

RESEARCH PAPER

Water relations traits of C₄ grasses depend on phylogenetic lineage, photosynthetic pathway, and habitat water availability

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Abstract

The repeated evolution of C₄ photosynthesis in independent lineages has resulted in distinct biogeographical distributions in different phylogenetic lineages and the variants of C₄ photosynthesis. However, most previous studies have only considered C₃/C₄ differences without considering phylogeny, C₄ subtype, or habitat characteristics. We hypothesized that independent lineages of C₄ grasses have structural and physiological traits that adapt them to environments with differing water availability. We measured 40 traits of 33 species from two major C₄ grass lineages in a common glasshouse environment. Chloridoideae species were shorter, with narrower and longer leaves, smaller but denser stomata, and faster curling leaves than Panicoideae species, but overall differences in leaf hydraulic and gas exchange traits between the two lineages were weak. Chloridoideae species had two different ways to reach higher drought resistance potential than Panicoideae; NAD-ME species used water saving, whereas PCK species used osmotic adjustment. These patterns could be explained by the interactions of lineage×C₄ subtype and lineage×habitat water availability in affected traits. Specifically, phylogeny tended to have a stronger influence on structural traits, and C₄ subtype had more important effects on physiological traits. Although hydraulic traits did not differ consistently between lineages, they showed strong covariation and relationships with leaf structure. Thus, phylogenetic lineage, photosynthetic pathway, and adaptation to habitat water availability act together to influence the leaf water relations traits of C₄ grasses. This work expands our understanding of ecophysiology in major C₄ grass lineages, with implications for explaining their regional and global distributions in relation to climate.

Key words: C₄ photosynthesis, C₄ subtype, drought resistance, leaf hydraulic conductance, osmotic adjustment, phylogeny, Poaceae, stomata, turgor loss point.

Introduction

The multiple origins of C₄ photosynthesis in Poaceae represent a classic example of convergent adaptive evolution (Edwards *et al.*, 2010). C₄ grasses evolved from 22–24 C₃ lineages under environmental conditions that promote photorespiration, including low atmospheric CO₂ and high temperatures in open habitats (Sage, 2004; Christin *et al.*, 2008;

Osborne and Freckleton, 2009; Edwards *et al.*, 2010; Grass Phylogeny Working Group II, 2012). In these environmental conditions, the C₄ pathway provides a higher photosynthetic efficiency and maximum carbon-fixation rate (Ehleringer and Björkman, 1977; Sage, 2004), and permits a more efficient hydraulic system (Osborne and Sack, 2012) than the

C₃ type. Both modelling and comparative experiments show hydraulic advantages of C₄ plants over their C₃ counterparts. For example, C₄ grasses can maintain a high photosynthetic rate at lower stomatal conductance (g_s), giving a higher water use efficiency (WUE) than their C₃ relatives (Osmond *et al.*, 1982; Taylor *et al.*, 2011). C₄ grasses also have smaller stomata at a given density, resulting in a lower maximum stomatal conductance to water vapour (g_{max}) (Taylor *et al.*, 2012), as well as a higher proportion of vascular bundle sheath tissue, which may offer hydraulic benefits (Christin *et al.*, 2013; Griffiths *et al.*, 2013). Therefore, during the onset of drought, C₄ plants can maintain g_s and photosynthetic rate, as well as leaf water potential (Ψ) and hydraulic conductance (Sperry, 2000; Taylor *et al.*, 2011; Osborne and Sack, 2012; Taylor *et al.*, 2014). However, under chronic drought, these advantages may be lost (Ibrahim *et al.*, 2008; Ripley *et al.*, 2010).

