoffoni, 2013). Reticulation could increase damage resistance by offsetting other leaf anti-herbivore defense strategies (Roth-Nebelsick *et al.*, 2001). More reticulation could provide higher damage resilience to herbivory or environmental stressors (e.g. wind-driven tearing) by providing alternate flow pathways that maintain hydraulic functioning (Sack *et al.*, 2008). More reticulation could also provide more deterrence against chewing/cutting damage through increased mechanical strength and resistance to tearing (Wagner, 1979; Choong *et al.*, 1992). Reticulation could also provide additional redundant pathways for latex flow, enabling successful deployment of defense compounds after wounding (Agrawal and Konno, 2009). All of these

strategies would provide an alternate solution offsetting investment in defense compounds. Thus, reticulation could be associated with traits related to secondary compound fractions (polyphenols, tannins, lignins), and toughness (force to punch or force to tear).

Reticulation could also directly provide mechanical strength to a leaf (Givnish, 1979; Givnish *et al.*, 2005; Sack and Frole, 2006; Niinemets *et al.*, 2007), with loops providing reinforcing cross-linkages that increase stiffness and strength (Niklas, 1999; Read and Stokes, 2006) as well as reducing the potential for tearing and crack propagation (Vincent, 1982). Moreover, because it is more carbon efficient to have a single primary vein supporting a thin layer of lamina (reinforced by reticulate veins than multiple primary veins supporting a thicker layer of lamina without such minor vein reinforcements, thinner leaves should be mechanically supported by a single large midvein and reticulate-veined lamina (Givnish, 1979). Such thin leaves are also found in the shade due to their higher light interception (Givnish, 1988). As a result, reticulation could be associated with variables related to leaf thickness, mechanical strength (force to punch and force to tear) and shaded light environment.

Vein networks also have constructions costs, particularly due to the lignification of the veins (Mooney and Gulmon, 1982). Lignin-rich veins have a higher carbon cost for construction relative to other tissues (Lambers and Poorter, 1992), and also displace photosynthetic tissue, potentially reducing benefits per unit cost. The lignification of veins (or the presence of costly bundle sheath extensions) is variable across species and light levels (Ohtsuka *et al.*, 2018), but in general veins have higher construction costs than other leaf tissues. The contribution of minor veins to leaf construction cost remains controversial, but is potentially large (Blonder *et al.*, 2011; Sack *et al.*, 2013; Carins Murphy *et al.*, 2016; John *et al.*, 2017). Thus, reticulation could be associated with traits related to structural investment, including thickness, leaf mass per area, dry matter content, and lignin concentration.

Lastly, reticulation may also not have functional benefits due to developmental constraints. Veins can complete development before leaf expansion ends (Sack and Scoffoni, 2013), such that cell

expansion coordinates venation patterns (Carins Murphy *et al.*, 2012; Jordan *et al.*, 2013) and potentially also leaf area (Carins Murphy *et al.*, 2016). Thus, variation in leaf form will also drive relationships between leaf shape, size, and reticulation traits. For example, larger leaves may have fewer veins per unit area and potentially fewer loops as well (Sack *et al.*, 2012; Blonder *et al.*, 2013), while more elongated leaves may also cause more elongated areoles (Blonder *et al.*, 2016). Thus reticulation could be associated with leaf area and leaf aspect ratio.

Evolutionary patterns of reticulation in angiosperms

There have been few studies pairing reticulation measurements with these potential predictors, limiting tests of these different functional hypotheses. Previous studies using qualitative metrics of reticulation have focused on its origin across extant basal or extinct plant clades (Alvin and Chaloner, 1970; Hickey and Wolfe, 1975; Takhtajan, 1980; Trivett and Pigg, 1996). Quantitative data exist for a few hundred angiosperm species (Price and Weitz, 2014), while other quantitative data exist for focal subsets of monocots (Givnish *et al.*, 2005; Horn *et al.*, 2009). Similarly, there have been studies focusing on freely ending veinlets, but only for small numbers of species (Fiorin *et al.*, 2016). Other intensive studies of loop topology have primarily examined mathematical questions, e.g. Ronellenfitsch *et al.* (2015).

There also may be constraints on the evolution of reticulation. Qualitative reticulation traits like 'open' and 'closed' venation are often used to assign taxonomic position in plant systematics (Ellis *et al.*, 2009), suggesting strong phylogenetic niche conservatism in network architecture. However a contrasting viewpoint is provided by intraspecific data from *Arabidopsis thaliana,* where small changes in developmental processes or mutations in single genes can yield wide variation in network architecture (Steynen and Schultz, 2003; Kang and Dengler, 2004; Carland *et al.*, 2015). As a result, there remains a key opportunity to better quantify the taxonomic levels and clades in which reticulation shows the most variation.

Present work

The aims of this study are to 1) quantitatively describe variation in reticulation, 2) test multiple ecological hypotheses for functions of reticulation, and 3) examine evolutionary pattern and process in reticulation. We explore several scale-dependent and scale-independent metrics for characterizing reticulation (Table 1), and then determine whether the above hypotheses are related to different functional traits or abiotic variables. Empirical data come from the dominant tree species occurring along a 3300 m humid tropical forest elevation gradient in western Amazonia and the Andes (Blonder *et al.*, 2017).

Materials and methods

Research site and sampling strategy - This study included 10 permanent 1-ha plots in the departments of Cusco and Madre de Dios in southeastern Peru (Malhi *et al.*, 2010; Malhi *et al.*, in review) (Table 2). Six of the plots are montane plots in the Kosñipata Valley of the Andes (elevation range 1500 - 3500 m asl), two are submontane plots located in the Pantiacolla front range of the Andes (range 600 - 900 m asl) and two plots are found in the Amazon lowlands in Tambopata National Park (range 200 - 225 m asl). Seasonal cloud immersion is common above 1500 m asl elevation (Halladay *et al.*, 2012). Plots were established in areas that have relatively homogeneous soil substrates and stand structure, as well as minimal evidence of human disturbance (Girardin *et al.*, 2014). All stems ≥10 cm diameter at breast height were tagged, sized, and identified to species-level.

From April to November 2013, we measured plant traits of biomass-dominant tree species in these plots as part of the CHAMBASA (CHallenging Attempt to Measure Biotic Attributes along the Slopes of the Andes) project. Sampling began at the highest plots in April and moved to the lowest by November. This design may lead to some phenology biases in trait measurements, but was necessary for logistical reasons. Based on data for the most recent census in each plot, we sampled species that most contributed to plot basal area as a proxy for abundance. We aimed to sample the minimum number of species that contributed to 80% of basal area, although in the diverse lowland forest

plots we only were able to sample species comprising 60-70% of plot basal area. Within each species, 3-5 individual trees were chosen for climbing (5 in upland sites and 3 in lowland sites). If 3 trees were not available in the chosen plot, we sampled additional individuals of the same species from an area immediately surrounding the plot. We sampled one sunlit canopy branch and one shaded (defined as occurring beneath other canopy layers) branch where possible, each at least 1 cm diameter, from each tree. From each branch, we measured 5 leaves from simple-leaved species, or 5 individual leaflets from compound-leaved species (both referred to as 'leaf' below) for trait measurements.

