# From one side to two sides: the effects of stomatal distribution on photosynthesis 

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#### Abstract

- The functions of stomata have been studied for a long time; however, a clear understanding of the influences of stomatal distribution on photosynthesis, especially the $\mathrm{CO}_{2}$ diffusion, is still unclear. - Here, we investigated the stomatal morphology, distribution on leaf surfaces, vein traits and gas exchange parameters of 61 species, of which 29 were amphistomatous, spanning 32 families. - Photosynthesis (A) was tightly coupled with operational stomatal conductance ( $g_{s}$ ) and mesophyll conductance $\left(g_{m}\right)$ regardless of whether phylogenetic relationships were accounted for. Although the enhancement of $g_{s}$ from ferns and gymnosperms to angiosperms could largely be explained by the increase in leaf vein density (VLA) and stomatal density (SD), the $g_{s}$ was decoupled from VLA and SD across angiosperm species. Instead, A in angiosperms was further influenced by the allocation of stomatal pores on leaf surfaces, which dramatically increased $g_{s}$ and $g_{m}$. Moreover, the ratio of $g_{s}$ to anatomically based maximum $g_{s}$ was, on average, 0.12 across species. - Our results show that the shift of stomatal pores from one leaf side to both sides played an important role in regulating $\mathrm{CO}_{2}$ diffusion via both stomata and mesophyll tissues. Modifications of stomata distribution have potential as a functional trait for photosynthesis improvement.


## Introduction

A fundamental challenge for land plants is to balance the benefit of $\mathrm{CO}_{2}$ uptake with the risk of desiccation resulting from concomitant transpirational water loss (de Boer et al., 2016a; Leakey et al., 2019). The capacity for exchanging $\mathrm{CO}_{2}$ or water vapor via stomata is quantified as stomatal conductance $\left(g_{s}\right)$, which is typically normalized by leaf area. In theory, $g_{s}$ is determined by stomatal density (SD) and the diffusion efficiency of single stomatal pores. In mature leaves, the stomatal anatomical traits are nearly fixed, and leaves regulate stomatal aperture to achieve a real-time $g_{\mathrm{s}}$ over a range between near-zero and anatomically-determined maximum stomatal conductance $\left(g_{\text {smax }}\right)$. In practice, $g_{\text {smax }}$ is calculated using SD and anatomical parameters of stomatal pores, and a high $g_{\text {smax }}$ has been suggested to be necessarily linked with a small stomatal size (SS) and a large SD (Franks \& Farquhar, 2001; Franks \& Beerling, 2009; Sack \& Buckley, 2016). Denser and smaller stomata in angiosperms than in ferns and gymnosperms have been observed in previous studies, indicating that angiosperms have evolved to contain many small stomata per leaf surface rather than a few large ones (Franks \& Beerling, 2009; McElwain et al., 2016; Xiong et al., 2018). However, the lack of
a significant relationship between stomatal characteristics (i.e. SD and SS) and operational $g_{s}$ across angiosperms suggests that other unknown traits might be involved in regulating $g_{s}$ (Russo et al., 2010; McElwain et al., 2016).

Beyond the density and size of stomata, the impacts of stomatal distribution on $g_{s}$ have been suggested by several recent studies (de Boer et al., 2016b; Muir, 2018; Drake et al., 2019). Species with an allocation of stomata on both the abaxial and adaxial leaf surfaces (amphistomatous species) tend to have a higher gas exchange capacity than species with stomata on only one side (hypostomatous species; Mott \& O’Leary, 1984; Beerling \& Kelly, 1996). Simulation results suggest that the high gas exchange capacity of amphistomatous plants may result from of the shortened $\mathrm{CO}_{2}$ diffusion pathway across the mesophyll (i.e. larger mesophyll conductance, $g_{\mathrm{m}}$ ) as well as the lowered boundary resistance (de Boer et al., 2012; Drake et al., 2019). However, this hypothesis has not been experimentally verified.

Regardless of stomatal characteristics and their distribution across leaf surfaces, the opening status of stomata during photosynthesis depends on the plant's capacity to replace the water that transpired to the atmosphere to prevent leaf dehydration. Maximum $g_{s}$ is therefore constrained by the plant's hydraulic
conductance, which is mainly related to leaf hydraulic conductance ( $K_{\text {leaf }}$ ) in most plants (Sack \& Holbrook, 2006). Indeed, a tight correlation between $K_{\text {leaf }}$ and photosynthesis has been widely observed (Brodribb et al., 2005, 2007; Nardini et al., 2014; Xiong et al., 2015b; Scoffoni et al., 2016; Xiong \& Nadal, 2020). $K_{\text {leaf }}$ is largely determined by leaf vein density because an increased amount of veins brings xylem tissues specialized for water transport closer to the evaporation sites inside the leaf (Brodribb et al., 2007; Buckley et al., 2015). Considering this, the largest $K_{\text {leaf }}$ would be achieved if vascular veins contacted all the living cells in the leaf, but no plant makes such an enormously expensive and complex architectural investment. Despite this, the coordination of stomatal conductance and leaf hydraulic conductance to maximize photosynthetic carbon gain across species has been often suggested (Brodribb et al., 2007; Scoffoni et al., 2016), but how the distribution of stomatal pores on leaf surfaces affects this relationship has been scarcely estimated. In this sense, Haworth et al. (2018), in a survey of 31 species, already showed that species with fast-responding stomata tended to more even distributions of stomata in both leaf surfaces (i.e. they tended to amphistomaty) and larger photosynthetic capacities. However, to what extent do these relationships depend on anatomical leaf traits and coordinate with mesophyll conductance and photosynthesis remains to be studied.
In this study, we investigated the stomatal morphological traits, leaf vein traits and gas exchange parameters of 61 species, including ferns, gymnosperms, and angiosperms, to address the following questions: first, is there a universal scaling relationship between $g_{\text {smax }}$ and operational $g_{s}$ across leaves with different stomatal distributions? Second, does photosynthesis increase in parallel to increases in $g_{\text {smax }}$ and leaf vein density (VLA)? And third, how does stomatal distribution from one leaf surface to both surfaces impact $\mathrm{CO}_{2}$ diffusion conductances and photosynthesis?

