New Phytologist

Research

Photosynthetic pathway and ecological adaptation explain stomatal trait diversity amongst grasses

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Received: *5 May 2011*
Accepted: *12 September 2011*

New Phytologist (2012) **193**: 387–396
doi: 10.1111/j.1469-8137.2011.03935.x

Key words: C_3 photosynthesis, C_4 photosynthesis, cell size, climate niche, evolution, functional diversity, stomata, stomatal development.

Summary

- The evolution of C_4 photosynthesis in plants has allowed the maintenance of high CO_2 assimilation rates despite lower stomatal conductances. This underpins the greater water-use efficiency in C_4 species and their tendency to occupy drier, more seasonal environments than their C_3 relatives.
- The basis of interspecific variation in maximum stomatal conductance to water (g_{max}), as defined by stomatal density and size, was investigated in a common-environment screening experiment. Stomatal traits were measured in 28 species from seven grass lineages, and comparative methods were used to test for predicted effects of C_3 and C_4 photosynthesis, annual precipitation and habitat wetness on g_{max}.
- Novel results were as follows: significant phylogenetic patterns exist in g_{max} and its determinants, stomatal size and stomatal density; C_4 species consistently have lower g_{max} than their C_3 relatives, associated with a shift towards smaller stomata at a given density. A direct relationship between g_{max} and precipitation was not supported. However, we confirmed associations between C_4 photosynthesis and lower precipitation, and showed steeper stomatal size–density relationships and higher g_{max} in wetter habitats.
- The observed relationships between stomatal patterning, photosynthetic pathway and habitat provide a clear example of the interplay between anatomical traits, physiological innovation and ecological adaptation in plants.

Introduction

The stomatal pores that perforate leaf surfaces are one of the best-characterized examples of the fundamental biological relationship between form and function (Hetherington & Woodward, 2003). The area and depth of each stomatal pore, together with the density of the stomata, determine the stomatal conductance to CO_2 and H_2O (Brown & Escombe, 1900; Parlange & Waggoner, 1970; Franks & Beerling, 2009a; Nobel, 2009), gaseous diffusion being regulated through turgor-mediated variation in the aperture of stomatal pores (Raschke, 1975; Buckley, 2005; Franks & Farquhar, 2007). The closure of stomata under dry atmospheric or soil conditions limits CO_2 diffusion from the atmosphere to chloroplasts, and means that stomatal physiology is inextricably linked to the physiology of photosynthesis (Farquhar & Sharkey, 1982). As a result, the patterning of stomata on leaf surfaces is correlated strongly with both hydrological conditions (Aasamaa *et al.*, 2001; Sack *et al.*, 2003; Franks *et al.*, 2009) and photosynthetic capacity (Franks & Beerling, 2009a,b).

The evolution of the C_4 pathway has caused radical increases in potential photosynthetic capacity. The C_4 syndrome is one of the most important functional innovations in plants, and is particularly prevalent in grasses, where it occurs in *c.* 18 lineages and is utilized by around half of all modern species (Kellogg, 1999; Sage, 2004; Christin *et al.*, 2008, 2009). The C_4 pathway operates as a CO_2-concentrating mechanism, elevating CO_2 concentrations locally around the carbon-fixing enzyme Rubisco, with the result that the rate of its carboxylase reaction is increased (Chollett & Ogren, 1975). In combination with the saturation of Rubisco in the bundle sheath, the C_4 pathway can also deplete CO_2 to lower concentrations within leaf airspaces before photosynthesis is limited (Björkman, 1970; Bauwe, 1986). This, in turn, allows the same rate of photosynthesis to be maintained with a lower stomatal conductance in C_4 than C_3 leaves (Björkman, 1970; Long, 1999). Each evolutionary origin of C_4 photosynthesis from a C_3 ancestor might therefore be expected to present an opportunity for an associated reduction in the maximum stomatal conductance, providing water-use benefits over C_3 sister taxa. However, this hypothesis remains untested.

Recent comparative studies of grasses have indicated that C_4 photosynthesis is an adaptation to low atmospheric CO_2 (Christin *et al.*, 2008; Vicentini *et al.*, 2008) and open habitats (Osborne & Freckleton, 2009), evolving at high temperatures and permitting the colonization of drier, more seasonal subtropical environments

(Edwards & Smith, 2010). This ecological transition from forested, higher rainfall environments to drier, more open habitats is also expected to have driven the evolution of stomatal patterning and maximum stomatal conductance (Hetherington & Woodward, 2003). Grasses exhibit further distinct traits relating to the efficiency and speed of guard cell movement (Franks & Farquhar, 2007), which are also thought to have facilitated adaptation to open environments (Hetherington & Woodward, 2003). However, the extent to which the diversity of stomatal traits among grasses is linked to habitat remains unknown.

We hypothesized that, across a diversity of independent evolutionary origins, C_4 grasses would consistently exhibit lower maximum stomatal conductance to H_2O (g_{max}) than C_3 grasses, associated with evolutionary shifts in stomatal patterning. Our recent work, which has emphasized the importance of controlling for phylogenetic diversity in comparisons of eco-physiological traits, has demonstrated that, on average, C_4 grasses across multiple lineages operate with lower stomatal conductance than species from C_3 sister lineages (Taylor *et al.*, 2010, 2011). Here, we use comparative methods to address the following questions. Is C_4 photosynthesis associated with reduced g_{max} compared with the C_3 type? Are differences in g_{max} between species associated with precipitation or habitat water availability? Amongst grass lineages, do pore size and density, which determine g_{max}, show consistent patterns associated with photosynthetic type and ecological niche?