All analyses were conducted on the same leaf (including reticulation measurements) except when other analyses were destructive, in which case a replicate leaf from the same branch was used. The full analysis pipeline and detailed methods for each trait are described in Malhi *et al.* (in review). Data from the campaign were accessed from the *GEMTraits* database and R package (Shenkin *et al.*, 2017) and are archived on Dryad (Blonder *et al.*, 2018).

Predictor variables (traits)

We measured a wide range of functional traits to determine their predictive capacity for minor vein reticulation. The following four traits were measured on the same leaf for which reticulation response variables were later measured.

Leaf dry matter content (LDMC; $g\ g^{-1}$) was approximated as dry mass divided by fresh mass. Lamina thickness (mm) was measured at midpoint of lamina avoiding primary and secondary veins with a micrometer (Tresna). Lamina area (cm^2) was calculated as the projected surface area of the whole leaf, including all leaflets if compound, measured at 300 pixels per inch resolution via digital scanner (Canon LiDE 110) and calculated from thresholded images in ImageJ. Leaf mass per area ratio (LMA) ($g\ m^{-2}$) was calculated as leaf dry mass divided by fresh leaf lamina area, not including the petiole or rachis. Light saturated maximum photosynthetic rate per unit area (A_{max}; $\mu mol\ m^{-2}\ s^{-1}$) was obtained with a portable photosynthesis system (LiCor, 6400XT) during morning times under

conditions of ambient humidity, saturating light (1200-1500 µmol m^{-2} s^{-1}), saturating [CO$_2$] (1000-1200 ppm), and ambient site temperature (16-26° interquantile range).

We also measured some traits on a different leaf from a branch of the same tree experiencing a similar light environment, because of the destructive nature of some of these measurements. Force to punch (kN m^{-1}) was measured as the normalized force required to puncture the lamina, measured on fresh leaves with a penetrometer built according to the specifications of Onoda *et al.* (2008), following the measurement guidelines of Pérez-Harguindeguy *et al.* (2013). Force to tear (kN m^{-1}) was measured as the normalized force required to tear the lamina, measured on fresh leaves with an apparatus designed by Hendry and Grime (1993), following the measurement guidelines of Pérez-Harguindeguy *et al.* (2013).

Carbon isotope ratio (δ^{13}C– the ratio of ^{13}C to ^{12}C relative to Vienna PeeDee Belemnite (VPDB) per mil (‰), used as a proxy for water use efficiency (Farquhar *et al.*, 1989)) was measured by continuous-flow gas-ratio mass spectrometry. Samples were combusted using an elemental analyzer (Costech) coupled to the mass spectrometer (Finnigan Delta PlusXL). Standardization is based on acetanilide for elemental concentration, NBS-22 and USGS-24 for δ^{13}C.

Phenol and condensed tannin concentrations (mg g^{-1} - dimensionless) were determined as the per- dry mass concentration of phenol-containing compounds of molecular weight < 500 and > 500, respectively. Branches of mature leaves were sealed in polyethylene bags in the field to maintain moisture, stored on ice in coolers, and transported to a local site for processing within 3 h. Small leaf discs (at least 30 per leaf) were immediately taken from approximately 6-12 randomly selected and cleaned leaves and transferred to -80°C cryogenic shipping containers. Frozen leaf disks were ground in 95% methanol on a high throughput tissue homogenizer. A portion of the solution was further diluted and incubated on an orbital shaker at room temperature (15-18°C) in the dark for 48 h to ensure proper phenol extraction. A second portion of the solution was further diluted in a 2 ml centrifuge tube containing 10 mg Polyvinylpyrrolidone (PVP) and incubated on ice for 30 min after vortexing. Following centrifugation, 75% of the supernatant was placed in a new centrifuge tube

containing another 10 mg PVP for a second precipitation step. The total phenol concentration in solution was determined colorimetrically using the Folin-Ciocalteu method. Phenol concentrations were measured in Gallic Acid Equivalents (GAE) relative to an eight-point Gallic acid standard curve. The tannin concentration was determined as the difference between total phenols and phenols in solution after PVP precipitation. Methods are described fully by Makkar *et al.* (1993); Ainsworth and Gillespie (2007).

Lignin concentration (100 x g g^{-1} – dimensionless) was determined as the per- dry mass concentration of fibers resistant to strong acids, determined in 0.5 g dry ground leaf tissue through using sequential digestion of increasing acidity in a fiber analyzer (Ankom Technology, Macedon NY). Methods follow Van Soest (1994).

Leaf aspect ratio (dimensionless) was calculated as leaf lamina length (not including petiole or rachis) divided by maximum leaf width, measured from a digital photograph in ImageJ. In the case of compound leaves, leaflets were used; in the case of palmate simple leaves, width was measured as lobe width.

Predictor variables (climate) – Climate data were obtained using quality-controlled and gap-filled data from weather stations co-located with plots. Data were obtained from site-specific papers on TAM05 and TAM06 (Malhi *et al.*, 2010), SPD01 and SPD02 (Huaraca Huasco *et al.*, 2014), TRU04 (Girardin *et al.*, 2013), ESP01 and WAY01 (Girardin CAJ, 2014), and ACJ01 (Oliveras *et al.*, 2014), while climate data from PAN02 and PAN03 were collected in 2013-2014 and were processed using the same techniques described for the other sites. Data from ACJ01, PAN02, PAN03, and TRU04 were available since 2012 or 2013 and since 2009 for all others. Mean annual temperature (MAT; °C) was calculated as the mean of daily average temperatures. Annual precipitation (mm yr^{-1}) was measured by weather station. Values are reported in Table 2.

Response variables (vein traits) - We chemically cleared, imaged, and hand-traced a leaf venation network for one leaf from a sampled branch a sunlit and (where possible) shaded light microenvironment from each tree, following standard protocol for dried leaves (Pérez-Harguindeguy *et al.*, 2013). Images were made at a spatial resolution of 179 pixels mm^{-1} and avoided major veins whenever possible. We traced an average of 36 ± 23 mm^2 of leaf area in order to capture a large number of areoles in each image (n=306 ± 305 s.d.). These images and tracing methods are fully described in Blonder *et al.* (2017). This large image extent was necessary to robustly estimate reticulation traits, and therefore necessarily included a small number of non-minor veins. We treated these equivalently to minor veins in tracing, as they often constituted integral boundaries of minor vein areoles, whose properties were of interest in this study.

We then calculated venation reticulation traits (Table 1) from these tracings, using MATLAB code available at https://github.com/bblonder/venation_programs/. Areoles that did not appear closed (i.e. partially lying outside the image) were not included in the analysis. We also added 1.0 to values of meshedness to make all values positive to permit log transformation in analyses. We corrected the two dimensional reticulation traits (vein density, vein loopiness) for bias due to shrinkage of dried leaf samples. Shrinkage corrections were not required for dimensionless traits. We estimated the fraction of leaf area that was lost upon drying (S; dimensionless). We calibrated a regression model for shrinkage using existing data for temperate and tropical species provided by Blonder *et al.* (2012) using both LMA and mean annual temperature (MAT) as predictors available in this dataset. The fitted model had coefficients S = $0.3496 - 0.0005162$ LMA $- 0.008316$ MAT (R^2=0.36, df=258, RMSE=0.12). We applied this model to each leaf in the current dataset to estimate S, which varied around a mean of 0.15 ± 0.04 s.d. We used these values to rescale vein density and loopiness values (with units mm^{-1} or mm^{-2} respectively) by a factor of $\sqrt{(1-S)}$ or $(1-S)$, following Blonder *et al.* (2017).