## Materials and Methods

## Plant materials

In this study, 61 species, including seven ferns, three gymnosperms, and 51 angiosperms, spanning 32 families were collected from the experimental fields of the University of Illes Balears, Mallorca, Spain, from June to August 2015 (see Supporting Information Table S1). Most of the species were grown under field conditions; however, eleven species were grown outdoors in pots (Table S1). According to stomatal allocation on the leaf surfaces, 29 and 32 species were identified as amphistomatous and hypostomatous respectively. Plants were irrigated during the whole experimental period. For each species, the samples were collected from at least three individual plants. To minimize the effects of leaf age and light environment, only new leaves developed in open habitats were sampled, and the climate conditions in the site during the leaves' developments are shown in Fig. S1. Additional information about the climate over the growing area can be found at http://plantmed.uib.es/paginas/ INTRANET.html. Although some of the species may be widely naturalized across the Mediterranean region, we would like to
note that some of the species selected for the current study may not have fully adapted to the local climate conditions, as many of the species originated outside of Mallorca (Table S1).

## Gas exchange

An open-flow gas exchange system (LI-6400XT; Li-Cor, Lincoln, NE, USA) was used to measure leaf gas exchange. Inside the gas exchange chamber (LI-6400-40), the reference $\mathrm{CO}_{2}$ concentration was adjusted to $400 \mu \mathrm{~mol} \mathrm{~mol}^{-1}$ with a $\mathrm{CO}_{2}$ mixture; photosynthetically active radiation (PAR) was set to $1500 \mu \mathrm{~mol} \mathrm{~m}^{-2} \mathrm{~s}^{-1}$ (10\% : 90\%, blue : red light); and block temperature was set at $25^{\circ} \mathrm{C}$. As the daytime air vapor pressure deficit (VPD) in this area is typically higher than 2.0 kPa between June and August (Fig. S1), the air VPD in the gas exchange chamber was maintained between 1.5 and 2.0 kPa to capture the daily maximum optimum stomatal conductance and photosynthesis. The flow rate was $400 \mu \mathrm{~mol} \mathrm{~s}^{-1}$ when the photosynthetic rate was higher than $5 \mu \mathrm{~mol} \mathrm{~m}^{-2} \mathrm{~s}^{-1}$, and was set to $150 \mu \mathrm{~mol} \mathrm{~s}^{-1}$ when the photosynthetic rate was lower than $5 \mu \mathrm{~mol} \mathrm{~m}^{-2} \mathrm{~s}^{-1}$. The measurements were made on sun-exposed leaves. After the leaf reached a steady state (i.e. the fluctuation of $g_{\mathrm{s}}$ was $<0.05 \mathrm{~mol} \mathrm{~m}^{-2} \mathrm{~s}^{-1}$ during a $10-\mathrm{min}$ period), usually after 15 to 25 min for the leaves under sunny conditions, the gas exchange parameters, steady-state fluorescence $\left(F_{s}\right)$ and maximum fluorescence ( $F^{\prime}{ }_{m}$ ) were recorded.

The actual photochemical efficiency of photosystem II ( $\Phi_{\text {PSII }}$ ) was calculated as follows:

$$
\Phi_{\mathrm{PSII}}=\frac{\left(F_{m}^{\prime}-F_{s}\right)}{F_{m}^{\prime}}
$$

The electron transport rate ( $/$ ) was then calculated:

$$
J=\Phi_{\text {PSII }} \cdot \operatorname{PAR} \cdot \alpha \beta,
$$

where $\alpha$ is the leaf absorptance and $\beta$ is the partitioning of absorbed quanta between photosystems II and I. Although intraspecific variation in $\alpha$ and $\beta$ has been reported (Muir et al., 2017), in this study, the values of 0.85 and 0.5 for $\alpha$ and $\beta$, respectively, were used in the calculations because it is difficult to obtain direct estimates of $\alpha$ and $\beta$ (e.g. by performing light response curve measurements under nonrespiration conditions) for all species under study.

The variable $J$ method (Harley et al., 1992) was used to calculate $g_{\mathrm{m}}$ and the $\mathrm{CO}_{2}$ concentration in chloroplasts $\left(C_{\mathrm{c}}\right) . C_{\mathrm{c}}$ and $g_{\mathrm{m}}$ were calculated as follows:

$$
\begin{gathered}
C_{c}=\frac{\Gamma *\left(J+8\left(A+R_{d}\right)\right)}{J-4\left(A+R_{d}\right)} \\
g_{m}=\frac{A}{C_{i}-C_{c}}
\end{gathered}
$$

where $\mathrm{C}_{\mathrm{i}}, \Gamma^{*}$ and $R_{\mathrm{d}}$ represent the intercellular $\mathrm{CO}_{2}$ concentration, $\mathrm{CO}_{2}$ compensation point in the absence of respiration, and daytime respiration rate, respectively. Unfortunately, $R_{\mathrm{d}}$ and $\Gamma^{*}$ were not directly measured as their estimation using the Laisk method is time consuming. In the current study, their values

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were assumed to be $40 \mu \mathrm{~mol} \mathrm{~mol}^{-1}$ and $1.0 \mu \mathrm{~mol} \mathrm{~m}^{-2} \mathrm{~s}^{-1}$ at $25^{\circ} \mathrm{C}$, respectively, through quantifying the respective effects of $R_{\mathrm{d}}$ and $\Gamma^{*}$ on $\mathrm{g}_{\mathrm{m}}$ estimation via a sensitivity analysis (Xiong et al., 2015a; Fig. S2). For each data point generated, we checked whether it met the criterion $10>d C_{\mathrm{c}} d d A>50$, as suggested by Harley et al. (1992). It is worth noting that $g_{\mathrm{m}}$ was estimated only for $C_{3}$ species.

## Leaf vein density and leaf thickness

To determine vein traits, one leaf from each of three individuals per species was chemically cleared in $15 \% \mathrm{NaOH}(\mathrm{w} / \mathrm{v})$ and bleach following our previous protocol (Xiong et al., 2018). The cleared leaves were stained with safranin and Fast Green (SigmaAldrich, St Louis, MO, USA). Leaves were scanned for quantification of leaf area and major vein length. To measure minor veins, a light microscope (U-TVO.5XC; Olympus, Tokyo, Japan) with a $\times 5$ objective lens and a digital camera were used, and pictures were taken at the top, middle and bottom of each leaf. Leaf area and vein length were manually measured by using ImageJ (https://imagej.nih.gov/ij/index.html). The leaf thickness was measured in situ at the middle of the leaves (avoiding midribs) using a DTG03 digital thickness gauge (Digital Micrometers Ltd, Sheffield, UK). For each leaf, ten adjacent locations were measured and then averaged.

