

# 1 **Scaling between stomatal size and density in forest plants**

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24 **The size and density of stomatal pores limit the maximum rate of leaf carbon gain and**  
25 **water loss ( $g_{\max}$ ) in land plants. Stomatal size and density are negatively correlated at**  
26 **broad phylogenetic scales, such that species with small stomata tend to have greater**  
27 **stomatal density, but the consequences of this relationship for leaf function has been**  
28 **controversial. The prevailing hypothesis posits that the negative scaling of size and density**  
29 **arises because species that evolved higher  $g_{\max}$  also achieved reduced allocation of**  
30 **epidermal area to stomata (stomatal-area minimization). Alternatively, the negative scaling**  
31 **of size and density might reflect the maintenance of a stable mean and variance in  $g_{\max}$**   
32 **despite variation in stomatal size and density, which would result in a higher allocation of**  
33 **epidermal area to achieve high  $g_{\max}$  (stomatal-area increase). Here, we tested these**  
34 **hypotheses by comparing their predictions for the structure of the covariance of stomatal**  
35 **size and density across species, applying macroevolutionary models and phylogenetic**  
36 **regression to data for 2408 species of angiosperms, gymnosperms, and ferns from forests**  
37 **worldwide. The observed stomatal size-density scaling and covariance supported the**  
38 **stomatal area increase hypothesis for high  $g_{\max}$ . Thus, contrary to the prevailing view,**  
39 **higher  $g_{\max}$  is not achieved while minimizing stomatal area allocation but requires**  
40 **increasing epidermal area allocated to stomata. Understanding of optimal stomatal**  
41 **conductance, photosynthesis, and plant water-use efficiency used in Earth System and crop**  
42 **productivity models will thus be improved by including the cost of higher  $g_{\max}$  both in**  
43 **construction cost of stomata and opportunity cost in epidermal space.**

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45 Stomatal pores are critical determinants of the function of plants and the composition of  
46 the atmosphere<sup>1</sup>. The stomatal conductance to diffusion of water vapor and CO<sub>2</sub> ( $g_s$ ) influences a  
47 broad spectrum of ecological processes at leaf, community, and ecosystem scales, including  
48 photosynthesis, net primary production, and water use efficiency<sup>2,3</sup>. Theoretically, stomata can  
49 regulate  $g_s$  through evolutionary or plastic shifts in stomatal size or numbers<sup>4</sup> or through short-  
50 term stomatal aperture changes<sup>5</sup>. The  $g_s$ , and its typical operational value ( $g_{op}$ ), can thus vary  
51 from near zero with stomata fully closed and  $g_{max}$  with stomata fully open. However,  $g_{op}$  and  $g_{max}$   
52 are correlated across plants measured under controlled conditions<sup>6,7</sup>, possibly to maintain the  
53 ratio  $g_{op}:g_{max}$  in which stomatal pore aperture is most responsive to guard cell turgor pressure<sup>8</sup>.  
54 There has been substantial debate about whether to increase  $g_{max}$ , plants must allocate a greater  
55 fraction of limited epidermal area to stomata, which might be costly. Stomatal optimization used  
56 to predict water use, productivity, and drought responses in critical global vegetation and crop  
57 models could be sensitive to costs of stomata, but they are rarely included<sup>9</sup>. Because of their  
58 importance in controlling leaf water and CO<sub>2</sub> fluxes, stomatal anatomy could be integrated with  
59 other plant traits to inform global vegetation and crop models<sup>10-13</sup> if we better understood what  
60 drives variation in these traits. Indeed, the anatomical traits stomatal density ( $D_s$ , number of  
61 pores per unit epidermal area) and size ( $A_s$ , area of guard cells surrounding each pore) have been  
62 widely used to study the adaptation and competition of plants because they are reliable indicators  
63 of  $g_{max}$  to water vapor and CO<sub>2</sub><sup>14-20</sup>. Yet, while the covariation of stomatal size and density  
64 within and across species has emerged as a critical constraint on  $g_{max}$  in controlled experiments  
65 and specific ecosystems<sup>21,22</sup>, these traits have engendered controversy when considering the  
66 diversity of species in natural vegetation at regional or global scales.

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68 Variation in stomatal size and density may be constrained to minimize leaf surface area  
69 (stomatal-area minimization) or leaves may allocate more area to increase stomatal conductance  
70 (stomatal-area increase). We adjudicate between these competing hypotheses by considering  
71 how they affect stomatal trait covariance differently and test competing predictions with a global  
72 data set of stomatal anatomy in forest plants. A leaf's  $g_{\max}$  is determined by stomatal anatomy:

$$g_{\max} = bmD_S A_S^{0.5}, \quad (1)$$

73 where  $b$  and  $m$  are biophysical and morphological constants, respectively<sup>23</sup> (see Methods for  
74 equations to calculate these constants). The fraction of epidermal area allocated to stomata ( $f_S$ ) is  
75 a fundamental physical constraint on  $g_{\max}$  because  $f_S$  cannot exceed unity, and

$$f_S = D_S A_S, \quad (2)$$

76 Therefore,  $D_S$  and  $A_S$  define both  $g_{\max}$  and  $f_S$ , two related traits that affect gas exchange,  
77 productivity, and fitness.