Phylogenetic analysis

To examine evolutionary patterns of reticulation, we built a phylogenetic tree for all species for which reticulation measurements were available, using the *phylomatic* function in phylocom 4.2 (Blonder *et al.*, 2017). Clades were assigned using the 'R20120829' plant megatree. Approximate crown ages were assigned via phylocom's *bladj* function, with constraints for internal nodes provided by Gastauer and Meira-Neto (2013).

Statistical analysis

We conducted all analyses in the R statistical environment (http://www.r-project.org). We conducted analyses for all angiosperms, and also for all angiosperms excluding palms (Arecaceae), because of the divergent morphology of this family.

To determine the overall reticulation trait space, we log-transformed and scaled all reticulation traits, then made a principal components analysis. Based on the variance explained by each principal component, we then used the scores along the leading components as orthogonal reticulation axes (PC1, PC2, and PC3). These transformed variables and scores also were used for all subsequent analyses.

To examine evidence for phylogenetic niche conservatism, we first performed a variance partitioning analysis across taxonomic scales using standard mixed modeling methods (Messier *et al.*, 2010). We also carried out an analysis of Blomberg's K, a metric of phylogenetic signal (Blomberg *et al.*, 2003), within the *phytools* R package. All other phylogenetic analyses were made with the *ape* package. To examine evidence for directional shifts in reticulation traits over evolutionary time, we also performed a regression analysis of family age and family-mean values of reticulation principal components. To examine variation in reticulation due to light microenvironment, we determined whether the difference between mean sun and shade leaf values, calculated for each species-site combination, was significantly different from zero according to a t-test.

To separate the independent effects of environment and traits on the reticulation trait space, we analyzed a series of Gaussian-family generalized linear mixed models within a Bayesian framework for each reticulation principal component. This approach allows for hierarchical grouping of predictor data and phylogenetic non-independence of data.

Before carrying out this analysis, we gap-filled the predictor data. Only 2.3% of the data were missing across all variables (Fig. S1), but 32.3% of cases had at least one missing observation of at least one predictor. As deleting all incomplete observations would have vastly reduced the sample size and also biased the analysis, we gap-filled missing values in the matrix of predictor variables. We used multiple imputation via chained equations with predictive mean matching using the 'mice' R package, which uses the distribution of observed data to predict the values of unobserved data. When data are missing at random, as is true here, this approach is asymptotically unbiased and asymptotically efficient (Buuren and Groothuis-Oudshoorn, 2011). We generated 10 independent imputations of the full dataset to account for uncertainty in the observed data. We then log-transformed a subset of highly skewed predictor variables: (LMA, lamina area, thickness, force to puncture, force to tear, leaf aspect ratio), and then re-centered and re-scaled all predictor variables to put them on common scales.

Mixed models were constructed, treating all of the trait and climate predictors as independent fixed effects, because collinearity among them was low (mean Pearson's rho among all predictor variables, 0.04 ± 0.24 s.d.). These models incorporated a random effect of site (capturing nesting of climate measurements within sites) as a diagonal variance matrix and a random effect of species (capturing the nesting of multiple branches for each species). We also incorporated a random effect of evolutionary distance (capturing the relatedness of species) using an inverted phylogenetic covariance matrix. Prior distributions for each effect were chosen as inverse-gamma with location and scale parameters set to 0.01 (Gelman, 2006). Models were implemented using a Markov-chain Monte Carlo algorithm in the *MCMCglmm* R package (Hadfield, 2010). Each model was run for a burn-in period of 100,000 iterations, after which posterior distributions were characterized by taking 1,000 samples, each 100 iterations apart in the chain. Chain mixing was sufficient in all cases. We then

repeated the modeling exercise for each imputation of the dataset, and generated final posterior distributions by concatenating posterior samples across all models. To facilitate interpretation, approximate p-values for each fixed effect a were calculated as $p=2\times\min[P(a>0), P(a<0)]$, with statistical significance inferred at $p<0.05$.

Because predictor variables were rescaled before analysis, the distribution of these fixed effects can be interpreted as effect sizes (s.d. s.d.$^{-1}$). That is, a coefficient with value +1 indicates that a 1 standard deviation increase in the predictor variable directly causes a 1 standard deviation increase in the response variable, after accounting for any other predictor variable, and for any effects of shared evolutionary history among species, or of hierarchical structure in the data.

Results

We obtained reticulation traits and paired predictor variables for 849 branches from 136 angiosperm species (Table 2), of which 12 were from palm taxa. Most species were sampled only at a single site, with only eight species sampled in more than two plots (Blonder *et al.*, 2017).

Reticulation trait space

We found wide variation in every reticulation trait, with variation spanning up to two orders of magnitude across all taxa (Table 3). Two of the most variable traits were vein loopiness (90% quantile range, 0.8 - 54.1 mm^{-2}) and freely ending veinlet ratio (90% quantile range, 0.086 - 0.43). Removing palms from this analysis had a negligible effect on trait ranges (Table S1).

A small number of orthogonal axes characterized these reticulation traits (Fig. 2). Three principal components (PC1-PC3) had variance fractions above 10%; these components explained 65.7%, 18.8%, and 10.9% of the variation in the data respectively (additional components explained only <2% each) (Fig. 1). Higher values along PC1 described more branching and less reconnecting

networks, with higher freely ending veinlet ratios and minimum spanning tree ratios. Higher values along PC2 described more circular and less elongated areoles. Higher values along PC3 described higher vein density and higher loopiness, as well as lower meshedness. When palms were excluded from this analysis, the overall shape of the trait space and variance fractions were largely unchanged (Fig. S2).

Patterns within clades

Species were not uniformly distributed within the reticulation trait space. While species were evenly spread along PC1 (skewness 0.65) and PC3 (skewness 0.28), variation along PC2 was uneven (skewness -5.9). PC2 was characterized by a small number of species with very high areole elongation indices, generally in Arecaceae (palms). Individual families sometimes occupied distinct subsets of the overall reticulation trait space, e.g. high loopiness within the Urticaceae, and high freely ending veinlet ratio within the Clusiaceae (Fig. 3).

Reticulation traits showed strong variation in their level of phylogenetic niche conservatism (Fig. 4A). A variance partitioning analysis indicated that some traits showed more than 50% of variation summed at the order or family level (areole elongation ratio, areole circularity ratio, vein density, vein loopiness, and PC2). In contrast, other traits traits showed more than 30% of variation summed at the interspecific or intraspecific level (meshedness ratio, PC3, minimum spanning tree ratio, freely ending veinlet ratio, areole loop index). Thus, some aspects of the network are evolutionary labile (those primarily relating to branching patterns), while others are not (those primarily related to areole shape and scale). An analysis of phylogenetic signal showed that PC1 had a value of Blomberg's $K=0.92$; PC2, $K=1.2$; PC3, $K=0.44$. Thus PC2, reflective of areole shape, showed conservatism ($K>1$) while PC1 and PC3, reflective of branching and scale, showed lability ($K<1$). However, some of the phylogenetic signal in these patterns was due to the inclusion of palms. When these taxa were removed from the analysis, nearly all of the order- and family-scale variation in PC2, areole elongation ratio, and areole circularity index was instead partitioned at genus scale (Fig.