Materials and Methods

Species sampling and phylogeny

Species (Supporting Information Fig. S1) were sampled from C_4 and closely related C_3 lineages on the basis of phylogenetic information that was available in 2007 (Barker *et al.*, 2001; Giussani *et al.*, 2001; Aliscioni *et al.*, 2003). Most groups included multiple species to allow for analysis within an ANOVA framework (Taylor *et al.*, 2010). Here, we combined previously unpublished data on stomatal traits with a new phylogeny based on three plastid regions: the coding genes *rbc*L and *ndh*F, and the region encompassing *trn*K introns and the *mat*K coding sequence. These markers were retrieved from GenBank and *de novo* sequencing was used to complete the dataset with, in most cases, the same accessions as were considered for the measurements of stomata.

Genomic DNA (gDNA) was isolated from seeds or dried plant tissues with the FastDNA Spin Kit (MP Biomedicals, Aurora, OH, USA). The three markers were then PCR amplified in multiple overlapping fragments of 600–800 bp with published and newly developed primers (Table S1). PCRs were carried out in a total volume of 50 μl, including *c.* 100 ng of gDNA template, 10 μl of 5 × GoTaq Reaction Buffer, 0.15 mM deoxynucleoside triphosphates (dNTPs), 0.2 μM of each primer, 2 mM of $MgCl_2$ and 1 unit of *Taq* polymerase (GoTaq DNA Polymerase; Promega, Madison, WI, USA). The PCR mixtures were incubated in a thermocycler for 3 min at 94°C, followed by 37 cycles consisting of 1 min at 94°C, 30 s at 48°C and 1 min at 72°C. This was followed by 10 min at 72°C. Successful amplifications were cleaned with an Exo-SAP treatment and sequenced using Big Dye 3.1 Terminator Cycle Sequencing chemistry (Applied Biosystems, Foster City, CA, USA). All sequences were deposited in GenBank. The three markers were aligned using ClustalW (Thompson *et al.*, 1994) and the alignments were then manually edited. The total length of the DNA markers exceeded 6000 bp per species (Table S2). A phylogenetic tree was obtained through Bayesian inference as implemented in MrBayes 3.1 (Ronquist & Huelsenbeck, 2003), under a general time-reversible substitution model with a gamma shape parameter and a proportion of invariant sites (GTR + G + I). Two different analyses, each of four parallel chains, were run for 10 000 000 generations, sampling a tree each 1000th generation after a burn-in period of 3 000 000. A consensus tree was computed on the 14 000 sampled trees (Fig. S1).

Plant material and growing conditions

Plants were raised primarily from seed. Seeds were surface sterilized before germination on water agar, and then allowed to establish in plugs of compost (John Innes Seed Compost) before transplanting into 4-l pots of topsoil (Lawnmix topsoil®; Dandy's Topsoil, Chester, UK). A minority of species were propagated vegetatively (*Arundo donax*, *Arundo formosana*, *Hakonechloa macra*) and transplanted directly into pots of topsoil. Plants were grown in a heated glasshouse in Sheffield, UK, between 21st May and 18th October 2007 (daily quantum input (mol m^{-2} d^{-1}): mean, 9.7; maximum, 24.7; minimum, 1.9; relative humidity (%): daily mean 64; maximum, 92; minimum, 28; temperature (°C): daily mean, 20; maximum, 28; minimum, 15; recorded using a DL2e datalogger with RHT2nl and QS2 sensors; Delta-T Devices Ltd, Cambridge, UK). Species were randomized within eight blocks and plants were watered to saturation at least twice weekly. No supplementary nutrients were provided.

Measurement of stomatal traits

The youngest fully emerged leaf was removed at the ligule from one tiller of each plant in each experimental block. Leaves were taped onto sheets of newspaper to prevent curling, and allowed to air dry in a flower press. Dental putty (President Plus-light body; Coltène/Whaledent Ltd, Burgess Hill, West Sussex, UK) impressions were taken from the mid-section of both surfaces of the preserved leaves, and nail varnish peels produced from the impressions were transferred onto Polysine microscope slides (SLS; Hessle, North Humberside, UK). Stomatal guard cell length, pore length and pore density were measured using a microscope, camera and image processing equipment (Leitz Laborlux S; Leica Quantimet 500 running Quantimet 500 Q win software, Leica Microsystems (UK) Ltd, Milton Keynes, Buckinghamshire, UK; Sanyo CCD, SANYO Sales & Marketing Europe GmbH, Watford, Hertfordshire, UK). On each slide, along a diagonal transect of the peel, five stomata were measured for guard cell and pore lengths at 400× magnification. The stomatal density on each leaf surface was determined as the mean number of stomata visible in five 0.25-mm^2 fields of view, sampled along the diagonal of each peel.

Calculation of g_{max}

Maximum stomatal conductance to water vapour (g_{max}) was calculated as the sum of the maximum conductance values for each side of each leaf (g_1 + g_2), based on the model of Brown & Escombe (1900) after Franks & Beerling (2009a). Alternative formulations of the Brown and Escombe model have been described by Weyers & Meidner (1990: pp. 56–57) (see also discussion in Franks & Farquhar, 2001). The equation for g of one side of the leaf is

$$g_i = \frac{d}{v} \cdot D \cdot \frac{a_{max}}{l + \frac{\pi}{2}\sqrt{\frac{a_{max}}{\pi}}}, \quad \text{Eqn 1}$$

where the subscript i indicates the relative conductance to water vapour: i = 1 for the side of the leaf with the minimum value of g and i = 2 for the side of the leaf with the maximum value of g. The diffusivity of water in air (d, $m^2\ s^{-1}$, at 25°C), the molar volume of air (v, $m^3\ mol^{-1}$, at 25°C) and π are physical and geometric constants. The stomatal density (D, m^{-2}) was measured as described above. The stomatal size (S = guard cell length × Σ guard cell widths, m^2) was calculated from our measurements of stomatal length. Following Franks & Beerling (2009a), we assumed that the depth of stomata (l, m) is equal to the guard cell width (i.e. guard cells are circular in cross-section). The maximum stomatal pore area (a_{max}, m^2) was predicted from its relationship with S, as measured from photomicrographs of fully open stomata on the leaves of 5-wk-old barley plants (grown in a glasshouse in 2-l pots of commercial compost and kept well watered). These had acclimated for several hours in full sun under water-saturated conditions. Leaf segments *c.* 3 cm in length were cut from mature leaves and placed directly onto the microscope stage. Within 2–3 min of excision, photomicrographs were collected using an inverted microscope equipped with a × 40 long-working-distance objective (Diaphot 200; Nikon Instruments Europe B.V., Amstelveen, the Netherlands).