78 Stomatal size and density covary negatively across species, reducing the variation in  $g_{\max}$   
79 and  $f_S$  compared to what it would be if these anatomical traits were uncorrelated<sup>4,22–27</sup>. Despite  
80 that the inverse relationship between stomatal size and density has been recognized since 1865  
81<sup>24</sup>, its evolutionary origin and its consequences have not been understood. The leading view,  
82 established by Franks and Beerling<sup>25</sup>, is that the size-density relationship arises due to the  
83 minimization of the area of epidermis allocated to stomata, such that the evolution of more  
84 stomata involves the reduction of stomatal size. By Eq. 2, a higher  $g_{\max}$  can be achieved with a  
85 smaller total stomatal area by increasing stomatal number and reducing stomatal size, because  
86 smaller stomata also have a shorter channel for diffusion. Thus, selection for higher  $g_{\max}$  would  
87 result in more numerous, smaller stomata, to minimize epidermal allocation to stomata.  
88 However, this hypothesis, and its implications that epidermal allocation to stomata is minimized,

89 and can be strongly reduced, have not been tested. Such reduction of allocation would greatly  
90 reduce the construction and maintenance cost of guard cells, and also the opportunity cost in  
91 other epidermal leaf structures, and in light penetration to the mesophyll. A counter-hypothesis  
92 to the ‘stomatal-area minimization’ is the ‘stomatal-area increase’ hypothesis in which selection  
93 for higher  $g_{\max}$  would lead to greater surface allocation, and thus incur substantial cost (Fig. 1). It  
94 is critical to determine which of these hypotheses is consistent with the observed stomatal size-  
95 density relationship. An implication of stomatal-area minimization is that extremely high  $f_s$  is  
96 rare because of costs associated with allocating too much epidermal area to stomata<sup>27–32</sup>, and  $g_{\max}$   
97 is ultimately constrained by the costs of high  $f_s$ . By contrast, an implication of the stomatal-area  
98 increase hypothesis is that on average high  $g_{\max}$  incur major costs in stomatal volume  
99 construction cost and in epidermal space.

100 Both the stomatal-area minimization and stomatal increase hypotheses for high  $g_{\max}$   
101 would be consistent with an inverse stomatal size-density relationship. We developed theory  
102 showing that the scaling exponents of the relationship would differ between the hypotheses using  
103 a new, additional criterion based on models of macroevolutionary landscapes<sup>26–29</sup> originally  
104 derived from quantitative genetics. Over macroevolutionary time, variation in phenotypic traits is  
105 usually constrained by some combination of physical, developmental, or ecological factors.  
106 Theoretically, this process can be described by an Ornstein-Uhlenbeck (OU) model in which trait  
107 variation expands through time until it reaches a stationary distribution around a long-term  
108 average<sup>27</sup>. In macroevolutionary models, the OU process describes the movement of the adaptive  
109 optima itself. Species should be close to their current adaptive optimum if there is sufficient  
110 genetic variation, so phenotypic constraint is caused by limits on movement in the adaptive  
111 optima, not limits on response to selection.

112 We propose that scaling between stomatal size and density does not reflect constraints on  
113 either trait alone, but rather the constraint on a composite trait associated with stomatal size and  
114 density. That is, if the composite traits  $f_S$  and/or  $g_{\max}$  were constrained following an OU process  
115 then constituent traits (size and density) should *appear to evolve* as if they are mutually  
116 constrained. This prediction is based on quantitative genetic theory which shows that a constraint  
117 on composite traits imposed by stabilizing selection limits variation in constituent traits<sup>30</sup>. We  
118 tested whether the observed size-density scaling across 2408 species was consistent with a  
119 stronger constraint on  $f_S$  or  $g_{\max}$ . Note that both  $f_S$  and  $g_{\max}$  equations have the form:

$$Z_S = \lambda D_S A_S^\beta, \quad (3)$$

120 where  $Z_S$  is a composite stomatal trait that is proportional to the product of constituent traits,  
121 stomatal density and stomatal size with scaling exponent  $\beta$  multiplied by a scalar  $\lambda$ . For  $g_{\max}$ ,  
122  $\lambda = bm$  and  $\beta = 0.5$  (Eq. 1); for  $f_S$ ,  $\lambda = 1$  and  $\beta = 1$  (Eq. 2). Next, we linearize Eq. 3 by log-  
123 transformation:

$$z_S = \log(\lambda) + d_S + \beta a_S. \quad (4)$$

124 Lowercase variables are the log-transformed versions of their uppercase counterparts. Log-  
125 transformation is also desirable here because  $D_S$  and  $A_S$  are log-normally distributed<sup>27</sup>. Using  
126 random variable algebra, the variance in  $z_S$  is defined as:

$$\text{Var}(z_S) = \text{Var}(d_S) + \beta^2 \text{Var}(a_S) + 2\beta \text{Cov}(d_S, a_S). \quad (5)$$

127 The scaling exponent  $\beta$  minimizes  $\text{Var}(z_S)$ , the most constrained composite trait, is:

$$\beta = -\frac{\text{Cov}(d_S, a_S)}{\text{Var}(d_S)} \quad (6)$$

128 The right-hand side of this equation is the negative of the ordinary linear regression slope of log-  
129 stomatal size against log-density, which means that  $\beta$  can be estimated using regression  
130 methods, but a negative relationship will result in positive value of  $\beta$ . The stomatal-area

131 minimization hypothesis predicts that  $\hat{\beta} = 1$  whereas the stomatal-area increase hypothesis  
132 predicts that  $\hat{\beta} = 0.5$ . Forward-time, individual based, macroevolutionary quantitative genetic  
133 simulations confirm that these predictions hold true regardless of underlying assumptions about  
134 mutational and genetic variance (Supplementary Information).

135 We estimated stomatal size-density scaling in 2408 forest plant species from new field-  
136 collected samples over 28 sites in China and global synthesis of data from the literature (Fig. 2)  
137 and estimated the scaling exponent  $\beta$  using OU phylogenetic multiple regression with group  
138 (Angiosperm, Pteridophyte, Gymnosperm) and growth form (tree, shrub, herb) as covariates (see  
139 Methods)

140 Our analysis shows that stomatal size-density scaling among forest plant species is  
141 consistent with selection for higher  $g_{\max}$  increasing stomatal area allocation, and not minimizing  
142 area allocation (Fig. 3). Given the variance in stomatal density, the covariance between size and  
143 density among forest species minimizes the variance in  $g_{\max}$ . There is no evidence that scaling  
144 differs between major groups, Angiosperms, Gymnosperms, and Pteridophytes (Fig. 3a; Table  
145 S1), but  $g_{\max}$  is 49% (17-88% 95% CI;  $P = 0.001$ ) and 14% (1-30% 95% CI;  $P = 0.04$ ) higher in  
146 Angiosperms than Gymnosperms and Pteridophytes, respectively (Table S2). Trees also have  
147 18% (8-28% 95% CI;  $P < 0.0001$ ) and 48% (39-59% 95% CI;  $P < 0.0001$ ) greater  $g_{\max}$  than  
148 shrubs and herbs, respectively (Table S2). The mean and variance in  $\log(g_{\max})$  are nearly  
149 invariant across latitude, temperature, and precipitation gradients, indicating that most of the  
150 variation in  $g_{\max}$  occurs within rather than between forest sites (Fig. 4).