S3). All other patterns remained consistent. Additionally, traits were observed to be much more evolutionary labile in non-palm taxa. PC1 had K=0.59; PC2, K=0.44; PC3, K=0.52. Patterns of trait clustering are apparent on a phylogenetic tree showing species-mean trait values (Fig. 4B).

We found that families with younger crown ages had lower values of PC1, and higher values of PC3, which was also reflected in higher values of areole circularity ratio, areole loop index, vein density, and loopiness (Fig. S4). However, variation in reticulation traits within families was high, leading to a weak overall trend for both all angiosperms and non-palm taxa.

Patterns across environment

There were no effects of light microenvironment on reticulation principal components, except for PC3 (p=0.049) (Fig. 5). Most individual reticulation traits showed a similar absence of pattern, with the exception of the two scale-dependent traits (vein density and vein loopiness) and areole loop index, for which sun leaves showed slightly higher trait values. When restricted to only non-palm taxa, results were largely consistent (Fig. S5). Thus, scale-independent reticulation traits appear to be invariant to light environment, while scale-dependent traits were weakly dependent.

There were strong direct relationships between reticulation traits and elevation when median values were analyzed at plot level. Higher elevation plots were associated with higher PC1 and PC2, and lower PC3 (all $R^2 > 0.46$), which was in turn driven by associations with lower areole loop index, higher minimum spanning tree ratio, higher freely ending veinlet ratio, lower vein density, and lower vein loopiness (all $R^2 > 0.58$) (Fig. 6). As such, there was a shift towards leaves with more branched veins, more elongated areoles, and lower vein densities at high elevations. These results were largely invariant when palms were excluded from the analysis (Fig. S6).

Predictors of reticulation

To better interpret these strong elevation and phylogenetic patterns, we used a generalized linear mixed model to interpret the climate and trait predictors of reticulation. After accounting for phylogenetic correlation structure, we found that different sets of predictors were associated with each reticulation axis (Fig. 7). Higher values of PC1 were associated with lower values of lignins, phenols, and LDMC, consistent with higher reticulation offsetting investment in secondary defense compounds and overall leaf structure. Higher values of PC2 were associated with lower values of force to punch, also consistent with higher reticulation offsetting investments in mechanical strength. Higher values of PC3 were associated with higher mean annual temperature, consistent with transpiration supply-demand matching. No other predictors were significantly associated with any reticulation axis. When restricted to non-palm taxa, results were qualitatively unchanged, except that higher values of PC2 were also associated with lower tannins (Fig. S7).

Discussion

While a large body of work has identified functions for leaf venation related to hydraulics (Brodribb *et al.*, 2010; de Boer *et al.*, 2012; Sack and Scoffoni, 2013; Brodribb *et al.*, 2016), our results also suggest a key role for structural and defensive functions of venation reticulation (Sack and Scoffoni, 2013). We found that PC1 (branching vs. reconnecting veins) reflected lower investment in defense compounds and PC2 (elongated vs. compact areoles) reflected lower investment in structure. In contrast, PC3 (high vs. low density veins) reflected higher site temperature, consistent with hydraulic functions. Indeed, an earlier study along this elevation gradient showed that vein density does respond strongly to temperature (R^2=0.5 at community-mean level) (Blonder *et al.*, 2017). However, the present study has now demonstrated that this finding is only a partial characterization for the phenotypic variation occurring along this elevation gradient. Other non-hydraulic aspects of network architecture, and consequently other non-climate predictors, play a more important role, and also have very strong community-weighted mean trait-environment relationships (e.g. R^2=0.7 for PC1 vs.

elevation). A more complete understanding of network architecture may require parsing tradeoffs amongst these multiple functions (de Boer *et al.*, 2016), i.e. the amount of hydraulic performance that can be sacrificed to enhance structural or defense performance.

There were only weak differences in traits between sunlit and shaded environments, especially for the scale independent traits. It is possible that this pattern was driven by low within-species replication (an average of 3.8 ± 2.8 s.d. sun and 2.8 ± 2.1 s.d. shade branches per species). However, the lack of effects of consistent directionality suggests that the absence of a pattern is biologically real. One possible explanation is that vein patterning is set early during leaf development, such that only scale-dependent traits can vary plastically via variation in cell expansion. This perspective is consistent with extant knowledge for vascular development and leaf cellular physiology (Sack and Scoffoni, 2013). However, we did not find consistent support for a link between leaf elongation and areole elongation in this dataset, which would also be expected under such a cellular expansion process. This pattern has only been shown in a small clade of Hawaiian species (Blonder *et al.*, 2016). It may be that leaf size and shape and venation patterns can become decoupled via multiple developmental processes.

Additionally, we found no evidence that higher reticulation leads to lower maximum per-mass photosynthesis rates, as might have been expected by an efficiency vs. redundancy tradeoff (Corson, 2010; Katifori *et al.*, 2010). This result probably arises because the increase in hydraulic conductance from increasing minor vein density is larger than the increase in cost from increasing the number of redundant veins and (thus reticulation traits like loopiness). It may be that the costs of redundant veins become more important only when considering larger-scale loops in the network, e.g. in primary and secondary veins.

Herbivory may also directly influence reticulation traits. Previous work has suggested that reticulation can enhance damage resistance. Leaf venation patterns have been demonstrated to influence leaf mechanical strength (Roth-Nebelsick *et al.*, 2001; Zhang *et al.*, 2004; Enrico *et al.*, 2016), which in turn are negatively related to herbivory (Coley, 1983; Nichols-Orians and Schultz,

1990; Pérez-Harguindeguy *et al.*, 2003; Grubb *et al.*, 2008). Additionally, reticulation can enhance damage resilience, as demonstrated by experimental wounding of species with or without loops in their veins (Katifori *et al.*, 2010). However, direct measurements of herbivore attack and success rates would be needed to determine whether reticulation axes are directly linked to performance in contemporary biotic contexts.

Quantifying variation in reticulation

Our results also help clarify the dimensionality of reticulation trait space. We found that three axes captured more than 95% of the variation for the 50 families on this Andes-Amazon transect. These axes also have a clear interpretation: the first described a continuum of scale-independent branching, the second axis a continuum of scale-independent areole compactness, and the third axis the scale-dependent density of the network. While the importance of network scale and minor vein density (PC3) in plant functioning and community assembly has been widely acknowledged (e.g. Sack and Scoffoni (2013)), we found that scale-independent axes of branching / reconnecting veins (PC1) and elongated / compact areoles (PC2), showed more variation in this dataset. Palms, which are dominant in several sites, occupy an extreme set of values along these axes. However the exclusion of palms did not qualitatively change our findings except those based on evolutionary position.