Leaf level values for g_{max} were calculated as the sum of one-sided values for each leaf (g_1 + g_2). The extent to which g_{max} was dominated by a single side of the leaf was quantified by the ratio of the smallest to the largest of the one-sided values (g_1 : g_2).

Characterization of hydrological niche

The realized precipitation niche of each species was described using geo-referenced species records obtained from the Global Biodiversity Information Facility (GBIF, http://www.gbif.org, accessed 26th September 2010). Species records were mapped onto 10' grid squares defined within the Climate Research Unit CL 2.0 global climatology (New *et al.*, 2002). Mean values for total annual precipitation, across the geographical range of each species, were calculated from precipitation values for 10' grid cells in which each species occurred. To account for habitat-scale variation in the hydrological niche, we also compiled a list of habitats from species descriptions in regional floras (Clayton, 1970, 1989; Launert, 1971; Gibbs Russell *et al.*, 1990; Western Australian Herbarium, 1998–; Cope, 1999, 2002; Van Oudtshoorn, 1999; Edgar & Connor, 2000; Malyschev & Peschkova, 2001; Tzvelev, 2001; Barkworth *et al.*, 2003; Chen *et al.*, 2006). We used these to classify species into two groups: those that were described explicitly as inhabiting wet habitats, for example, bogs, rivers, streams and water bodies ('wet'), and those that were not ('mesic-dry').

Comparative methods

Analyses were carried out using species means, which were calculated from between two and eight replicates. To allow for the use of ANCOVA designs combining both discrete and continuous independent variables, we employed a phylogenetic generalized least-squares approach (PGLS, Grafen, 1989; Martins & Hansen, 1997). Correlation structures that accounted for phylogenetic covariance between species means were generated, based on pairwise shared distances on the phylogenetic tree, using Pagel's λ (Pagel, 1999; Freckleton *et al.*, 2002; Freckleton, 2009). Optimum values of λ were identified, and models were evaluated using a maximum likelihood modelling approach, implemented in R (Freckleton *et al.*, 2002; *pglm3.3* code available on request from R. P. Freckleton, University of Sheffield, UK). Phylogenies were edited, and the phylogenetic covariance matrix was generated using the R package *ape* (Paradis *et al.*, 2004). To evaluate the robustness of predictions to the comparative method used, for those models of stomatal traits in which a simple ANOVA design was applicable, an Ornstein–Uhlenbeck (OU) approach, implemented in the R package *ouch* (Butler & King, 2004; King & Butler, 2009), was used to generate independent estimates of mean trait values (Table S3). Selective regimes along our phylogeny, applied in the OU models, were estimated in R via maximum likelihood using the *ace* function in *ape*, selecting the best-fitting model from symmetrical, all-rates-different and equal-rates models on the basis of the Akaike information criterion (AIC). Pagel's correlation analysis, as implemented in Mesquite (Maddison & Maddison, 2010), was used to test for independence in the evolution of pairs of discrete traits. The likelihood test statistic was computed on the basis of 30 initial likelihood searches and 1000 simulations.

Results

Precipitation and habitat

Precipitation niches were, on average, significantly drier for species with the C_4 photosynthetic type than those with C_3 (Fig. 1; Table 1, model A). This difference was not affected by the habitat occupied by each species, nor was there a significant difference in precipitation niches between species preferring mesic-dry vs wet habitats (Table 1, model A). In this model of precipitation niche as affected by photosynthetic type and habitat, the estimate of phylogenetic covariance (Pagel's λ) was zero, that is, the modelled effects were independent of phylogenetic distance. Mean values for the precipitation niche of the C_3 and C_4 groups predicted by the PGLS model were highly consistent with optimum trait values obtained using the OU approach (± 6%;

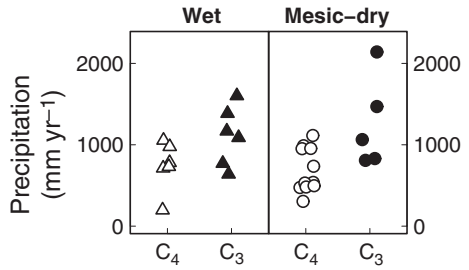

Fig. 1 Species values for precipitation niche by habitat type (wet, triangles; mesic-dry, circles) for C_3 (closed symbols) and C_4 (open symbols) grasses used in the screening experiment.

Table S3). The independent evolution of photosynthetic type and habitat preference along our phylogenetic tree was confirmed using Pagel's 1994 test (difference in log-likelihoods, 0.27; P (traits independent) = 0.857). Contrasts between species of wet and mesic-dry habitats occurred within both C_3 and C_4 clades.

Stomatal patterning

The allometry of individual stomata in grasses, with their distinctive dumb-bell-shaped guard cells, was derived from a variety of published photomicrographs and scale drawings (Fig. 2). The width of grass stomata is approximately equal to 0.25 × stomatal length (Fig. 2a), whereas the guard cell width, and hence the pore depth (l), is approximately equal to 0.5 × stomatal width. We found that a_{max} was approximately 0.4 × S when measured for fully turgid barley leaves (Fig. 2b,c).