## Stomatal traits

The stomatal morphological traits were estimated following our previous method (Xiong et al., 2018). Six small leaf discs (c. $10 \times$ 10 mm ) at the centre of each leaf (three leaves from three plants per species) were removed; however, only four small leaf discs per leaf were collected for leaves of Taxus baccata and Metasequoia glyptostroboides due to the leaves being extremely small in size. Leaf discs were cleared with $10 \% \mathrm{NaOH}(\mathrm{w} / \mathrm{v})$ hydrotrope solution for 24 h , and were placed in $50 \%$ ethanol solution overnight. If necessary, leaf discs were bleached in $10 \% \mathrm{H}_{2} \mathrm{O}_{2}$ to remove background color. A 5\% solution of safranin (in ethanol) was used to stain the leaves. Images of both the abaxial and adaxial sides were taken using a light microscope (U-TVO.5XC; Olympus, Tokyo, Japan). Stomatal density (SD, $\mathrm{mm}^{-2}$ ), guard cell length (GL), width of the entire stoma at the center of the stoma (SW), stoma pore length (PL), pore width at center of the stoma (PW) and guard cell width (GW) at the center of the stoma were manually recorded using ImageJ. In the current study, stoma size ( $\mathrm{SS}, \mu_{\mathrm{m}}{ }^{2}$ ) was measured by defining an ellipse with its major axis equal to GL and its minor axis equal to SW; and maximum stomatal pore area ( $\alpha_{\max }, \mu \mathrm{m}^{2}$ ) was defined as an ellipse with its with major axis equal to PL and minor axis equal to PW.

Maximum theoretical stomatal conductance, as defined by stomatal anatomy ( $g_{\text {smax }}, \mathrm{mol} \mathrm{m}^{-2} \mathrm{~s}^{-1}$ ), was estimated for each species using a double end-corrected version of the equation by Franks \& Farquhar (2001) (see also Sack \& Buckley, 2016):

$$
g_{\mathrm{smax}}=\frac{d \cdot \mathrm{SD} \cdot a_{\max }}{\nu\left(P D+\frac{\pi}{2} \sqrt{\frac{a_{\max }}{\pi}}\right)}
$$

where $d$ is the diffusivity of water in air $\left(24.9 \times 10^{-6} \mathrm{~m}^{2} \mathrm{~s}^{-1}\right.$, at $\left.25^{\circ} \mathrm{C}\right), v$ is the molar volume of air $\left(22.4 \times 10^{-3} \mathrm{~m}^{3} \mathrm{~mol}^{-1}\right.$, at $\left.25^{\circ} \mathrm{C}, 101.3 \mathrm{kPa}\right), \mathrm{PD}$ is the stomatal pore depth, which is equal to GW in the current study, and $\pi$ is the mathematical constant. The $g_{\text {smax }}$ value for each leaf was calculated as the sum of abaxial and adaxial $g_{\text {smax }}$ values.

## Phylogenetic analysis

We conducted a phylogenetic analysis of leaf vein, stomatal and physiological traits using species-mean values. Firstly, a phylogeny was constructed with Phylocom's Phylomatic tool (Webb et al., 2008) using the R20120829 tree. The branch lengths were then adjusted using the default ages file (Wikström et al., 2001). In order to compute the phylogenetic covariances, we used the most common model for the evolution of continuously valued traits: the Brownian model (Blomberg et al., 2003). In this model, the expected variance for the trait value at a given tip is directly proportional to the summed branch length from the tree root to that tip, and, therefore, the expected covariance between two values at the tips is directly proportional to the shared history of the taxa represented by the two tips. The phylogenetic signal in each trait was assessed with Blomberg et al.'s $K$ (Blomberg et al., 2003) and Pagel's $\lambda$ (Pagel, 1999) using the R package PHYtools (Revell, 2012). $K$ measures the extent to which a trait displays phylogenetic signal, where $K=0$ indicates no phylogenetic signal, $K=1$ suggests that the trait distribution perfectly conforms to the Brownian model, and $K>1$ indicates stronger similarities among closely related species than expected by the Brownian model. The $\lambda$ parameter reveals whether the phylogeny correctly predicts the patterns of covariance among species for a given trait, and its value can differ for different traits on the same phylogeny. Pagel's $\lambda$ statistic varies between 0 (no phylogenetic signal) and 1. We also calculated the phylogenetic independent contrast (PIC) of leaf vein, stomatal and physiological traits using Phytools. The PIC method uses phylogenetic information to account for the fact that species in a comparative analysis are related to each other and thus may share similarities due to their shared ancestry (Felsenstein, 1985).

## Statistics

Standardized major axis (SMA) analysis was performed to estimate the best fitting lines $(\alpha=0.05)$ for the $\log 10$-transformed key trait-trait relationships using the R package Smatr-3 (Warton et al., 2012). We tested for differences in slopes and intercepts between amphistomatous and hypostomatous species. All the analyses were performed in R (R Core Team, 2018) using the TIDYVERSE, SMATR-3 and PHYTOOLS packages.

## Results

## Trait variation across species

Leaf photosynthetic, venation and stomatal traits varied substantially across the species selected for this study (Fig. 1; Table S2).

We found 20 -fold and 31 -fold variation in $A$ and $g_{s}$ across species, respectively. The values of $A$ varied from 2.2 to $44.8 \mu \mathrm{~mol} \mathrm{~m} \mathrm{~m}^{-2} \mathrm{~s}^{-1}$, and the values of $g_{\mathrm{s}}$ varied from 0.02 to $0.70 \mathrm{~mol} \mathrm{~m}^{-2} \mathrm{~s}^{-1} \quad(P<0.001$; Table S2). Species varied by 16.8 - to 34.3 -fold in stomatal density, stomatal size and anatomical maximum stomatal conductance $\left(g_{\text {smax }}\right)$. The stomatal density on the adaxial leaf surface varied from 0 to 308 stomata $\mathrm{mm}^{-2}$, and the adaxial : abaxial stomatal density ratio varied between 0 and 0.98 (unitless). However, no differences in stomatal size were observed between the adaxial and abaxial surfaces of amphistomatous leaves (Fig. S3a), and the stomatal density ratio showed a bimodal distribution (Fig. S3b). The species also showed significant variation in VLA and leaf thickness, which varied by 40.2fold and 10.5 -fold, respectively.