Variation generally was phylogenetically conserved at the genus level and above, but also varied consistently at community scale along the elevation gradient. This suggests that reticulation traits drive species sorting into local communities across environments, and/or that historical biogeographic processes have led to the radiation of clades with certain reticulation traits only in some regions.

We also found only limited evidence for directional shifts in reticulation traits over evolutionary time, as most family-age vs. reticulation trait relationships were not statistically significant. While increases in vein density have been observed in more recently derived clades

within the angiosperms (Boyce *et al.*, 2009a), reticulation metrics showed weak patterns. Thus it appears that high values of reticulation traits are possible in both new and old angiosperm clades.

The strong niche conservatism of reticulation is surprising, given the apparent ease of evolution in these traits. The transition between purely branching and reconnecting venation is apparently not difficult (Takhtajan, 1980) and has occurred independently in both directions many times over macroevolutionary time (Wagner, 1979). Rapid transitions in reticulation are seen via qualitative metrics within the monocots (Givnish *et al.*, 2005) and with quantitative traits within the Hawaiian silversword alliance (Asteraceae) (Carlquist, 1959; Blonder *et al.*, 2016). Transitions may occur quickly because reticulation can be under simple genetic control. In *Arabidopsis thaliana,* knockouts of single genes are sufficient to shift from a wildtype reticulate pattern to an open pattern (Steynen and Schultz, 2003; Carland and Nelson, 2004). Similarly, studies of vascular network formation in the same species show that small developmental changes in auxin gradients and canalization can lead to dramatically different reticulation trait values (Berleth *et al.*, 2000). We do not yet understand how to reconcile these observations with this study.

There are likely to be additional axes required to fully characterize reticulation trait space. The traits we surveyed are focused only on the minor veins, and do not contain information related to branching angles and diameters, or to the nesting of loops across vein orders, which are also important components of transportation network architecture. Loops in secondary or tertiary veins may show different patterns than the minor veins. Potential benefits include reinforcement of leaf edges against tearing (Niklas, 1999), or tolerance of large-scale hydraulic failure (Sack *et al.*, 2008). Potential carbon costs arise because of the large mass investment in major veins (John *et al.*, 2017). Such variation could be measured via recently proposed hierarchical metrics that quantify how areoles are nested within other areoles (Katifori and Magnasco, 2012; Mileyko *et al.*, 2012; Ronellenfitsch *et al.*, 2015).

There have been many hypotheses for the functions of leaf venation (Sack and Scoffoni, 2013). This study now demonstrates that multiple hypotheses are supported: our data shows that

venation can have structural and defensive functions, as well as hydraulic functions. Thus, patterns of reticulation may indicate the outcome of selection for multiple functions. However, patterns of reticulation still remain unmeasured for the vast majority of plant taxa, despite the importance of venation networks in mediating plant carbon uptake and water loss worldwide. Systematic studies of reticulation across the evolutionary tree will further unravel the drivers and consequences of leaf venation network architecture.

the Carnegie Institution for Science and a grant from the National Science Foundation (DEB-1146206). Y.M. was also supported by the Jackson Foundation. S.D. and L.E. were supported by the Leverhulme Trust (UK) the Inter-American Institute for Global Change Research, and FONCyT and CONICET (Argentina).

Author contributions

Y.M. conceived and received funding for the field campaign. N.S., L.P.B., A.S., and T.E.B.E. led the implementation of the field campaign. G.P.A., S.D., B.J.E., and Y.M. designed the field campaign. B.B., N.S., Y.V.T., and P.O.C.P. contributed venation analyses. B.B. led the statistical analysis and writing. All authors contributed to generating other datasets, to writing, and to fieldwork.

Data accessibility

Data are deposited in the Dryad repository: https://doi.org/10.5061/dryad.33bf108 (Blonder et al., 2018). Venation network images are archived at http://www.clearedleavesdb.org/ (collection 'Kosñipata Valley (CHAMBASA elevation transect)') and can be searched using the branch and tree codes in the data file. Voucher specimens are viewable at http://spectranomics.ciw.edu/ using the 'Carnegie codes' in the data file.

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Table 1. Summary of quantitative reticulation traits for the minor veins. Some traits are dimensional, i.e. scale-dependent, while others are dimensionless, i.e. scale-independent ratios. Example references that either defined or measured each metric are also given.

Type	Trait name	Definition	Higher values indicate	Example reference
Scale-dependent	Vein density	Length of veins per unit leaf area	More veins	(Brodribb *et al.*, 2007)
Scale-dependent	Loopiness	Number of areoles per unit lamina area	More looping	(Blonder *et al.*, 2011)
Scale-independent	Areole loop index	Number of areoles per unit lamina area per squared vein density	More redundant pathways per vein	(Blonder *et al.*, 2013)
Scale-independent	Areole circularity ratio	Median of the distribution across areoles of 4π times the area divided by squared perimeter	Areoles with less in-folding	(Friel, 2000)
Scale-independent	Areole elongation ratio	Median of the distribution across areoles of major axis length to minor axis length	Longer, more 'stretched' areoles	(Blonder *et al.*, 2016)
Scale-independent	Minimum spanning tree ratio	Summed length of the minimum spanning tree connecting all vein segments divided by the summed length of all vein segments. The minimum spanning tree is defined as a subset of vein segments that maintains connectivity between all vein intersections but has less or no more total length than any other subset of vein segments.	More tree-like pathways	(Price and Weitz, 2014)
Scale-independent	Freely ending veinlet ratio	Number of vein segments that are only connected to other vein segments at one end divided by number of all vein segments	More non-redundant pathways per vein	(Kang and Dengler, 2004)
Scale-independent	Meshedness ratio	Number of loops in the network divided by the maximum possible number of loops given the number of vein intersections	Less tree-like pathways	(Price and Weitz, 2014)

Table 2. Summary of angiosperm branch collections at each site along the elevation gradient.

Plot code	Latitude (°)	Longitude (°)	Elevation (m)	Mean annual temperature (°C)	Mean annual precipitation (mm)	# Species Sampled	# Families Sampled	# Branches Sampled
TAM-06	-12.8385	-69.2960	215	24.4	1900	22	13	82
TAM-05	-12.8309	-69.2705	223	24.4	1900	25	16	91
PAN-02	-12.6495	-71.2626	595	23	2366	12	10	55
PAN-03	-12.6383	-71.2744	859	21.9	2835	14	10	59
SPD-02	-13.0491	-71.5365	1494	18.8	5302	23	17	92
SPD-01	-13.0475	-71.5423	1713	17.4	5302	26	21	114
TRU-04	-13.1055	-71.5893	2719	13.5	2318	15	11	95
ESP-01	-13.1751	-71.5948	2868	13.1	1560	15	11	107
WAY-01	-13.1908	-71.5874	3045	11.8	1560	13	9	78
ACJ-01	-13.14689	-71.6323	3537	9.0	1980	10	9	76

Table 3. Ranges of variation for all unscaled reticulation traits and ecological predictors.