Because the degree of amphistomy varied between species, and some species had stomata on one side of their leaves only, we tested the effects of photosynthetic type, habitat and phylogeny on stomatal patterning by focusing on the side of the leaf that had the greatest calculated conductance capacity (g_2). An initial examination of differences in S and D indicated that species belonging to the *Aristida* and Chloridoideae clades tended to have smaller values of S than other C_4 species (Fig. 3). By contrast, six of the seven species with $S > 300$ μm^2 were members of the Paniceae tribe, and all of the species from the tribe Andropogoneae and subfamily Arundinoideae exhibited values for D that were greater than the median value for the dataset (Fig. 3).

Phylogenetic covariance in each of these stomatal patterning traits was supported by separate tests for the effects of photosynthetic type on $\log_e D$ and $\log_e S$; in each case, there was evidence for a strong phylogenetic signal (Table 1, models B and C). After accounting for these phylogenetic effects, there was no significant difference in S between C_3 and C_4 species, but a significant 40% difference in the mean values of D (Table 1, model B) between C_3 (mean, 173 mm^{-2}; SEM, 126–238 mm^{-2}) and C_4 (mean, 124 mm^{-2}; SEM, 94–163 mm^{-2}) species. Although the OU method predicted a slightly larger difference in S than the PGLS method, parameter estimates for both S and D were comparable between the two methods (Table S3).

An inverse relationship, linearized by log transformation, is typically reported between S and D at the between-species level, and our data matched this expectation (Fig. 4a). After correction

Table 1 Phylogenetic generalized least-squares models used to explore differences in precipitation and habitat classification between C_3 and C_4 species, and the influence of photosynthetic type, precipitation and habitat classification on stomatal traits

Precipitation and habitat

(A) $\log_e$ rain = 7.12 − 0.67 C_4 − 0.17 wet + 0.25 C_4 wet (AIC = 40.9)
$\lambda \approx 0$, L_1 ($\lambda_0 - \lambda_0$) ≈ 0, P = 1

	$F_{1,24}$	P
C_4	10.9	0.003
Wet	0.03	0.869
C_4 wet	0.52	0.479

Stomatal patterning

(B) $\log_e D$ = 5.16 − 0.34 C_4 (AIC = 41.7)
λ = 0.86, L_1 ($\lambda_{0.86} - \lambda_0$) = 10.08, P = 0.002

	$F_{1,26}$	P
C_4	5.9	0.023

(C) $\log_e S$ = 5.32 − 0.05 C_4 (AIC = 35.4)
λ = 0.65, L_1 ($\lambda_{0.65} - \lambda_0$) = 6.49, P = 0.011

	$F_{1,26}$	P
C_4	3.8	0.063

(D) $\log_e S$ = 7.90 − 0.47 $\log_e D$ − 0.71 C_4 + 2.96 wet + 0.07 $\log_e D$ C_4 − 0.54 $\log_e D$ wet − 1.62 C_4 wet + 0.32 $\log_e D$ C_4 wet (AIC = 23.4)
λ = 0.44, L_1 ($\lambda_{0.44} - \lambda_0$) = 2.56, P = 0.109

	$F_{1,20}$	P
$\log_e D$	17.27	< 0.001
C_4	5.18	0.034
Wet	2.00	0.173
$\log_e D$ C_4	2.54	0.127
$\log_e D$ wet	3.08	0.094
C_4 wet	0.15	0.703
$\log_e D$ C_4 wet	0.204	0.656

(E) $\log_e S$ = 7.63 − 0.43 $\log_e D$ − 0.34 C_4 + 2.48 wet − 0.45 $\log_e D$ wet (AIC = 18.9)
λ = 0.43, L_1 ($\lambda_{0.43} - \lambda_0$) = 3.50, P = 0.061

	$F_{1,23}$	P
$\log_e D$	18.33	< 0.001
C_4	5.79	0.025
Wet	2.29	0.144
$\log_e D$ wet	5.44	0.029

(F) $\log_e S$ = 9.09 − 0.72 $\log_e D$ − 1.63 C_4 + 0.26 $\log_e D$ C_4 (AIC = 21.2)
λ = 0.58, L_1 ($\lambda_{0.58} - \lambda_0$) = 7.88, P = 0.005

	$F_{1,24}$	P
$\log_e D$	19.79	< 0.001
C_4	3.99	0.057
$\log_e D$ C_4	1.16	0.292

Maximum leaf stomatal conductance (g_{max})

(G) $\log_e g_{max}$ = 0.59 − 0.33 C_4 (AIC = 33.2)
λ = 0.70, L_1 ($\lambda_{0.70} - \lambda_0$) = 7.70, P = 0.006

	$F_{1,26}$	P
C_4	7.11	0.013

(H) $\log_e g_{max}$ = − 0.42 − 0.0007 rain + 0.67 C_4 + 1.73 wet − 0.0008 rain C_4 − 0.0011 rain wet − 1.50 C_4 wet + 0.0012 rain C_4 wet (AIC = 34.8)
λ = 0.55, L_1 ($\lambda_{0.55} - \lambda_0$) = 4.53, P = 0.033

	$F_{1,20}$	P
Rain	2.90	0.104
C_4	4.45	0.048
Wet	5.15	0.034
Rain C_4	0.146	0.707

Table 1 (Continued)