Overall, our tests of phylogenetic signal using Blomberg's $K$ and Pagel's $\lambda$ were highly consistent (Table 1). We found statistical evidence of phylogenetic signal for all tested traits, although the phylogenetic signal of leaf thickness was relatively
low ( $K=0.51, P=0.035 ; \lambda=0.88, P=0.037$ ). As shown in Figs 1 and 2, $A, g_{s}$, VLA, and stomatal density significantly increased from ferns and gymnosperms to angiosperms; by contrast, stomatal size declined from ferns and gymnosperms to angiosperms. However, there was no clear difference in leaf thickness among ferns, gymnosperms and angiosperms, despite the high diversity in angiosperms (Fig. 1). Stomata are found only on the abaxial leaf surface in ferns and gymnosperms (i.e. they are hypostomatous); however, stomatal distribution in angiosperms was shown to be highly variable (Fig. S4). Across the studied species, $A$ evolved in tight coordination with shifts in $g_{s}$, - that is, shifts up or downwards in $A$ along branches of the phylogenetic tree corresponded to similar shifts in $g_{\mathrm{s}}$ (Fig. 2), as already shown by e.g. Haworth et al. (2018). Moreover, the abaxial : adaxial stomatal density ratio showed strong coevolution with $A$ and $g_{\mathrm{s}}$ (Fig. 2). We found strong coordination of stomatal density, stomatal size and VLA (Figs 2, 3).


Fig. 1 Variation in gas exchange (a, b), leaf vein density (c), stomatal traits ( $d, e$ ), and leaf thickness ( $f$ ) across growth forms. A, light-saturated photosynthetic rate; $g_{s}$, light-saturated stomatal conductance; VLA, leaf vein density; and $T_{\text {leaf, }}$, leaf thickness. For the box plot, the lower and upper hinges correspond to the first and third quartiles (interquartile range, IQR), the horizontal lines inside the boxes are the medians, the upper/lower whisker extends from the hinge to the largest/smallest value no further than $1.5 \times I Q R$ from the hinges, and data beyond the end of the whiskers are plotted individually (grey circles).

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## Trait correlations

PIC correlations were mostly similar to species-wise correlations (Table 2), which suggests that the relationships found were mostly not driven by phylogenetic relatedness (Table 2). As expected, the strong relationships between $A$ and $g_{s}$ (Pearson: $r=0.92, P<0.001$ and PIC: $r=0.95, P<0.001$; Table 2 and Fig. 3) and between $A$ and $g_{\mathrm{m}}$ (Pearson: $r=0.89$, $P<0.001$ and PIC: $r=0.89, P<0.001$; Table 2 and Fig. 3) were found across species. The analysis also showed that $g_{s}$ was positively correlated with abaxial : adaxial stomatal density ratio (Pearson: $r=0.76, P<0.001$ and PIC: $r=0.84, P<0.001$ ), but negatively correlated with stomatal size (Pearson: $r=-0.43$, $P<0.001$ and PIC: $r=-0.59, P<0.001)$. Although $g_{s}$ was found to be independent of stomatal density ( $r=0.20$, $P>0.05$ ), the PIC of $g_{s}$ and stomatal density were correlated (PIC: $r=0.33, P<0.01$ ) across the estimated species. Both stomatal density (Pearson: $r=0.62, P<0.001$ and PIC: $r=0.71, P<0.001$ ) and size (Pearson: $r=-0.55, P<0.001$ and PIC: $r=-0.63, P<0.001$ ) were tightly correlated with VLA. Leaf thickness was found to be independent of almost all of the traits under study here, except the stomatal density ratio (Pearson: $r=-0.26, P<0.05$ and PIC: $r=-0.31, P<0.05$ ).

## Hypostomatous vs amphistomatous

$A(P<0.001), g_{\mathrm{s}}(P<0.001)$ and $g_{\mathrm{m}}(P<0.001)$ were larger in amphistomatous than in hypostomatous species (Fig. 4; Fig. S4). Although the average values were different, there was a high degree of overlap in stomatal density ( $P<0.05$ ), stomatal size ( $P$ $<0.05$ ), vein density ( $P<0.05$ ) and leaf thickness ( $P<0.05$ ) values between amphistomatous and hypostomatous species (Fig. 4). Only in angiosperm herbs were these parameters clearly different between hypostomatous and amphistomatous leaves (Fig. S4). The correlations between stomatal traits and VLA are less affected by stomatal distribution (Fig. 5); however, the correlations between $g_{s}$ and leaf anatomical traits were strongly impacted by stomatal distribution (Fig. 6). At a given stomatal density, size or VLA, $g_{s}$ was higher in amphistomatous than in hypostomatous species. Moreover, the correlation between $\mathrm{g}_{s}$ and anatomical maximum stomatal conductance $\left(g_{\text {smax }}\right)$ was different for amphistomatous and hypostomatous species (Fig. 7). The $g_{s}: g_{s m a x}$ ratio was 0.18 for amphistomatous species (with Q1 and Q3 values of 0.09 and 0.24 , respectively) and was 0.07 for hypostomatous
species (with Q1 and Q3 values of 0.04 and 0.12 , respectively). We also found a positive correlation between $g_{s}$ and the adaxial : abaxial stomatal density ratio, and the correlation was less affected by stomatal density (Fig. 8). A similar correlation was observed between $g_{\mathrm{m}}$ and the adaxial : abaxial stomatal density ratio (Fig. 9).

## Discussion

The predominant role of modern angiosperms comparing with relative to other terrestrial plant groups such as ferns and gymnosperms has been suggested to be at least partially related to their higher assimilation rates (Brodribb \& Feild, 2010; Gago et al., 2019). The early evolution of photosynthesis is primarily promoted through an increase in both vein and stomatal density; however, further enhancement of photosynthesis in angiosperms is related to the redistribution of stomatal pores across the leaf surface.