Type	Variable	Units	5% Quantile	50% Quantile	95% Quantile
Predictor (climate)	Light environment	-	Shade	Sun	Sun
Predictor (climate)	Mean annual precipitation	mm	1560	1980	5302
Predictor (climate)	Mean annual temperature	°C	9	17.4	24.4
Predictor (trait)	Amax	nmol g s^{-1}	0.849	7.607	18.94
Predictor (trait)	δ ^{13}C	per mil	-33.4	-30.3	-26.83
Predictor (trait)	Force to punch	kN m^{-1}	0.295	0.875	2.51
Predictor (trait)	Force to tear	kN m^{-1}	0.379	0.722	4.88
Predictor (trait)	Lamina area	cm^2	9.354	58.224	632.01
Predictor (trait)	Lamina thickness	mm	0.141	0.264	0.61
Predictor (trait)	LDMC	-	0.24	0.425	0.69
Predictor (trait)	Leaf aspect ratio	-	1.626	2.456	4.21
Predictor (trait)	Lignin	mg g^{-1}	9.064	23.76	39.55
Predictor (trait)	LMA	g m^{-2}	64.39	106.9	189.11
Predictor (trait)	Phenols	mg g^{-1}	0	88.145	166.01
Predictor (trait)	Tannins	100 g g^{-1}	0	38.52	83.55
Response (trait)	Areole circularity ratio	-	0.623	0.754	0.86
Response (trait)	Areole elongation ratio	-	1.373	1.502	1.77
Response (trait)	Areole loop index	-	0.023	0.074	0.15
Response (trait)	Freely ending veinlet ratio	-	0.086	0.212	0.43
Response (trait)	Meshedness ratio	-	-0.019	0.051	0.11
Response (trait)	Minimum spanning tree ratio	-	0.603	0.68	0.86
Response (trait)	Vein density	mm^{-1}	5.342	11.221	18.69
Response (trait)	Vein loopiness	mm^{-2}	0.882	9.154	54.06