151 Our results overturn the prevailing view that size-density scaling results in minimized  
152 stomatal area allocation. Instead, we find that negative covariance between stomatal size and  
153 density is consistent with stomatal area allocation increasing with  $g_{\max}$ , and, furthermore that

154 limits on the fraction of epidermis allocated to stomatal ( $f_s$ ) are a secondary consequence of  
155 limits on  $g_{\max}$ . Our results differ from previous studies because we adopted a new theoretical  
156 framework based on quantitative genetic and macroevolutionary theory. Previous analyses<sup>31</sup>  
157 estimated scaling exponents close to 1 for hypostomatous leaves using standardized major axis  
158 (SMA) regression, which finds the scaling exponent which minimizes residual variance in both  
159  $d_s$  and  $a_s$ . SMA regression, adopted from allometry, does not enable analysis of how stomatal  
160 size-density scaling shapes covariance between constituent traits (see Supplementary  
161 Information). Although estimated scaling using standard phylogenetic regression approaches (see  
162 Methods), it is more appropriate to interpret the results not as minimizing residual variance, but  
163 rather estimating the  $\beta$  consistent with the covariance structure of stomatal size and density (Fig.  
164 1).

165 Our results have at least three important implications for understanding the evolutionary  
166 consequences of the stomatal size-density scaling relationship. First, size-density scaling implies  
167 that extreme values of  $g_{\max}$  do not provide competitive advantage across environments. Low  $g_{\max}$   
168 limits plants productivity by reducing the CO<sub>2</sub> supply for photosynthesis under favorable  
169 conditions. Excessively high  $g_{\max}$  may be rare because selection on  $g_{\max}$  most likely ensures a  
170 proper  $g_{\text{op}}:g_{\max}$  ratio that situates leaf  $g_{\text{op}}$  in a region of maximal control to respond rapidly to  
171 changing environments<sup>8</sup>. High anatomical  $g_{\max}$  may also increase the risk of hydraulic failure<sup>32</sup>  
172 especially under transient conditions, thereby setting a physical upper limit to leaf gas exchange.  
173 Other possible costs include interference of stomatal movements and diffusion, as well as  
174 energetic costs<sup>31,33</sup>. The scaling between size and density is consistent with excessive  $g_{\max}$   
175 incurring a major cost that cannot be obviated by stomatal area-minimization. Future work  
176 should prioritize identifying the fitness costs and evolutionary constraints that prevent higher



177  $g_{\max}$  from evolving. Second, if  $g_{\max}$  is the primary constraint, this implies that plants could  
178 allocate a greater fraction of their epidermal area to stomata than they currently do without  
179 incurring a major cost. For example, we predict that if stomatal size and density could be  
180 manipulated independently, anatomies with the same  $g_{\max}$ , but different  $f_s$ , would have similar  
181 fitness. Another important corollary is that smaller stomata were not directly required to increase  
182  $g_{\max}$  in angiosperms. All three major land plant lineages have similar variance in  $g_{\max}$  (Fig. 3b);  
183 gymnosperms and pteridophytes have lower average  $g_{\max}$  because they have on average a lower  
184  $d_s$  for a given  $a_s$ , not because of differences in the scaling relationship. Increased  $g_{op}$  may be an  
185 indirect consequence of higher stomatal density being linked to increases in leaf water transport  
186 capacity, for example, by decreasing the distance between vein and stomata, allowing stomata to  
187 stay open<sup>40</sup>. The primary constraint on maximum stomatal conductance appears to be selection  
188 itself, implying that vegetation and crop models should incorporate costs of extreme trait values  
189 in their predictions.

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## 191 **Methods**

### 192 *Stomatal trait data from global forests*

193 The stomatal dataset of global forests involved the information of a total of 2408 plant  
194 species from natural forests. The data consists of novel field data collected from Chinese forest  
195 communities and a compilation of published literature values.

196 Our field data were collected from 28 typical forest communities occurring between 18.7 °N and  
197 53.3 °N latitude in China. The field sites were selected to cover most of the forest types in the  
198 northern hemisphere, including cold-temperate coniferous forest, temperate deciduous forest,  
199 subtropical evergreen forest, and tropical rain forest (Fig. 2). In total, we sampled 28 forest sites.  
200 We used the Worldclim database<sup>34</sup> to extract additional data on mean annual temperature  
201 (MAT) and precipitation (MAP) over the period 1960-1990 using latitude and longitude. Among  
202 these forests, mean annual temperature (MAT) ranged from -5.5-23.2 °C, and mean annual  
203 precipitation (MAP) varied from 320 to 2266 mm. The field investigation was conducted in July-  
204 August, during the peak period of growth for forests. Sampling plots were located within well-  
205 protected national nature reserves or long-term monitoring plots of field ecological stations, with  
206 relatively continuous vegetation. Four experimental plots (30 × 40 m) were established in each  
207 forest.

208 Leaves from trees, shrubs, and herbs were collected within and around each plot. For trees,  
209 mature leaves were collected from the top of the canopy in four healthy trees and mixed as a  
210 composite sample. Eight to 10 leaves from the pooled samples were cut into roughly 1.0 × 0.5  
211 cm pieces along the main vein, and were fixed in formalin-aceto-alcohol (FAA) solution (5 ml  
212 38 % formalin, 90 ml 75 % ethanol, 5 ml 100 % glacial acetic acid, and 5 ml 37 % methanol)<sup>35</sup>.

213 In the laboratory, three small pieces were randomly sampled, and each replicate was  
214 photographed twice using a scanning electron microscopy (Hitachi SN-3400, Hitachi, Tokyo,  
215 Japan) on the lower surface at different positions. We focused on the lower epidermis<sup>22</sup>, because  
216 a previous study has demonstrated that most of leaf upper epidermis has no stomata for forest  
217 plants<sup>36</sup>.