	Rain wet	1.66	0.213
	C_4 wet	1.14	0.299
	Rain C_4 wet	1.25	0.277
(I)	$\log_e g_{max}$ = 0.50 − 0.32 C_4 + 0.29 wet + 0.03 C_4 wet (AIC = 33.1)		
	λ = 0.55, L_1 ($\lambda_{0.55} - \lambda_0$) = 4.18, P = 0.041		
		$F_{1,24}$	P
	C_4	7.0	0.014
	Wet	4.67	0.041
	C_4 wet	0.002	0.964
Asymmetry between leaf surfaces (g_1 : g_2)			
(J)	$\log_e g_{max}$ = 0.14 + 0.89 $g_1 : g_2$ + 0.00005 C_4 − 0.66 $g_1 : g_2$ C_4 (AIC = 27.5)		
	λ = 0.81, L_1 ($\lambda_{0.81} - \lambda_0$) = 11.31, P < 0.001		
		$F_{1,24}$	P
	$g_1 : g_2$	10.26	0.004
	C_4	4.88	0.037
	$g_1 : g_2$ C_4	2.16	0.154

Headers indicate relevant sections in the Results section. C_4, effect of C_4 relative to C_3; wet, effect of wet habitat relative to mesic-dry; rain, linear response to precipitation niche (mm yr^{-1}); $g_1 : g_2$, linear response to the ratio of minimum : maximum one-sided leaf stomatal conductance.

for phylogenetic covariance, we found no significant interaction terms in a maximal model of $\log_e S$ as a function of $\log_e D$ × photosynthetic type × habitat (Table 1, model D). A minimal model, produced using AIC as a criterion for the stepwise exclusion of terms, indicated that habitat preference had a significant effect on the slope of the $\log_e S$–$\log_e D$ relationship (Table 1, model E), which was shallower amongst species from mesic-dry environments (Fig. 4b). The photosynthetic type had no significant effect on the slope of the $\log_e S$–$\log_e D$ relationship in either model, but there were significant differences in the intercept between C_3 and C_4 species in both cases (Table 1, models D and E). The minimal model suggested that, for species with high D, habitat was relatively unimportant in determining S, which differed primarily between C_3 and C_4 species (Fig. 4b). Amongst species with low D (first quartile, 114 mm^{-1}), habitat preference accounted for substantial differences in S: predicted S amongst C_4 species from mesic-dry environments was 74% of that in wet environments, whereas, for C_3 species from mesic-dry environments, predicted S was 67% of that in wet environments. Phylogenetic covariance was similar between the maximal and minimal models and did not have a significant impact on the fit of either model (Table 1, models D and E). The reduced importance of the phylogenetic covariance in these models, relative to those for the individual stomatal traits, may be a result of the strong influence of habitat on stomatal patterning. When habitat effects were not included in the initial model of $\log_e S$–$\log_e D$ × photosynthetic type, accounting for phylogenetic covariance significantly improved the model (Table 1, model F).

Maximum leaf stomatal conductance (g_{max})

The tendency for C_4 species to show lower D, and lower values of S for a given D on the side of the leaf with the greatest

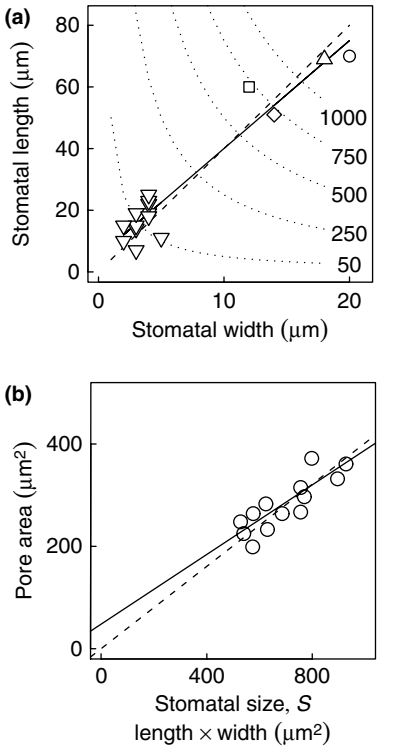

Fig. 2 (a) Stomatal size (S), as defined by guard cell length (L) and width (W). Data are values for species, based on measurements from the following: line drawings in Metcalfe (1960; triangles, apex down); photomicrographs in Flint & Moreland (1946; circle) and Kaufman *et al.* (1970; triangle, apex up); images of stomata from Franks & Farquhar (2007; square); and photomicrographs of barley stomata (P. J. Franks, unpublished; diamond). Dotted lines show isoclines for different values of S. Solid line shows the predicted relationship L = 3.5W + 5.0, estimated using least squares. Dashed line shows the simplified relationship, L = 4W, used for modelling purposes. (b) Relationship between pore area (a) and stomatal size (S) based on measurements from 13 images of stomata similar to (c). Solid line shows the predicted relationship a = 0.33S + 47.6, estimated using least squares. Dashed line shows the simplified relationship, a = 0.4S, used for modelling purposes. (c) Photomicrograph of an open stomatal pore on an attached leaf of barley (P. J. Franks, unpublished).

conductance value (g_2), suggested that C_4 species should generally have lower values of g_{max} (g_1 + g_2). A phylogenetically corrected model of $\log_e g_{max}$ × photosynthetic type confirmed this expectation, showing a significant difference between C_3 and C_4 species (Table 1, model G). The model predicted that, on average, g_{max} was 29% lower in C_4 (mean, 1.29 mol m^{-2} s^{-1}; SEM, 1.06–1.58 mol m^{-2} s^{-1}) than in C_3 (mean, 1.80 mol m^{-2} s^{-1}; SEM, 1.43–2.27 mol m^{-2} s^{-1}) species. The best-fitting value of λ for