Figure 1. Minor vein reticulation trait space, as seen via principal components analysis of reticulation traits for the angiosperms. **A, B)** Biplot of variable rotations 1 vs. 2 and 2 vs. 3. Individual leaves are shown as gray dots; axes as red arrows, with the overall trait space boundaries delineated via a purple convex hull. Labels indicate % variance explained by each axis. **C, D)** Examples of venation networks corresponding to the same biplots. Grid cells show a randomly chosen venation network from a leaf with principal component scores within the grid ranges. The size of each image is 2.79 x 2.79 mm.
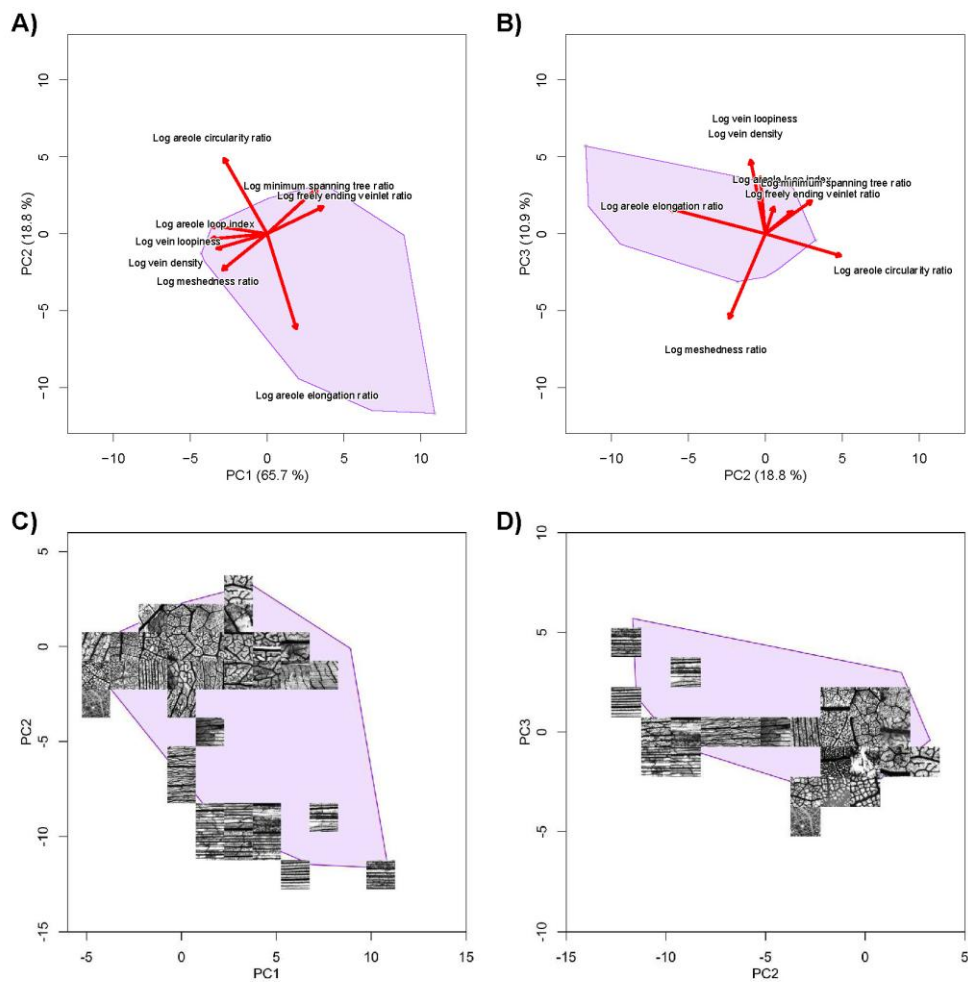
Figure 2. Illustration of major axes of variation in reticulation traits along low (left) and high (right) values of principal component axes. **A)** Higher values of PC1 indicate networks with more branching for a given amount of reconnecting. **B)** Higher values of PC2 indicate networks with more elongated areoles. **C)** Higher values of PC3 indicate networks with higher vein density.
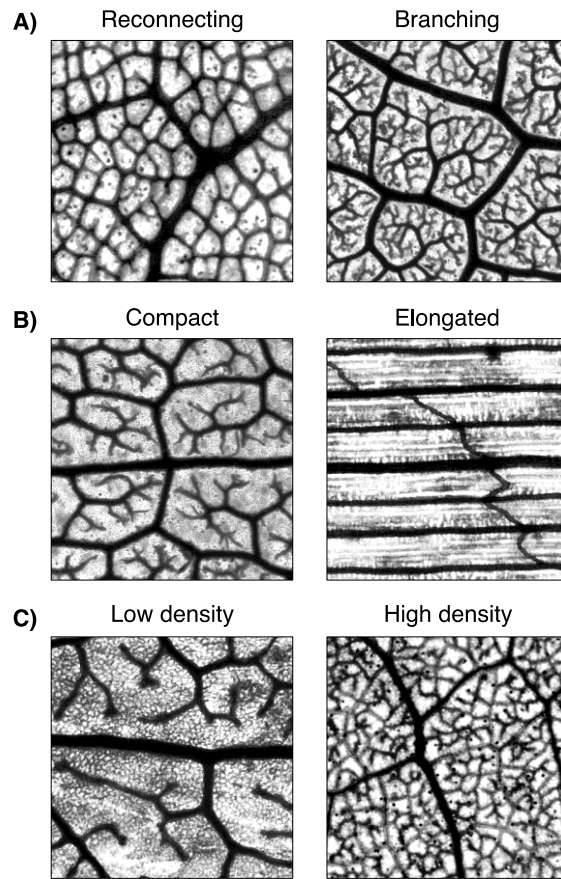
Figure 3. Variation in reticulation traits, grouped by family. The overall reticulation trait space for the first two principal components is shown in purple. Each family's distribution is shown via black points indicating individual leaves and red point their centroid and with red convex polygon outline.
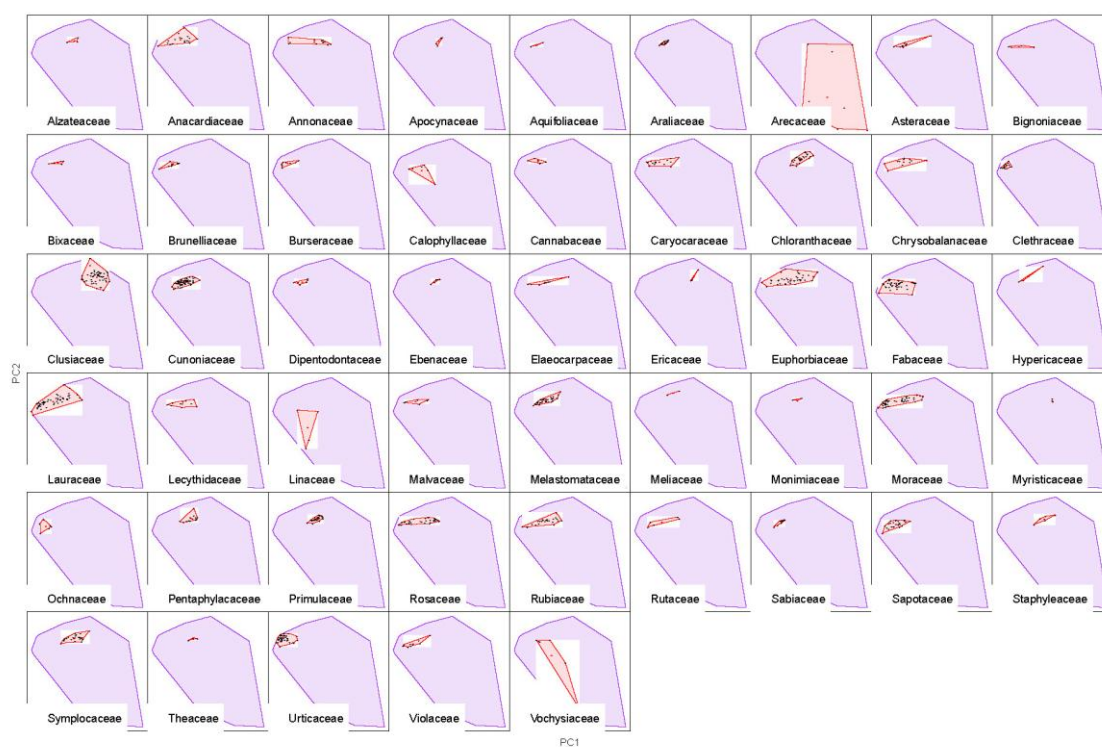
Figure 4. A) Hierarchical variance partitioning of reticulation traits among taxonomic scales. **B)** Distribution of reticulation principal components across the phylogenetic tree. Tips are labeled with circles whose color indicates mean values for PC1, PC2, and PC3.