218 In each photograph, the number of stomata was recorded, and  $D_S$  was calculated as the  
219 number of stomata per unit leaf area. Simultaneously, five typical stomata were selected to  
220 measure stomatal size using an electronic image analysis equipment (MIPS software, Optical  
221 Instrument Co. Ltd., Chongqing, China).

222 Peer-reviewed papers on leaf stomata were collected using an all-databases search of Web  
223 of Science ([www.webofknowledge.com](http://www.webofknowledge.com)) from 1900 to 2018 using “forest” and “stomata” as a  
224 topic, in line with the principle of “nature forest, non-intervention, species name”. There were a  
225 total of 90 papers (see Supporting Table S3) which met our requirements, yielding  $D_S$  and  $L$   
226 measurements from 413 plant species (Fig. 2) from which we calculated  $g_{\max}$  and  $f_S$ .  $f_S$  is  
227 proportional to the stomatal pore area index (SPI), which defined as the product of  $D_S$  and  
228 stomatal length ( $L$ ) squared<sup>37</sup>, because  $A_S = mL^2$ <sup>23</sup>.

229 We calculated  $g_{\max}$  (Equation 1) to water vapor at a reference leaf temperature ( $T_{\text{leaf}} = 25^\circ$   
230 C) following Sack and Buckley<sup>23</sup>. They defined a biophysical and morphological constant as:

$$b = \frac{D_{\text{wv}}}{v}$$
$$m = \frac{\pi c^2}{j^{0.5}(4hj + \pi c)}$$

231  $b$  is the diffusion coefficient of water vapor in air ( $D_{wv}$ ) divided by the kinematic viscosity of  
232 dry air ( $\nu$ ).  $D_{wv} = 2.49 \times 10^{-5} \text{ m}^2 \text{ s}^{-1}$  and  $\nu = 2.24 \times 10^{-2} \text{ m}^3 \text{ mol}^{-1}$  at  $25^\circ\text{C}$ <sup>38</sup>. For kidney-  
233 shaped guard cells,  $c = h = j = 0.5$ ; for dumbbell-shaped guard cells in the Poaceae,  $c = h =$   
234  $0.5$  and  $j = 0.125$ . We used the species average  $g_{\max}$  and  $f_s$  for all analyses.

### 235 *Phylogenetic regression*

236 By positing that the least variable composite of stomatal size and density indicates the trait  
237 with the most constraint (Fig. 1), we identify a new way to estimate the scaling exponent  $\beta$  (Eq.  
238 6) using linear regression estimates, but these estimates should still account for phylogenetic  
239 nonindependence. We used the Plant List (<http://www.theplantlist.org>) to confirm species names,  
240 then we assembled a synthetic phylogeny using S.PhyloMaker<sup>39</sup>. We fit phylogenetic regression  
241 models using the **phylolm** version 2.6 package in R<sup>40</sup>. As we derived in the main text, the  
242 scaling exponent  $\beta$  can be estimated from the slope of the regression of  $a_s$  on  $d_s$ , where  $\hat{\beta} =$   
243  $-\text{slope}$ . We estimated separate scaling exponents for major groups, Angiosperms, Pteridophytes,  
244 and Gymnosperms. We also estimated different intercepts, corresponding with different average  
245  $g_{\max}$  values, for functional types (herbs, shrubs, and trees) and grasses, because of their unique  
246 stomatal anatomy. We used the “OUrandomRoot” model of trait evolution. 95% confidence  
247 intervals for all parameters were estimated from 1000 parametric bootstrap samples generated by  
248 simulating from the best-fit model and re-fitting.  $P$ -values for coefficients are based on  $t$ -tests.  
249 We used the same methods to test whether  $g_{\max}$  (log-transformed for homoskedasticity) was  
250 affected by  $|\text{latitude}|$ , MAP, MAT, group (Angiosperms, Pteridophytes, Gymnosperms), and/or  
251 functional type (herb, shrub, tree). One gymnosperm species, *Torreya fargesii*, had substantially  
252 lower stomatal size than would be predicted from its density (Fig. 3a). These results of the paper  
253 did not change if this outlier was excluded because the confidence intervals for stomatal-density

254 scaling are very wide for Gymnosperms regardless. Therefore, we excluded this species from  
255 statistical analyses but show it in the figure for completeness. All data were analyzed in R  
256 <sup>41</sup>version 4.0.5