## High stomatal and vein density as the primary strategy in photosynthesis increase

The strong limiting role of $g_{s}$ on photosynthesis has been widely confirmed by previous studies (Scoffoni et al., 2016; Tosens et al., 2016; Xiong et al., 2018), and here we further demonstrated that $A$ evolved in tight coordination with shifts in $g_{s}$ (Fig. 2). Achieving high $A$ requires foliage that has stomatal valves to provide enough $\mathrm{CO}_{2}$ for the photosynthetic apparatus, and a hydraulic system (i.e. leaf hydraulic conductance, $K_{\text {leaf }}$ ) to water the desiccation-prone photosynthetic cells. For vascular plants, VLA has been widely used as a robust indicator of $K_{\text {leaf }}$, due to its important role in determining hydraulic conductance both inside and outside of xylem (Brodribb et al., 2007; Boyce et al., 2009; Brodribb \& Feild, 2010; Sack et al., 2013; Sack \& Scoffoni, 2013; Buckley et al., 2015). The leaves of ferns and gymnosperms had very low VLA values ( $1.8 \mathrm{~mm} \mathrm{~mm}^{-2}$ on average); by contrast, angiosperm leaves are endowed with VLA values of $10 \mathrm{~mm} \mathrm{~mm}^{-2}$ on average, and up to $20 \mathrm{~mm} \mathrm{~mm}^{-2}$. Similar to VLA in determining maximum liquid water conductance, maximum water vapor diffusion conductance is proposed to be determined by stomatal density and pore size (Franks \& Beerling, 2009). The fact that the stomata in ferns and gymnosperms are few in number but very large in size leads to their low $g_{s}$ relative to angiosperms. The low $g_{s}$ in ferns and gymnosperms couples low VLA with a few large stomata, while the high $g_{s}$ in

Table 1 Phylogenetic conservatism indices for leaf traits.

| Traits | VLA | SD | SS | SR | $g_{\text {smax }}$ | $A$ | $g_{s}$ | $T_{\text {leaf }}$ | $g_{\mathrm{m}}$ |
| :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- |
| Blomberg's K | $1.65^{* * *}$ | $0.85^{* * *}$ | $4.57^{* * *}$ | $0.94^{* * *}$ | $0.77^{* * *}$ | $0.67^{* * *}$ | $0.65^{* *}$ | $0.51^{*}$ | $0.58^{* *}$ |
| Pagel's $\lambda$ | $0.96^{* * *}$ | $0.59^{* * *}$ | $0.99^{* * *}$ | $0.99^{* * *}$ | $0.48^{* * *}$ | $0.73^{* * *}$ | $0.70^{* * *}$ | $0.88^{*}$ | $0.66^{* *}$ |

VLA, leaf vein density $\left(\mathrm{mm} \mathrm{mm}^{-2}\right)$; SD, stomatal density $\left(\mathrm{mm}^{-2}\right)$; SS, stomatal size ( $\mu \mathrm{m}^{2}$ ); SR, adaxial : abaxial stomatal density ratio (unitless); $g_{\text {smax }}$ anatomical maximum stomatal conductance ( $\mathrm{mol} \mathrm{m}^{-2} \mathrm{~s}^{-1}$ ); A, light-saturated photosynthetic rate ( $\mu \mathrm{mol} \mathrm{m} \mathrm{m}^{-2} \mathrm{~s}^{-1}$ ); $\mathrm{g}_{\mathrm{s}}$ light-saturated stomatal conductance ( $\mathrm{mol} \mathrm{m}{ }^{-2} \mathrm{~s}^{-1}$ ); $T_{\text {leaf, }}$ leaf thickness $(\mu \mathrm{m}) ; g_{\mathrm{m}}$, mesophyll conductance $\left(\mathrm{mol} \mathrm{m}^{-2} \mathrm{~s}^{-1}\right.$ ).
${ }^{*}, P<0.05 ;{ }^{* *}, P<0.01 ;{ }^{* * *}, P<0.001$.



## SD units

Fig. 2 Phylogenetic diversification of leaf vein density (VLA), stomatal density (SD), stomatal size (SS), adaxial : abaxial stomatal density ratio (SR), anatomical maximum stomatal conductance ( $g_{\text {smax }}$ ), light-saturated photosynthetic rate (A), light-saturated stomatal conductance ( $g_{s}$ ), and leaf thickness $\left(T_{\text {leaf }}\right)$. The phylogenetic tree for 61 species estimated in this study is shown on the left, and the heat map for leaf traits is shown on the right (red, green, and purple branches represent fern, gymnosperm, and angiosperm, respectively). On the heat map, each trait was standardized to have the same variance and mean before analysis.


Fig. 3 Correlations between light-saturated photosynthetic rate $(A)$ and $\mathrm{CO}_{2}$ diffusion conductances. (a) A vs stomatal conductance ( $g_{s}$ ); (b) phylogenetic independent contrast $A$ vs phylogenetic independent contrast $g_{s}$; (c) A vs mesophyll conductance ( $g_{\mathrm{m}}$ ); and (d) phylogenetic independent contrast $A$ vs phylogenetic independent contrast $g_{m}$. In panels ( a ) and ( c ), both axes are on a logarithmic scale to compress the enormous range of traits observed in our species sample. Lines were fitted using a linear model and the shaded areas around the lines indicate the $95 \%$ of confidence intervals.

Table 2 Correlation matrix between leaf traits.