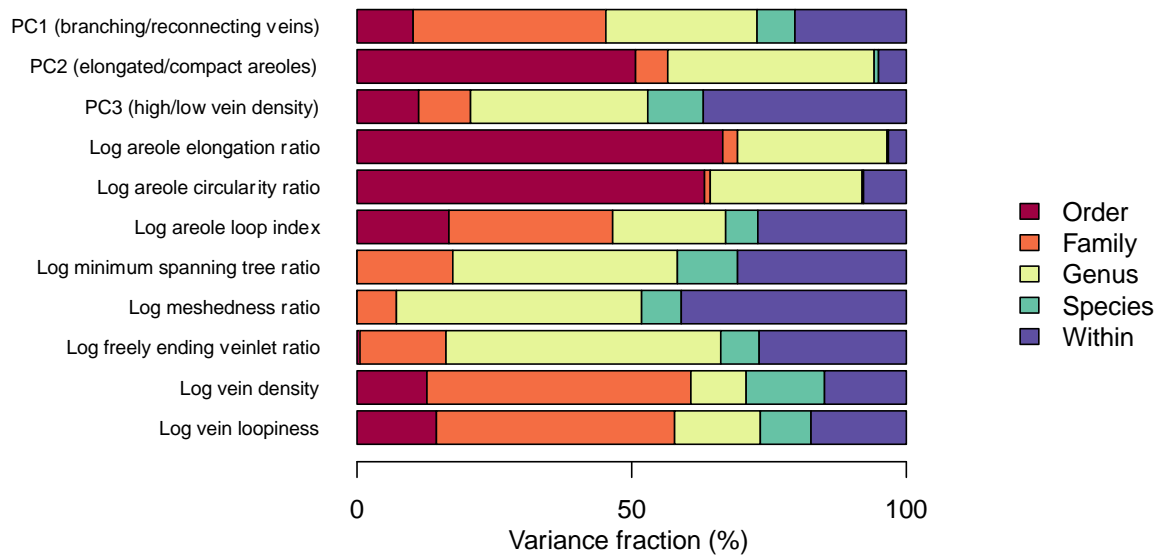
Figure 5. Effect of light availability on reticulation traits. Purple lines indicate the distribution of sun mean minus shade mean values within each species, across all species-sites combinations for the whole dataset. The null expectation of zero is shown as a black line. The mean of the observed distribution is shown as a solid red vertical line if significantly different from zero and dashed gray if not.
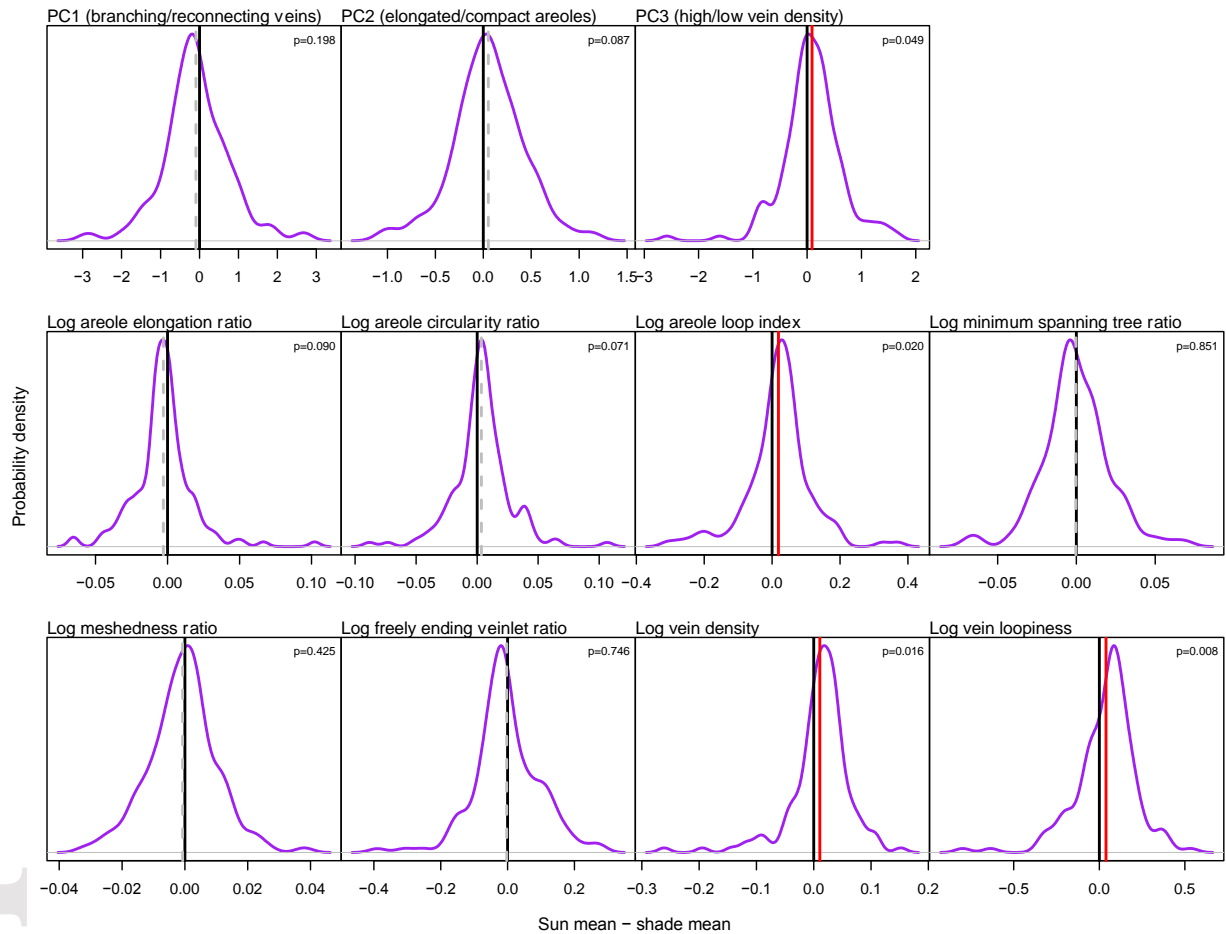
Figure 6. Elevation trends in principal component and individual reticulation traits for the entire dataset. Gray points represent individual leaves; open purple circle site medians. Regression lines (red) for site-median data are drawn when statistically significant.
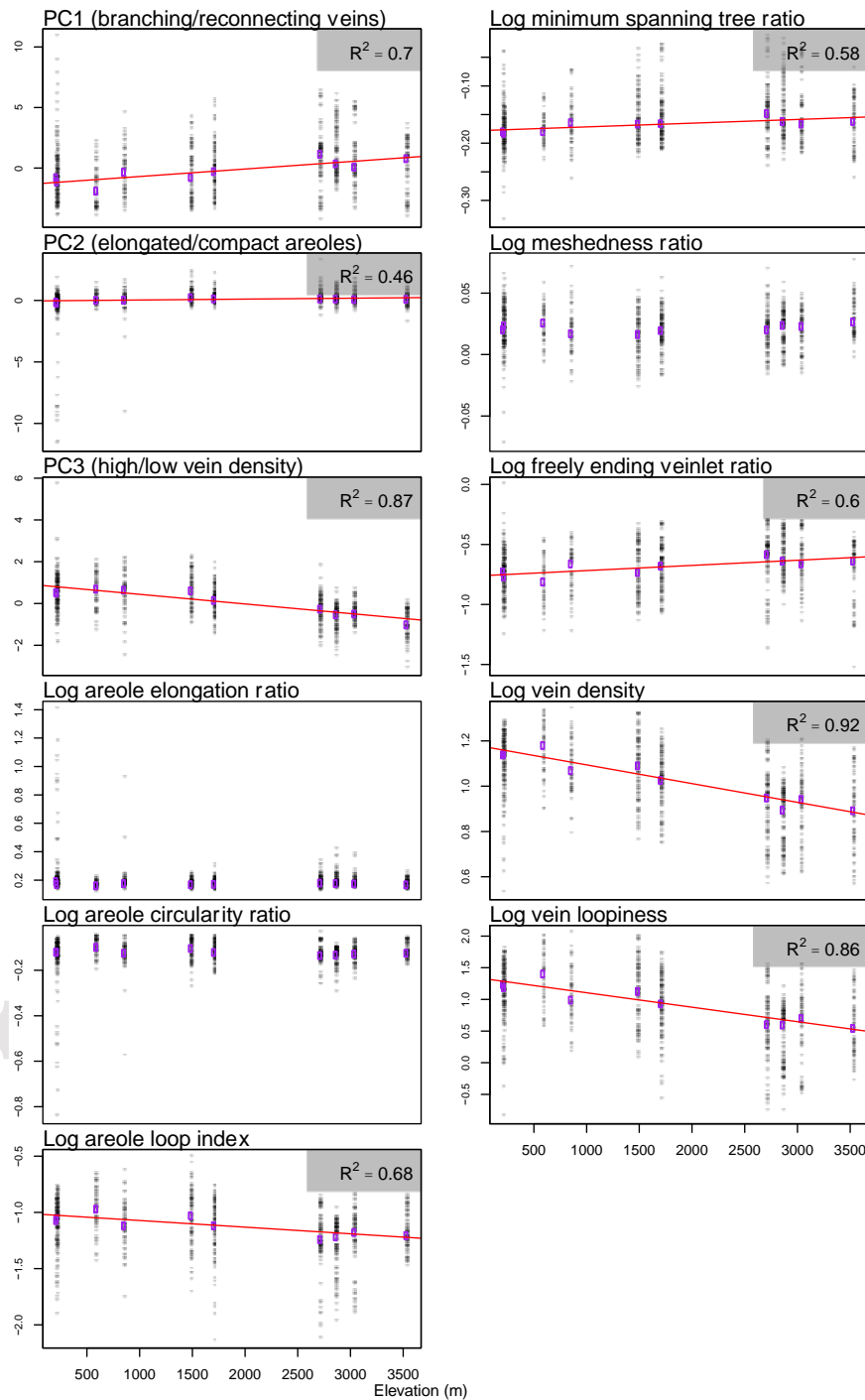
Figure 7. Relationships between reticulation trait axes and multiple trait and climate predictor variables. Boxes indicate the posterior distribution of fixed effects for Bayesian GLMM models. The analysis is scaled such that a value of +1 indicates that a 1 standard deviation change in a predictor results in a 1 standard deviation change in the reticulation principal component, after accounting for all phylogenetic effects and hierarchical structure in the data. Distributions are concatenated across ten resampled imputations of the dataset. Significant fixed effects are highlighted in dark blue and otherwise shown in light gray. Estimates are shown for **A)** PC1, **B)** PC2, and **C)** PC3.