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## 277 References

- 278 1 Berry, J. A., Beerling, D. J. & Franks, P. J. Stomata: key players in the earth system, past  
279 and present. *Curr. Opin. Plant Biol.* **13**, 232-239 (2010).
- 280 2 Cramer, W. *et al.* Global response of terrestrial ecosystem structure and function to CO<sub>2</sub>  
281 and climate change: results from six dynamic global vegetation models. *Glob. Change*  
282 *Biol.* **7**, 357-373 (2001).
- 283 3 Haworth, M., Elliott-Kingston, C. & McElwain, J. C. Stomatal control as a driver of plant  
284 evolution. *J. Exp. Bot.* **62**, 2419-2423 (2011).
- 285 4 Jordan, G. J., Carpenter, R. J., Koutoulis, A., Price, A. & Brodribb, T. J. Environmental  
286 adaptation in stomatal size independent of the effects of genome size. *New Phytol.* **205**,  
287 608-617 (2015).
- 288 5 Hetherington, A. M. & Woodward, F. I. The role of stomata in sensing and driving  
289 environmental change. *Nature* **424**, 901-908 (2003).
- 290 6 Franks, P. J. *et al.* Sensitivity of plants to changing atmospheric CO<sub>2</sub> concentration: from  
291 the geological past to the next century. *New Phytol.* **197**, 1077-1094 (2013).
- 292 7 Haworth, M., Elliott-Kingston, C. & McElwain, J. C. Co-ordination of physiological and  
293 morphological responses of stomata to elevated CO<sub>2</sub> in vascular plants. *Oecologia* **171**,  
294 71-82 (2013).
- 295 8 Franks, P. J., Leitch, I. J., Ruszala, E. M., Hetherington, A. M. & Beerling, D. J.  
296 Physiological framework for adaptation of stomata to CO<sub>2</sub> from glacial to future  
297 concentrations. *Philos. T. R. Soc. B.* **367**, 537-546 (2012).
- 298 9 Deans, R. M., Brodribb, T. J., Busch, F. A. & Farquhar, G. D. Optimization can provide  
299 the fundamental link between leaf photosynthesis, gas exchange and water relations. *Nat.*  
300 *Plants* **6**, 1116-1125 (2020).
- 301 10 Ordoñez, J. C. *et al.* A global study of relationships between leaf traits, climate and soil  
302 measures of nutrient fertility. *Glob. Ecol. Biogeogr.* **18**, 137-149 (2009).
- 303 11 Yuan, Z. Y. & Chen, H. Y. H. Global-scale patterns of nutrient resorption associated with  
304 latitude, temperature and precipitation. *Glob. Ecol. Biogeogr.* **18**, 11-18 (2009).
- 305 12 Díaz, S. *et al.* The global spectrum of plant form and function. *Nature* **529**, 167-171  
306 (2016).
- 307 13 Freschet, G. T. *et al.* Climate, soil and plant functional types as drivers of global fine-root  
308 trait variation. *J. Ecol.* **105**, 1182-1196 (2017).
- 309 14 Brown, H. T. & Escombe, F. Static diffusion of gases and liquids in relation to the  
310 assimilation of carbon and translocation in plants. *Proc. R. Soc. Lond.* **67**, 124-128  
311 (1901).
- 312 15 Parlange, J.-Y. & Waggoner, P. E. Stomatal dimensions and resistance to diffusion. *Plant*  
313 *Physiol.* **46**, 337-342 (1970).
- 314 16 Franks, P. J. & Farquhar, G. D. The effect of exogenous abscisic acid on stomatal  
315 development, stomatal mechanics, and leaf gas exchange in *Tradescantia virginiana*.  
316 *Plant Physiol.* **125**, 935-942 (2001).
- 317 17 Vatén, A. & Bergmann, D. C. Mechanisms of stomatal development: an evolutionary  
318 view. *EvoDevo* **3**, 11 (2012).
- 319 18 McElwain, J. C., Yiotis, C. & Lawson, T. Using modern plant trait relationships between  
320 observed and theoretical maximum stomatal conductance and vein density to examine  
321 patterns of plant macroevolution. *New Phytol.* **209**, 94-103 (2016).

- 322 19 Conesa, M. À., Muir, C. D., Molins, A. & Galmés, J. Stomatal anatomy coordinates leaf  
323 size with Rubisco kinetics in the Balearic *Limonium*. *AoB Plants* **12** (2019).
- 324 20 Murray, M. *et al.* Consistent relationship between field-measured stomatal conductance  
325 and theoretical maximum stomatal conductance in c3 woody angiosperms in four major  
326 biomes. *Int. J. Plant Sci.* **181**, 142-154 (2020).
- 327 21 Wang, R. *et al.* Latitudinal variation of leaf stomatal traits from species to community  
328 level in forests: linkage with ecosystem productivity. *Sci. Rep.* **5**, 14454 (2015).
- 329 22 Liu, C. *et al.* Variation of stomatal traits from cold temperate to tropical forests and  
330 association with water use efficiency. *Funct. Ecol.* **32**, 20-28 (2018).
- 331 23 Sack, L. & Buckley, T. N. The developmental basis of stomatal density and flux. *Plant*  
332 *Physiol.* **171**, 2358-2363 (2016).
- 333 24 Weiss, A. G. *Untersuchungen über die Zahlen-und Grössenverhältnisse der*  
334 *Spaltöffnungen*. Vol. 4 125-196 (Jahrbücher für Wissenschaftliche Botanik, 1865).
- 335 25 Franks, P. J. & Beerling, D. J. Maximum leaf conductance driven by CO<sub>2</sub> effects on  
336 stomatal size and density over geologic time. *Proc. Natl. Acad. Sci. USA.* **106**, 10343-  
337 10347 (2009).
- 338 26 Pennell, M. W. & Harmon, L. J. An integrative view of phylogenetic comparative  
339 methods: connections to population genetics, community ecology, and paleobiology. *Ann*  
340 *N.Y. Acad. Sci.* **1289**, 90-105 (2013).
- 341 27 Hansen, T. F. Stabilizing selection and the comparative analysis of adaptation. *Evolution*  
342 **51**, 1341-1351 (1997).
- 343 28 Uyeda, J. C. & Harmon, L. J. A novel bayesian method for inferring and interpreting the  
344 dynamics of adaptive landscapes from phylogenetic comparative data. *Syst. Biol.* **63**, 902-  
345 918 (2014).
- 346 29 Boucher, F. C., Démery, V., Conti, E., Harmon, L. J. & Uyeda, J. A general model for  
347 estimating macroevolutionary landscapes. *Syst. Biol.* **67**, 304-319 (2017).
- 348 30 Walsh, B. & Blows, M. W. Abundant Genetic Variation + Strong Selection =  
349 Multivariate Genetic Constraints: A Geometric View of Adaptation. *Annu. Rev. Ecol.*  
350 *Evol. S.* **40**, 41-59 (2009).
- 351 31 de Boer, H. J. *et al.* Optimal allocation of leaf epidermal area for gas exchange. *New*  
352 *Phytol.* **210**, 1219-1228 (2016).
- 353 32 Henry, C. *et al.* A stomatal safety-efficiency trade-off constrains responses to leaf  
354 dehydration. *Nat. Commun.* **10**, 3398 (2019).
- 355 33 Farquhar, G. D., Cowan, I. R. & Zeiger, E. *Stomatal Function*. (Stanford University  
356 Press, 1987).
- 357 34 Hijmans, R. J., Cameron, S. E., Parra, J. L., Jones, P. G. & Jarvis, A. Very high resolution  
358 interpolated climate surfaces for global land areas. *Int. J. Climatol.* **25**, 1965-1978 (2005).
- 359 35 He, N. *et al.* Variation in leaf anatomical traits from tropical to cold-temperate forests and  
360 linkage to ecosystem functions. *Funct. Ecol.* **32**, 10-19 (2018).
- 361 36 Muir, C. D. Light and growth form interact to shape stomatal ratio among British  
362 angiosperms. *New Phytol.* **218**, 242-252 (2018).
- 363 37 Sack, L., Cowan, P. D., Jaikumar, N. & Holbrook, N. M. The ‘hydrology’ of leaves: co-  
364 ordination of structure and function in temperate woody species. *Plant Cell Environ.* **26**,  
365 1343-1356 (2003).
- 366 38 Monteith, J. L. & Unsworth, M. H. *Principles of environmental physics: plants, animals,*  
367 *and the atmosphere*. (Elsevier/Academic Press, 2013).