|  | VLA | SD | SS | SR | $g_{\text {smax }}$ | A | $g$ s | $T_{\text {leaf }}$ | $g \mathrm{~m}$ |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| VLA |  | 0.71 *** | $-0.63 * * *$ | $0.13{ }^{\text {ns }}$ | 0.71 *** | $0.18{ }^{\text {ns }}$ | $0.21{ }^{\text {ns }}$ | $-0.02^{\text {ns }}$ | $0.04{ }^{\text {ns }}$ |
| SD | 0.62 *** |  | -0.80 *** | 0.32* | $0.94 * * *$ | $0.28{ }^{*}$ | 0.33 ** | $-0.21^{\mathrm{ns}}$ | $0.22^{\text {ns }}$ |
| SS | $-0.55^{* * *}$ | -0.70 *** |  | $-0.64 * * *$ | $-0.70^{* * *}$ | -0.53 *** | $-0.59{ }^{* * *}$ | 0.27* | $-0.45{ }^{* *}$ |
| SR | $-0.02^{\text {ns }}$ | $0.11^{\text {ns }}$ | $-0.47^{* * *}$ |  | $0.19{ }^{\text {ns }}$ | 0.82 *** | $0.84{ }^{* * *}$ | -0.31* | 0.82*** |
| $g_{\text {smax }}$ | $0.64 * *$ | 0.93 *** | $-0.64 * * *$ | $0.00{ }^{\text {ns }}$ |  | $0.15{ }^{\text {ns }}$ | $0.19^{\text {ns }}$ | $-0.07^{\text {ns }}$ | $0.09{ }^{\text {ns }}$ |
| A | $0.14{ }^{\text {ns }}$ | $0.13{ }^{\text {ns }}$ | $-0.41^{* * *}$ | $0.76{ }^{* * *}$ | $0.02{ }^{\text {ns }}$ |  | $0.95{ }^{* * *}$ | $-0.23{ }^{\text {ns }}$ | 0.89 *** |
| $g$ s | $0.17^{\text {ns }}$ | $0.20{ }^{\text {ns }}$ | $-0.43^{* * *}$ | 0.76 *** | $0.08{ }^{\text {ns }}$ | 0.92 *** |  | $-0.24{ }^{\text {ns }}$ | 0.81 *** |
| $T_{\text {leaf }}$ | $-0.01^{\text {ns }}$ | $-0.11^{\mathrm{ns}}$ | $0.14{ }^{\text {ns }}$ | -0.26 * | $-0.05^{\text {ns }}$ | $-0.19^{\text {ns }}$ | $-0.16^{\text {ns }}$ |  | $-0.18^{\mathrm{ns}}$ |
| $g \mathrm{~m}$ | $0.07^{\text {ns }}$ | $0.09{ }^{\text {ns }}$ | $-0.39^{* *}$ | $0.75{ }^{* * *}$ | $-0.01^{\text {ns }}$ | $0.89{ }^{* * *}$ | $0.88{ }^{* * *}$ | $-0.11^{\text {ns }}$ |  |

Values represent Pearson correlation ( $r$; bottom left half of table) and phylogenetically independent contrast (PIC $r$; upper right half of table) for the association between traits. VLA, leaf vein density $\left(\mathrm{mm} \mathrm{mm}^{-2}\right)$; SD, stomatal density ( $\mathrm{mm}^{-2}$ ); SS, stomatal size ( $\mu \mathrm{m}^{2}$ ); SR, adaxial : abaxial stomatal density ratio (unitless); $g_{\text {smax }}$, anatomical maximum stomatal conductance ( $\mathrm{mol} \mathrm{m}^{-2} \mathrm{~s}^{-1}$ ); A, light-saturated photosynthetic rate ( $\mu \mathrm{mol} \mathrm{m}^{-2} \mathrm{~s}^{-1}$ ); $g_{\mathrm{s}}$, light-saturated stomatal conductance ( $\mathrm{mol} \mathrm{m} \mathrm{m}^{-2} \mathrm{~s}^{-1}$ ); $T_{\text {leaf, }}$, leaf thickness ( $\mu \mathrm{m}$ ).
${ }^{*}, P<0.05 ;{ }^{* *}, P<0.01 ;{ }^{* * *}, P<0.001$; ns, not significant $(P>0.05)$.
angiosperms is associated with high VLA and dense but small stomata (Fig. 1), indicating that both VLA and stomata co-vary with photosynthetic increase from ferns and gymnosperms to angiosperms.

Beyond VLA and stomata, leaf thickness has been considered to be another factor related to $g_{s}$, because leaf thickness was assumed to influence the water transport pathway length between veins and stomata (Brodribb et al., 2007; de Boer et al., 2012,

2016a). However, we found no correlation between leaf thickness and $g_{s}$ when phylogeny was considered (Table 2), which suggests a lack of functional association. These results support the 'vein density hypothesis' that proposes a high vein density, resulting in high water supplement capacity of angiosperms compared with ferns and/or gymnosperms, and further support the idea that coevolution of vein density and stomata dramatically increased $g_{\mathrm{s}}$ and thus $A$ from ferns to angiosperms.


Fig. 4 Leaf traits of amphistomatous and hypostomatous plants. A, light-saturated photosynthetic rate; $g_{s}$, light-saturated stomatal conductance; $g_{\text {smax }}$, anatomical maximum stomatal conductance; $S D$, stomatal density; $S D_{\text {abaxial, }}$ stomatal density on abaxial surface; $S D_{\text {adaxial, }}$ stomatal density on adaxial surface; SR, adaxial : abaxial stomatal density ratio; SS, stomatal size; $T_{\text {leafr }}$ leaf thickness; VLA, leaf vein density. The lower and upper hinges of the box plot correspond to the first and third quartiles (interquartile range, IQR), the horizontal lines inside the boxes are the medians, the upper/lower whisker extends from the hinge to the largest/smallest value no further than $1.5 \times$ IQR from the hinges, and the data beyond the end of the whiskers are plotted individually (grey circles). ${ }^{*}, P<0.05 ;{ }^{* *}, P<0.005 ;{ }^{* * *}, P<0.001$.

## Ratio of $g_{s}$ to $g_{\text {smax }}$

In the present study, the average $g_{\mathrm{s}}: g_{\text {smax }}$ ratio was 0.12 across 61 species, including seven ferns, three gymnosperms, and 51 angiosperms, spanning 32 families (Table S1), and the ratio is somewhat lower than the values reported in previous studies (Dow et al., 2014; McElwain et al., 2016). Dow et al. (2014) reported a value of 0.31 for Arabidopsis grown in a growth chamber, and McElwain et al. (2016) reported a value of 0.25 across 18 species grown in a glasshouse, including one fern, five gymnosperms and 12 angiosperms. Many sources of biological and methodological variation among these studies could have caused the differences in results. Firstly, the numbers of the species under study were different - the number of investigated species in our study was three times larger than in previous studies. In fact, the $g_{s}: g_{\text {smax }}$ ratio ranged from 0.007 to 0.86 , which covered the ranges of the previous studies (Table S2). The different growth and measurement conditions in these studies may be another reason, as $g_{s}$ is sensitive to environmental conditions (Buckley, 2019). For instance, we performed the gas exchange measurement under high VPD ( 1.5 to 2.0 kPa ) conditions to capture the in situ maximum operational $g_{s}$ under local climate conditions; however, the VPD in the gas exchange chambers was lower than 1.0 kPa for Arabidopsis grown in the controlled conditions in the Dow et al. (2014) study. In addition, different
instruments were used to measure $g_{s}$ - the LiCor-6400 was used in the current study and in the study by Dow et al. (2014), but the SC-1 leaf porometer and the CIRAS 2 gas exchange system were used in the study by McElwain et al. (2016).