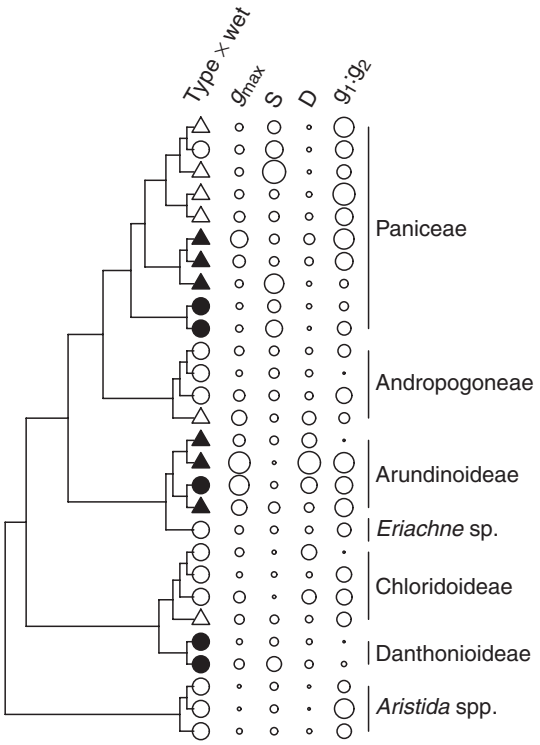

Fig. 3 Distribution across phylogeny of the maximum stomatal conductance (g_{max}, 0.73–4.30 mol m^{-2} s^{-1}), average size of stomata (*S*, 87–577 μm^2), average density of stomata (*D*, 59–511 mm^{-2}) and the ratio of the minimum to maximum conductance for the two sides of leaves (g_1 : g_2, 0–0.9). The size of the circular symbols varies in proportion to the trait values, within the ranges specified. Photosynthetic type (C_3, closed symbols; C_4, open symbols) and habitat (wet, triangles; mesic-dry, circles) are indicated at the tips of the phylogeny. Values of g_{max} are the sum of conductances for the two separate sides of the leaf.

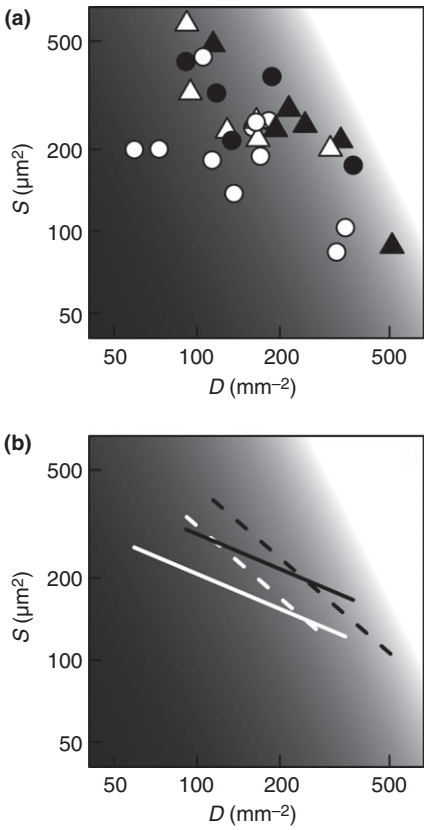

Fig. 4 Log–log relationship between stomatal size (*S*) and density (*D*) for C_3 and C_4 grass species. Values are for the side of the leaf with the greatest calculated *g*; background shading indicates *g* over the range zero (black) to 3.4 mol m^{-2} s^{-1} (no shading). Wet (triangles) vs mesic-dry (circles) habitat preferences and photosynthetic types (C_3, closed symbols; C_4, open symbols) are highlighted. (a) Mean values for species. (b) Predicted relationships based on a minimized linear model (Akaike information criterion, AIC), accounting for the effects of phylogeny, photosynthetic type (colour scheme as in (a)) and habitat preference (solid lines, mesic-dry; dashed lines, wet) (Table 1, model E).

this model was relatively high (0.70) and resulted in a significant improvement in model likelihood (Table 1, model G). Estimated optimum trait values for g_{max} in an equivalent OU model were 15% and 20% higher, respectively, for C_3 and C_4 species, but fell within the estimated standard error of the means based on the PGLS model (Table S3).

For the $\log_e$ *S*–$\log_e$ *D* relationship, we found that the phylogenetic dependence of *S* and *D* was diminished in importance when considering the effects of habitat. We therefore tested for the effects of habitat on g_{max}, and asked whether their inclusion in our models reduced the importance of phylogenetic covariance effects on model likelihood. When $\log_e$ g_{max} was modelled as a function of precipitation niche, photosynthetic type and habitat, precipitation was not significant in explaining variance in g_{max} (Table 1, model H). By contrast, and consistent with our analysis of the $\log_e$ *S*–$\log_e$ *D* relationship, both photosynthetic type and habitat had significant and independent effects on g_{max} (Table 1, model H). The estimated value of λ for this model was lower than that for the model without habitat (0.55), but, again, provided a significant improvement in model log-likelihood (Table 1, model H). The effects of C_4 photosynthesis and habitat classification on g_{max} were therefore detected against a background of significant phylogenetic covariance in this trait.

As the precipitation niche was strongly dependent on the photosynthetic pathway (Fig. 1), we explored the relative effects of these two factors on g_{max}. On the basis of the AIC criterion, no terms could be dropped from our initial model (Table 1, model H), meaning that each factor had an effect on g_{max} that could not be explained adequately by the other. When precipitation niche was excluded from the model, C_4 species had significantly lower g_{max} values than C_3 species, and there was an increase in the *F* value for photosynthetic pathway (Fig. 5; Table 1, model I). This suggests that the overall difference in g_{max} between photosynthetic types may be partially explained by differences in precipitation niches between C_3 and C_4 species. The difference in g_{max} attributed to photosynthetic type in the model excluding precipitation niche, remained independent of the significant difference in g_{max} observed between species from wet and mesic-dry habitats (Fig. 5; Table 1, model I). Optimum trait values for g_{max} from an OU model were consistently larger, but, again, within 20% of those predicted by the PGLS model (Table S3).