- 368 39 Qian, H. & Jin, Y. An updated megaphylogeny of plants, a tool for generating plant  
369 phylogenies and an analysis of phylogenetic community structure. *J. Plant Ecol.* **9**, 233-  
370 239 (2015).
- 371 40 Ho, L. & Ané, C. A linear-time algorithm for Gaussian and non-Gaussian trait evolution  
372 models. *Syst. Biol.* **63**, 397-408 (2014).
- 373 41 R Core Team. *R: A Language and Environment for Statistical Computing*. (R Foundation  
374 for Statistical Computing, 2019)  
375

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384 should contact to N.P. He ([henp@igsnr.ac.cn](mailto:henp@igsnr.ac.cn)). There are no conflicts of interest to declare.

### 385 **Author contributions**

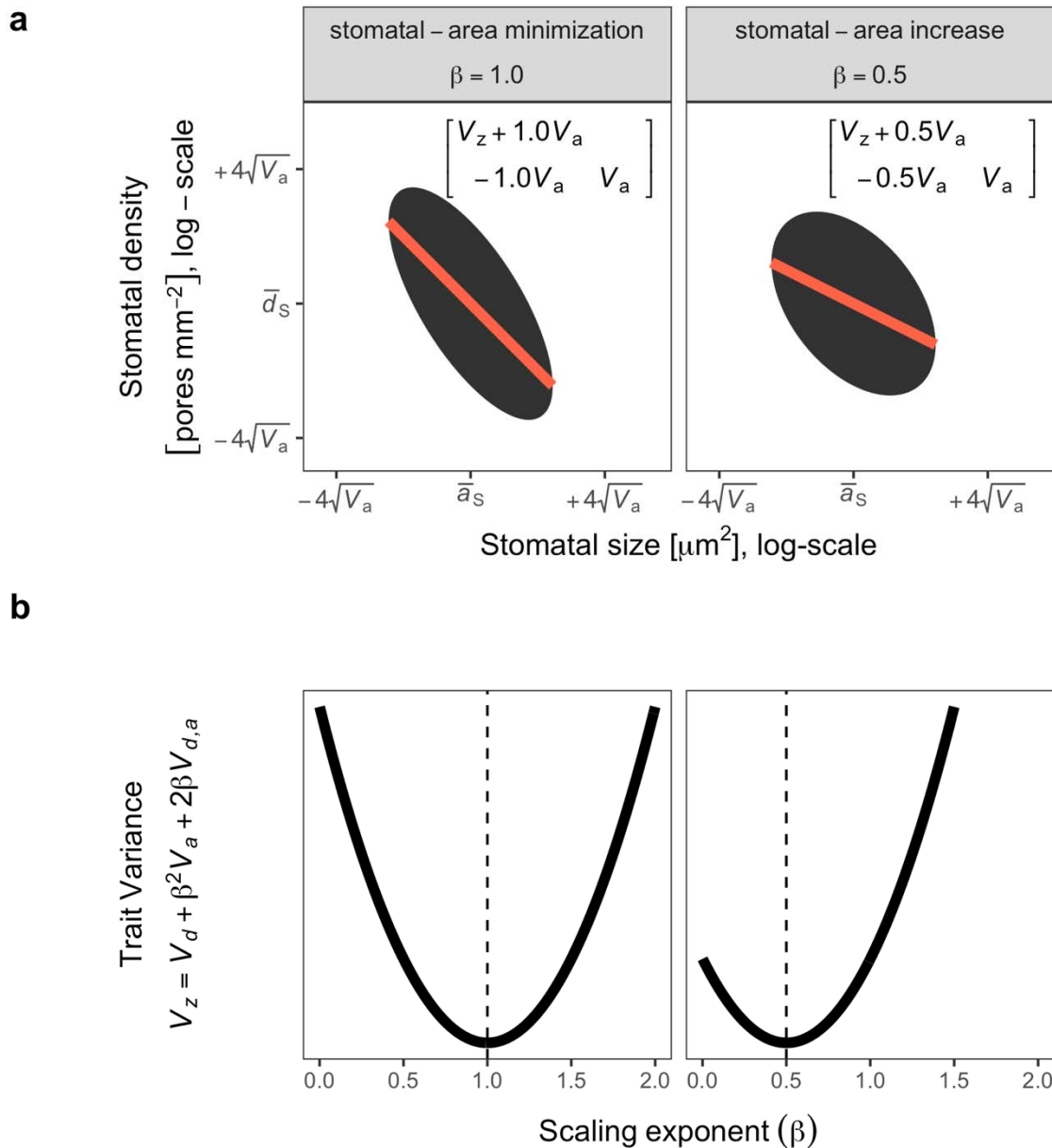
386 N.H. and G.Y. designed field sampling; N.H., C.D.M, and C.L. conceived the initial ideas; C.L.,  
387 N.H., Y.L. J.Z., M.L. and L.X collected the data; C.L. writed the first draft, and C.D.M. made  
388 major revision on it, and C.D.M. contributed the final mathematical derivations, data analysis,  
389 and wrote the final manuscript; L.S., H.J.B., C.L., N.H., G.Y., and X.H. revised the manuscript.  
390 All authors gave final approval for publication.