Despite the common growth conditions as well as the consistent VPD in the leaf chamber, we showed that the $g_{s}: g_{\text {smax }}$ ratio for amphistomatous leaves was significantly higher than the ratio for hypostomatous leaves (Fig. 7b), and that the relationship between $g_{\text {s }}$ and $g_{\text {smax }}$ scaled up across hypostomatous species but not across amphistomatous species (Fig. 7a). Clearly, at a given $g_{\text {smax }}$ level, the $g_{\mathrm{s}}$ value of amphistomatous species was much higher than hypostomatous species which, again, demonstrated that the shift of stomata distribution from one leaf surface to both surfaces improved the efficiency of individual stomata. Furthermore, we showed that the ratio $g_{\mathrm{s}}: g_{\text {smax }}$ varied among ecotypes. Overall, grasses had the highest $g_{s}: g_{\text {smax }}$ ratio, and evergreens had the lowest (Fig. S5). A lack of correlation between $g_{\mathrm{s}}$ and $g_{\text {smax }}$ across amphistomatous species may because of the asymmetric stomatal distribution across the two surfaces, as we found that $g_{s}$ was positively correlated with stomatal ratio (Fig. 8). Furthermore, the stomatal pores on each surface may differ at a functional level, as the internal and external environments of stomata are different. However, whether the abaxial pores have the same efficiency as the adaxial ones is currently unclear, and further investigations are required to address this issue.


Fig. 5 Correlations between stomatal traits and leaf vein density (VLA). (a) VLA vs stomatal density; (b) VLA vs stomatal size; (c) stomatal density vs stomatal size. The ellipsoid for the broad trend across amphistomatous or hypostomatous species corresponds to the $95 \%$ confidence region of the linear statistical trend. Both axes are on a logarithmic scale to compress the enormous range of the traits observed in our species sample. The slope and intercept of amphistomatous and hypostomatous species were compared using the standardized major axis (SMA) method. ${ }^{*}, P<0.05$; ns, not significant $(P>0.05)$.

## Boosting photosynthesis by shifting stomatal distribution from one side to two sides

In this study we have shown, in a larger set of species than in previous investigations (e.g. McElwain et al., 2016; Haworth et al., 2018), that the increase in photosynthesis from ferns and


Fig. 6 Relationship between light-saturated stomatal conductance $\left(g_{s}\right)$ and leaf anatomical traits. (a) $g_{s}$ vs stomatal density, (b) $g_{s}$ vs stomatal size, and (c) $g_{s}$ vs leaf vein density (VLA). The ellipsoid for the broad trend across amphistomatous or hypostomatous species corresponds to the $95 \%$ confidence region of the linear statistical trend. Both axes are on a logarithmic scale to compress the enormous range of the traits observed in our species sample. The slope and intercept of amphistomatous and hypostomatous species were compared using the standardized major axis (SMA) method. ${ }^{* *}, P<0.01 ;{ }^{* * *}, P<0.001$.
gymnosperms to angiosperms was associated with the coevolution of stomata and vein density. However, we found a significant variability in $g_{\mathrm{s}}$ in angiosperms (Fig. 1), and the variability of $g_{\mathrm{s}}$ among angiosperms cannot be fully explained by stomatal density, size and/or VLA (Fig. 6). Further investigation of stomatal distribution showed that, although $g_{\text {smax }}$ increased linearly with


Fig. 7 (a) The relationship between light-saturated stomatal conductance ( $g_{s}$ ) and anatomical maximum stomatal conductance ( $g_{\text {smax }}$ ), and (b) the ratio $g_{s}$ : $g_{\text {smax. }}$ In (a) the ellipsoid for the broad trend across amphistomatous or hypostomatous species corresponds to the $95 \%$ confidence region of the linear statistical trend. The slope and intercept of amphistomatous ( $R^{2}=0.005 ; P=0.711$ ) and hypostomatous ( $g_{\mathrm{s}}=0.13 g_{\text {smax }}+0.061 ; R^{2}=0.208$; $P=0.009$ ) species were compared using the standardized major axis (SMA) method. ${ }^{* * *}, P<0.001$. In panel (b), the lower and upper hinges of the box plot correspond to the first and third quartiles (interquartile range, IQR), the horizontal lines inside the boxes are the medians, the upper/lower whisker extends from the hinge to the largest/smallest value no further than $1.5 \times I Q R$ from the hinges, and the circles represent the mean value for each species. The difference between amphistomatous and hypostomatous species was estimated using one-way ANOVA. ${ }^{* *}, P<0.01$.


Fig. 8 The effect of adaxial : abaxial stomatal density ratio (SR) on light-saturated stomatal conductance ( $g_{s}$ ) and anatomical maximum stomatal conductance ( $g_{\text {smax }}$ ). The point size indicates the stomatal density of the species, and the correlations were tested using the standardized major axial (SMA) method. In panel (a), the line was fitted using a linear model, and the shaded areas around the lines indicate the $95 \%$ confidence intervals.

VLA, the operational $g_{s}$ value was strongly affected by stomatal distribution (Figs 7, S6). For hypostomatous angiosperms, the rate of increase of $g_{s}$ was lower than the rate of increase of VLA, which suggests that vapor conductance via stomata on the leaf surface, rather than liquid conductance, plays the role of the limiting step for water transport in the hypostomatous species with high VLA. Indeed, previous studies have suggested that under current ambient $\mathrm{CO}_{2}$ concentration conditions, a VLA of $8.0 \mathrm{~mm} \mathrm{~mm}^{-2}$ is high enough to support transpiration under nonstress conditions (Brodribb \& Feild, 2010; de Boer et al., 2012). In addition, high vein density enhances water transport in leaves, but trade-offs associated with vein production include the displacement of photosynthetic tissue by veins, the investment in thick lignified cell walls and the metabolism of living cells which support the veins.