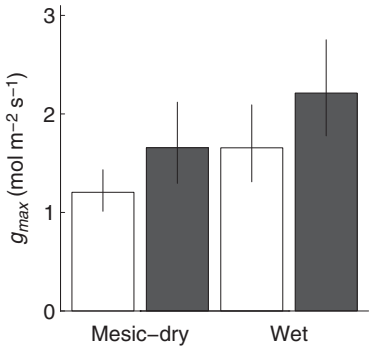

Fig. 5 Response of stomatal capacity (g_{max}) to photosynthetic type (closed bars, C_3; open bars, C_4) and habitat preference, after accounting for phylogenetic covariance. Back-transformed mean ± SEM was estimated using a phylogenetic least-squares model of $\log_e$-transformed values (Table 1, model I). Precipitation was not accounted for explicitly, but covaried with photosynthetic type, as shown in Fig. 1.

Asymmetry between leaf surfaces (g_1 : g_2)

The whole-leaf value of g_{max} comprises the sum of the predicted conductances for the two sides of the leaf (g_1 + g_2); therefore, the degree of amphistomy, that is, the equivalence in stomatal distribution/patterning between the sides of the leaf, measured here as g_1 : g_2, might be associated with g_{max}. If, for example, g_2 is similar between species, and g_1 varies, then g_1 : g_2 would be strongly associated with g_{max}. Alternatively, if increased g_1 was offset by a compensatory decrease in g_2, then g_{max} would be constant over the range of g_1 : g_2 from zero to unity. In the context of our comparisons, g_1 : g_2 might be associated with differences in g_{max} between photosynthetic types in two ways. First, either photosynthetic type might be more commonly associated with a specific range of g_1 : g_2 values. Second, if the range of g_1 : g_2 values is similar, an overall difference in g_{max} might result if the relationship between g_{max} and g_1 : g_2 differs between photosynthetic types. The median and range for g_1 : g_2 were similar amongst species within each photosynthetic type (C_3: median, 0.52; range, 0–0.82; C_4: median, 0.56; range, 0–0.91). However, although there were two species from each photosynthetic type with g_1 = 0, all of the remaining C_4 species (15/17, 88%) had g_1 : g_2 > 0.38, compared with just over one-half of the C_3 species (6/11, Fig. 6). Values of g_{max} for species with g_1 = 0 overlapped (Fig. 6) and, when $\log_e$ g_{max} × photosynthetic type was re-tested with g_1 : g_2 included as a linear covariate, there was a substantial, but nonsignificant, shift in the slope of the $\log_e$ g_{max}–g_1 : g_2 relationship between photosynthetic types (Table 1, model J), the slope being steeper amongst C_3 than C_4 grasses (Fig. 6). However, *t*-tests of coefficient values indicated that none of the coefficients for this model were significantly different from zero ($t_{24} \leq 1.53$, $P \geq 0.501$), perhaps as a result of the uneven distribution of C_4 species along the g_1 : g_2 axis. Although C_4 photosynthesis was clearly associated with an average reduction in g_{max}, this analysis provides some support for the hypothesis that the difference is greatest amongst species exhibiting greater degrees of amphistomy. As with the other models of g_{max} presented, correction for phylogenetic covariance provided a significant improvement in the fit of the model to the data (Table 1, model J).

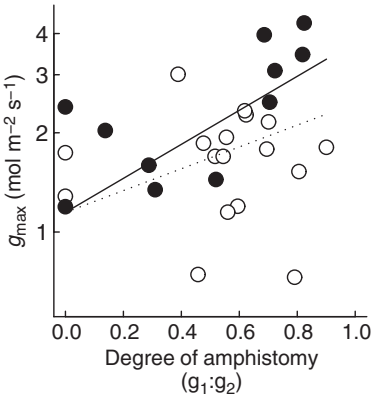

Fig. 6 Response of stomatal capacity (g_{max}) to the ratio of the minimum to maximum one-sided conductance values in C_3 (closed symbols) and C_4 (open symbols) grass species. Lines (solid, C_3; dotted, C_4) show the linear model fit after correction for phylogenetic covariance (Table 1, model I).

Discussion

Our analyses support an adaptive hypothesis of stomatal evolution in grasses. First, the results indicate the correlated evolution of g_{max} and photosynthetic pathway. In keeping with previous work, our results also show that C_4 species tend to inhabit drier precipitation niches (Edwards & Still, 2008; Edwards & Smith, 2010). However, there is little evidence that g_{max} is influenced by precipitation niche independently of photosynthetic type. By accounting statistically for the effects of photosynthetic pathway, precipitation niche and habitat wetness, our analyses support a relationship between stomatal traits and the physiological contrast between C_3 and C_4 grasses.

Overall, it was found that g_{max} is lower in C_4 than in C_3 species, mirroring the previously reported lower operating leaf conductance observed for C_4 species (Taylor *et al.*, 2010). This finding, of constitutive differences in g_{max} between C_3 and C_4 species, is consistent with well-established physiological differences between the two photosynthetic types. The role played by stomatal patterning as described by the *S*–*D* trade-off, in determining this difference, is also consistent with previous studies investigating the trade-off between CO_2 uptake and water loss (Hetherington & Woodward, 2003; Franks & Beerling, 2009a; Franks *et al.*, 2009).