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392 **Supplementary information**

393 **Figures**



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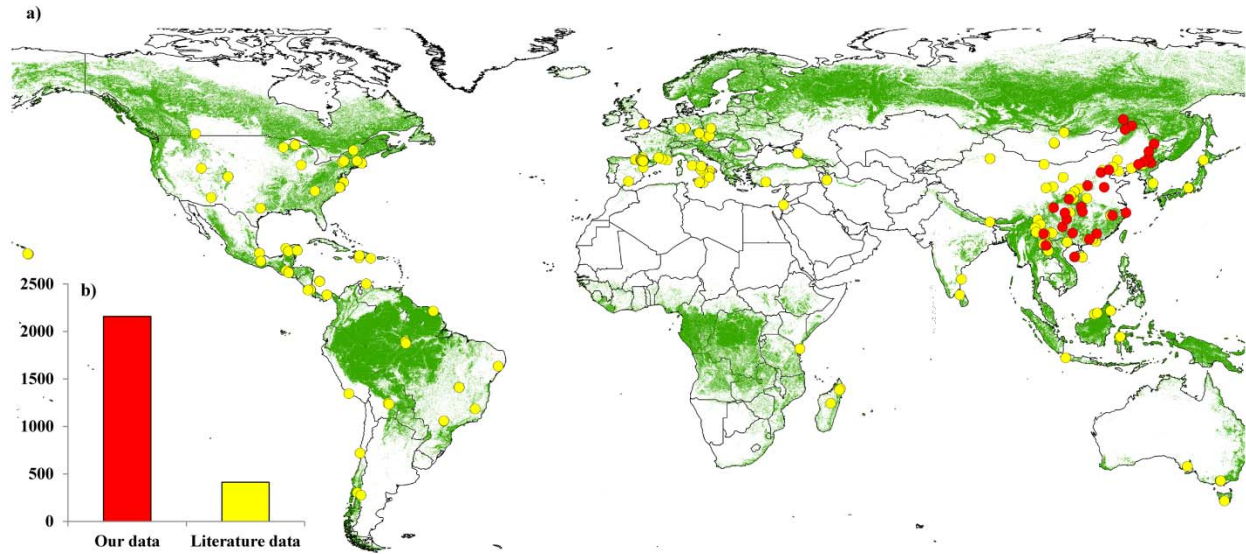
395 **Fig. 1 | Competing hypotheses for the origin of negative stomatal size-density scaling make**

396 **different predictions about the trait covariance structure.** Maximum stomatal conductance

397 ( $g_{\max}$ ) and the fraction of epidermal area allocated to stomata ( $f_s$ ) are composite traits determined

398 by stomatal density and size. On a log-scale, they are the sum of log-stomatal density ( $d_s$ ) and

399 log-stomatal size ( $a_s$ ) times a scaling exponent ( $\beta$ ), 0.5 for  $g_{\max}$  and 1.0 for  $f_s$  (see Methods).  
400 There are infinite number of other composite traits, which we denote  $z_s$ , associated with different  
401 values of  $\beta$  (Eq. 4). **a.** A unique stomatal size-density covariance structure is associated with the  
402  $\beta$  that minimizes the variance in the resulting composite trait. If the means ( $\bar{d}_s, \bar{a}_s$ ) and variances  
403 ( $V_d, V_a$ ) of stomatal density and size, respectively, can be measured, the covariance between  
404 them ( $V_{d,a}$ ) is equal to  $-\beta V_a$ . Under the stomatal-area increase (left panel) and stomatal-area  
405 minimization (right panel) hypotheses,  $\beta$  should be 0.5 and 1, respectively. The ellipse is the  
406 0.95 quantile of covariance ellipse associated with the covariance matrix (upper right corner of  
407 the plot); the orange line is the scaling exponent fit through the constituent trait means. **b.** The  
408 covariance structure depicted in **a** is that which minimizes the variance in a composite trait ( $V_z$ )  
409 given a hypothesized scaling exponent.