At a given VLA, the shift of distribution of stomata from one leaf surface to both leaf surfaces (amphistomatous) significantly increased leaf $\mathrm{CO}_{2}$ diffusion conductance (both $g_{\mathrm{s}}$ and $g_{\mathrm{m}}$ ) and enhanced photosynthesis (Figs 8, 9). One reason for the high $g_{s}$ in amphistomatous species is that the water transport pathway from vein to stomata is shorter, resulting in a larger leaf hydraulic capacity in amphistomatous than in hypostomatous species if the leaf thickness is similar (Muir, 2015). In fact, Scoffoni et al. (2016) have demonstrated that the leaf hydraulic conductance is mainly determined by the water transport outside-xylem pathway properties. Similar leaf thickness in both hypostomatous and amphistomatous species were found in the current study and in a previous study by Muir (2015). However, this result is somewhat biased by the fact that several ecological groups used here contained only hypostomatous or amphistomatous species (Fig. S4).


Fig. 9 (a) The relationship between adaxial : abaxial stomatal density ratio ( SR ) and mesophyll conductance and $\mathrm{CO}_{2}\left(\mathrm{~g}_{\mathrm{m}}\right)$, and (b) the $\mathrm{g}_{\mathrm{m}}$ values (the species mean values) of amphistomatous and hypostomatous species. In (a) the point size indicates the stomatal density of the species, the correlations were tested using the standardized major axial (SMA) method, and the shaded areas around the lines indicate the $95 \%$ confidence intervals. In panel (b), the lower and upper hinges of the box plot correspond to the first and third quartiles (interquartile range, IQR), the horizontal lines inside the boxes are the medians, the upper/lower whisker extends from the hinge to the largest/smallest value no further than $1.5 \times I Q R$ from the hinges, and the circles represent the mean value for each species. The difference between amphistomatous and hypostomatous species was estimated using one-way ANOVA. **, $P<0.01$.

In fact, in angiosperm herbs and, to a lesser extent, deciduous species - but not in evergreens - leaf thickness was larger in amphistomatous than hypostomatous species, as found by Parkhurst (1978). Another reason is that too high a density of stomata in hypostomatous species may potentially constrict $g_{\mathrm{s}}$ by increasing overlaps between diffusion shells of neighboring stomatal pores (Lehmann \& Or, 2015) and/or hamper effective stomatal opening and closing responses due to the fact that guard cell movements depend partly on the mechanical advantage of neighboring dermis cells (Franks \& Farquhar, 2007; Dow et al., 2014; de Boer et al., 2016b). Although it has rarely been confirmed by experimental investigations, it has been suggested that shifting stomatal distribution from one side to both sides of the leaf may also have advantages for $g_{\mathrm{m}}$, a major photosynthetic limitation factor (Parkhurst, 1978; Flexas et al., 2008; de Boer et al., 2012; Drake et al., 2019). In this study, we demonstrated that the $g_{\mathrm{m}}$ values of amphistomatous species were larger than those of hypostomatous species and, in addition, that $g_{\mathrm{m}}$ increased linearly with the adaxial : abaxial stomatal density ratio (Fig. 9). The shorter $\mathrm{CO}_{2}$ diffusion pathways inside amphistomatous leaves, as $\mathrm{CO}_{2}$ enters the leaves from both sides, potentially minimizes the $\mathrm{CO}_{2}$ concentration gradient between carboxylation sites in chloroplasts and leaf surfaces. In fact, Muir (2018) found that
amphistomaty is common under high light environments, where the $\mathrm{CO}_{2}$ concentration in the chloroplasts is the major photosynthetic limiting factor. Recently, the competition hypothesis for epidermal space between veins (mainly the vascular bundle sheath extensions) and stomata has been highlighted by Baresch et al. (2019). Allocating the stomatal pores to both surfaces in amphistomatous species potentially helps to lift the constraint of competition with veins for epidermal space. Moreover, distribution of stomata over both leaf surfaces reduces the leaf boundary layer resistance as the leaves have twice the transpiring surface area, which is of particular importance for large leaves and in environments with low air flow (Foster \& Smith, 1986).

Some of the studied species that originated from other parts of the world may not adapt to Mediterranean climate (high light, high daytime temperature and low air humidity), and, hence, the high stomatal ratio may not directly linked to high photosynthesis as those species might be stressed. In fact, several previous studies argued that species with high photosynthesis under high light are likely to be the same species that are naturally selected to be amphistomatous (Mott et al., 1982; Jordan et al., 2014; Muir, 2015, 2018), and other studies suggested that amphistomaty might be part of a leaf syndrome associated with high photosynthetic capacity (Beerling \& Kelly, 1996; Smith et al., 1997;

Smith et al., 1998; Oguchi et al., 2018; Muir, 2019). It is worth mentioning that photosynthesis can also be influenced by other traits, such as mesophyll tissue properties, photosynthetic biochemistry, and carbohydrate exportation, and hence the differences in photosynthesis between hypostomatous and amphistomatous leaves may also reflect the differences in some of those traits. More work is therefore needed on the high photosynthesis of amphistomatous leaves.

## Concluding remarks

In summary, the increase in photosynthetic rate from ferns and gymnosperms to angiosperms is related to the increase in stomata and vein density; however, the shift in allocation of stomatal pores from one side (hypostomatous) to both sides (amphistomatous) of the leaf contributes to the photosynthesis variations in angiosperms. We demonstrate that amphistomatous species have advantages in $\mathrm{CO}_{2}$ diffusion from leaf surface to chloroplasts via improved $g_{\mathrm{s}}$ and $g_{\mathrm{m}}$.

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## Author contributions

DX and JF planned and designed the research. DX performed experiments and conducted fieldwork. DX wrote the manuscript with input from JF.

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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 Climate conditions (2015) during the development of the leaves.

Fig. S2 Sensitivity analysis to quantify the $\mathrm{CO}_{2}$ compensation point in the absence of respiration $\left(\Gamma^{*}\right)$ and daytime respiration rate $\left(R_{\mathrm{d}}\right)$ on mesophyll conductance $\left(\mathrm{g}_{\mathrm{m}}\right)$ estimation.

Fig. S3 Relationship between adaxial stomatal size and abaxial stomatal size, and a density histogram of stomatal ratio.

Fig. S4 Differences of gas exchange, leaf vein density, and stomatal traits between amphistomatous and hypostomatous plants for each growth form.

Fig. $\mathbf{S} 5$ Variation in the ratio of stomatal conductance $\left(g_{s}\right)$ to anatomical maximum $g_{s}\left(g_{\text {smax }}\right)$ across growth forms.

Fig. S6 Correlation between stomatal conductance and leaf vein density (VLA) across all species and in hypostomatous species only.

Table S1 The species list used in this study.
Table S2 Trait variation across species.
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