The physiological trade-off between carbon fixation and water loss differs dramatically between C_3 and C_4 species (Björkman, 1970). This trade-off has driven adaptive shifts in *S* and *D* amongst C_3 species since the origins of terrestrial plants (Franks & Beerling, 2009a). Overall, *S* and *D* are negatively correlated, such that higher g_{max} is associated with smaller *S* and higher *D* (Fig. 4; Franks & Beerling, 2009a; Franks *et al.*, 2009). However, reduced *S* and increased *D* can also lead to lower g_{max} if the reduction in *S* is sufficiently large, as observed for plants grown under treatment with the drought stress hormone abscisic acid (ABA) (Franks & Farquhar, 2001). The adaptation and evolution of g_{max} is therefore complex, and further work is necessary to

elucidate the drivers and evolutionary directions of the pattern in S, D and g_{max} observed in this study.

The effects of the photosynthetic pathway on S and D were on the margins of statistical significance, but the phylogenetic signal was strongly supported in the model of each trait. We inferred that adaptive changes in g_{max} have resulted from various combinations of stomatal patterning traits, against a background of phylogenetic signals in S, D and g_{max}. The nonsignificant difference in the response of g_{max} to the degree of amphistomy observed between the photosynthetic types was also detected after correction for significant phylogenetic covariance. These results suggest constraints on the extent to which S, D and, perhaps, $g_1 : g_2$ can vary within an individual lineage, and indicate that the proximate developmental mechanisms determining g_{max} may depend critically on the phylogenetic group. Amongst C_4 clades, for example, low g_{max} in *Aristida* species is associated with low pore density, whereas, in Chloridoideae, it is associated with small pore size (Fig. 3). Based on these differences in trait values, it seems likely that the mechanistic underpinning of differences in g_{max} is a further example of a similar functional outcome achieved through alternative evolutionary routes in different C_4 lineages (Sinha & Kellogg, 1996; Kellogg, 1999; Christin *et al.*, 2007, 2009).

More generally, it has been proposed that, whenever the CO_2 supply becomes less limiting for photosynthesis, the high energetic costs of operating stomata should select against high D (Franks & Beerling, 2009a). In C_4 leaves, physiological adaptations have reduced the limitation of CO_2 uptake by CO_2 supply, and our results indicate that D on the surface with the highest conductance (g_2) has declined. This overall decline in D may, however, be linked with a well-characterized difference between leaves of C_3 and C_4 photosynthetic types: the smaller distance between vascular bundles observed in C_4 species (Ueno *et al.*, 2006), which is associated with the lower mesophyll to bundle sheath ratios diagnosing Kranz anatomy (Hattersley, 1984). As most stomata in grasses occur in rows between the vascular bundles (Metcalfe, 1960), the reduced distance between these in C_4 species limits the proportion of the leaf surface area over which stomata can be distributed. It is interesting to note that, although not formally tested in this small dataset, the frequency with which C_4 species showed a more even partitioning of g_{max} between the two sides of the leaf was higher, a phenomenon which might arise as a result of physical constraints on the development of stomata on any one leaf surface.

Our analysis suggests that there are subtle differences in effect between photosynthetic pathway and habitat in their influence on stomatal traits. Independent of the effects of photosynthetic type, we found that g_{max} was lower in species from dry-mesic habitats than in those from wet habitats. This is consistent with the hypothesis that stomatal patterning has evolved under selection from the degree of habitat wetness towards more or less conservative use of water. The interspecific pattern shown here, of a shallower relationship between S and D amongst species from mesic-dry habitats when compared with those from wet habitats, replicates the results of a recent intraspecific study of the impacts of water availability on *Eucalyptus* (Franks *et al.*, 2009). The similarity in the outcomes of these two studies is remarkable given the potential for impacts of gross leaf morphology, for example, architectural traits associated with leaf rolling (Redmann, 1985; Heckathorn & DeLucia, 1991; Maricle *et al.*, 2009), on stomatal patterning in comparisons of grass species from a variety of habitats.

The extent to which operational differences in leaf conductance between C_3 and C_4 species depend on the anatomy of stomatal patterning, as opposed to the physiological behaviour of stomatal aperture, which is considered to differ between C_3 and C_4 species (e.g. Jones, 1992), remains to be tested. However, our results indicate that evolutionary shifts in stomatal patterning comprise an important element in our understanding of the physiological impacts of the C_4 syndrome.

Conclusions

We have shown that g_{max}, as determined by the size and density of stomata, is lower among C_4 than among C_3 grass species, a trend associated with a clear distinction between these photosynthetic types in terms of their precipitation niche. We have also shown that g_{max} is lower in grass species from mesic-dry habitats than in those from wet habitats. Our results are consistent with the hypothesis that interspecific diversity in g_{max} amongst grasses has arisen as a result of phylogenetic divergence in stomatal patterning, evolution of the C_4 photosynthetic pathway and adaptation to habitat wetness. These results provide an excellent example of correlated evolution in physiological traits, showing how selection on physical form is mediated by physiological function.

Acknowledgements

This work was supported by the Natural Environment Research Council (NERC) standard grant NE/DO13062/1 awarded to C.P.O. and F.I.W., a Royal Society University Research Fellowship awarded to C.P.O., and National Science Foundation (NSF) grant IOS-0843231 awarded to E.J.E. P.J.F. gratefully acknowledges support from the Australian Research Council (ARC) Discovery Projects and Future Fellowships programs. We thank Guillaume Besnard for access to unpublished sequences, Elizabeth Kellogg for DNA samples, and Vernon Visser, Hui Liu and Russell Hall for critical comments on the manuscript. We thank Rob Freckleton for advice on the phylogenetic comparative methods.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Consensus tree for 28 grass species based on three plastid markers: coding genes *rbcL* and *ndhF*, and the region encompassing *trnK* introns and the *matK* coding sequence.

Table S1 Primers used for the amplification of plastid markers

Table S2 Vouchers and accession numbers for the taxa used in the phylogenetic analyses

Table S3 Comparison of estimated mean trait values based on phylogenetic least squares with Pagel's λ (PGLS λ, described in Table 1) and Ornstein–Uhlenbeck (OU) models

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