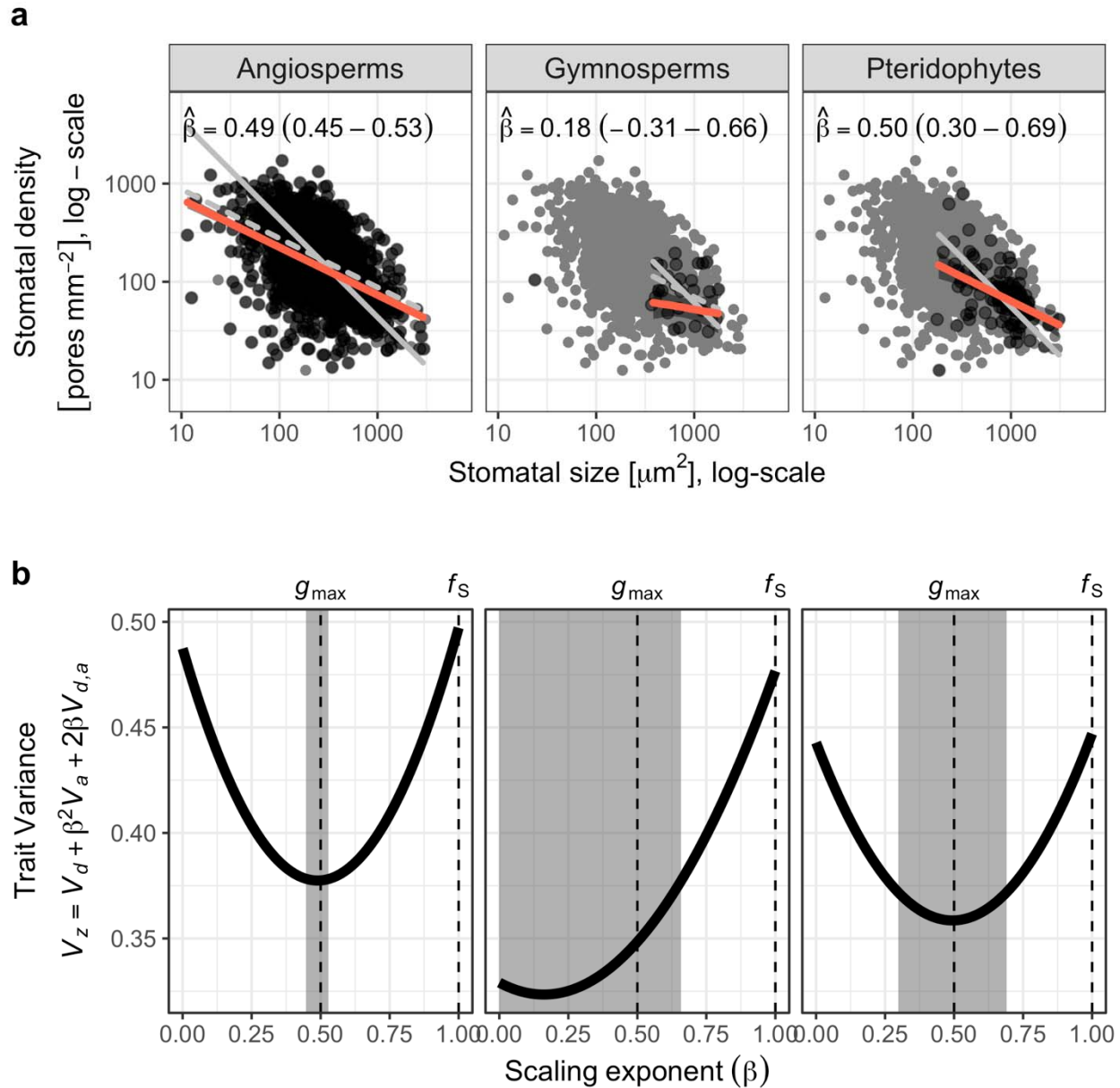


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411

412 **Fig. 2 | Geographic distribution of sampling sites (a) and the number of plant species (b) in**

413 **this study.**



414

415 **Fig. 3 | Stomatal size-density scaling is consistent with stomata-area increase but not area-**

416 **minimization. a.** In both angiosperms (left panel) and pteridophytes (right panel), the scaling

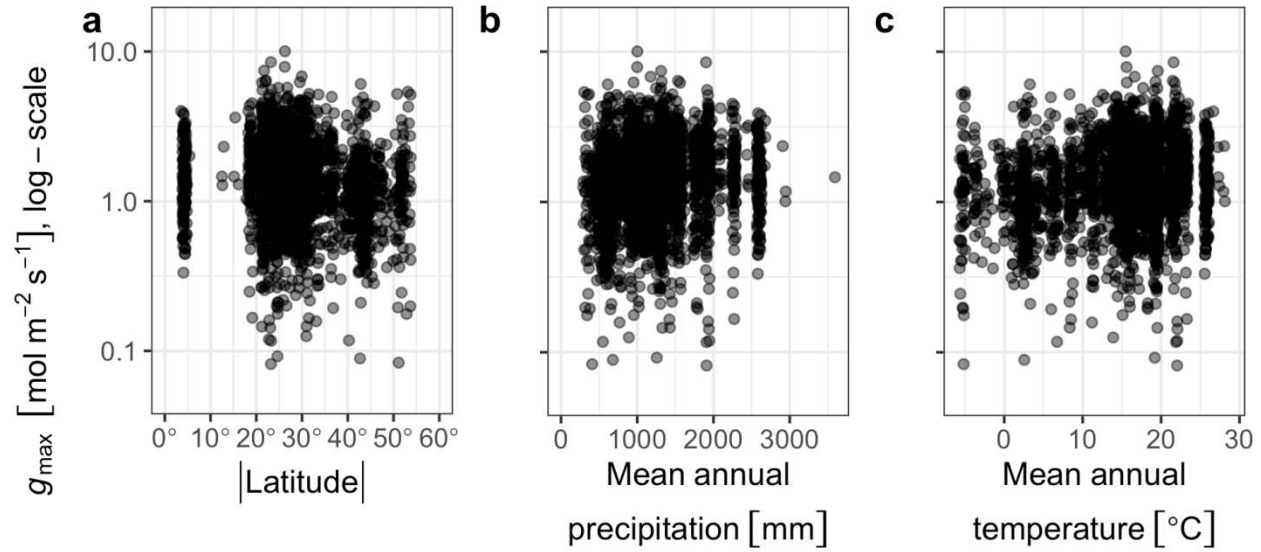
417 exponent ( $\beta$ ) estimated as the phylogenetic linear regression slope of stomatal size against

418 density (Methods) is close to 0.5 as predicted by the stomatal-area increase hypothesis, but much

419 less than 1.0, as predicted by the stomatal-area minimization hypothesis. For comparison, thin

420 gray lines in the background show predicted slopes for each group when  $\beta = 1.0$  (solid line) and

421  $\beta = 0.5$  (dashed line). The bootstrap 95% confidence intervals are in parentheses and shown  
422 graphically by the width of the grey rectangle in **b**. Dark points represent species mean trait  
423 values from the focal group; grey background points are from all groups for comparison. Orange  
424 line and ribbon are the estimated phylogenetic regression line and the 95% bootstrap confidence  
425 intervals. Scaling in gymnosperms (middle panel) is not significantly different from 0 or 0.5, but  
426 the confidence intervals do not include 1.0. **b**. The variance of the composite trait ( $V_z$ ) is  
427 minimized near  $\beta = 0.5$ , as predicted under the stomatal-area increase hypothesis (dashed-line  
428 under  $g_{\max}$ ) but not where  $\beta = 1.0$  as predicted by the stomatal-area minimization hypothesis  
429 (dashed-line under  $f_s$ ).



430

431 **Fig. 4 | Anatomical maximum stomatal conductance varies little with latitude, mean annual**

432 **precipitation, or mean annual temperature.** Each point is the species' mean |latitude| (a.),

433 mean annual precipitation (b.), or mean annual temperature (c.) on the  $x$ -axis and the maximum

434 stomatal conductance ( $g_{\max}$ ) on the  $y$ -axis (log-scale).

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