Phylogeny and variants of the C₄ photosynthetic pathway have long been known to have tight associations in Poaceae. Three subtypes within C₄ grasses are classically defined according to the different enzymes that catalyse the decarboxylation of C₄ acids: NADP-ME, NAD-ME, and PCK. Previous studies on the biogeography of C₄ grasses have shown that NAD-ME species occur more in drier places, whereas NADP-ME species prefer wetter areas (Hattersley, 1992; Taub, 2000). However, NAD-ME and NADP-ME species originate almost exclusively from specific lineages, and only PCK species exist across multiple lineages (Sage *et al.*, 1999; Liu *et al.*, 2012). In particular, the predominantly NAD-ME grass lineage Chloridoideae is typically associated with drier environments than the largely NADP-ME lineage Panicoideae (Hartley, 1958a, b; Hartley and Slater, 1960; Taub, 2000; Edwards *et al.*, 2010; Visser *et al.*, 2012; Visser *et al.*, 2014). Investigation of the interaction between C₄ subtype and phylogenetic lineage in these taxa has shown that plant traits and habitat water availability (precipitation gradients and habitat wetness) are explained well by phylogeny (Liu *et al.*, 2012). However, these tests only included morphological traits at the genus level, and whether the observed differences in C₄ subtype distributions are physiologically determined or interact with phylogenetic lineage or habitat water availability remains unclear. More ecophysiological traits related to plant hydraulics are therefore needed to study drought adaptations among C₄ subtypes.

Most studies on plant hydraulic traits have looked at woody species, especially focusing on the trade-off between hydraulic efficiency and safety (Sack and Holbrook, 2006; Sperry *et al.*, 2008; Meinzer *et al.*, 2010). Reports on the leaf hydraulic conductance (K_{leaf}) of grasses are rare and only investigate single species (Martre *et al.*, 2001; Holloway-Phillips and Brodribb, 2011). At the leaf scale, plants in dry areas adjust both the concentration of cell osmotic solutes and the elasticity of cell walls to gain greater drought tolerance (Lambers *et al.*, 1998). Pressure–volume (PV) curves describe how Ψ changes with the relative water content (RWC) of living leaves during desiccation, providing important hydraulic traits, including the turgor loss point (TLP) (Scholander *et al.*, 1965; Tyree and Richter, 1982; Bartlett *et al.*, 2012). A recent meta-analysis found that osmotic adjustment is more important

for setting the TLP than is elasticity, and that less elastic leaves (represented by small cells or high leaf density) contribute to dehydration resistance by maintaining a higher RWC at TLP, thereby reducing the risk of cellular dehydration (Bartlett *et al.*, 2012). Consistent with this observation, C₄ grasses from dry areas typically have higher leaf dry matter content (LDMC) than those from wet areas (Carmo-Silva *et al.*, 2009). However, whether osmotic or elastic adjustments contribute more to drought adaptations among C₄ lineages is unknown.

Following up on recent work elucidating the biogeographical and morphological divergences between the two largest subfamilies of C₄ grasses, Chloridoideae and Panicoideae (Liu *et al.*, 2012), we carried out a species-level study of traits that are involved in plant water relations and drought tolerance in a common glasshouse environment. Our questions included: (i) Does phylogenetic lineage interact with C₄ subtype or habitat water availability in explaining plant water relations traits? (ii) Do species of Chloridoideae differ from Panicoideae in ecophysiological traits to cope with their naturally drier habitats? Owing to the logistic constraints of working with large numbers of species, we confined our analysis to traits that are constitutively expressed under mesic conditions. Previous work has shown that the hydraulic traits measured for plants growing in a moist environment can predict the range limits of species along aridity gradients (Baltzer *et al.*, 2008; Blackman *et al.*, 2012).

Materials and methods

Species sampling

Species were chosen from the two largest C₄ grass subfamilies, Chloridoideae and Panicoideae, including six main lineages. Cynodonteae, Eragrostideae, and Zoysieae in Chloridoideae; and Paniceae, Paspaleae, and Andropogoneae in Panicoideae (Grass Phylogeny Working Group II, 2012). All three C₄ subtypes (NADP-ME, NAD-ME, and PCK) were included to balance phylogenetic and photosynthetic groups. It is increasingly recognized that the expression of C₄ decarboxylases may be flexible within species (Furbank, 2011), and that PCK may be used in a secondary pathway with important physiological functions (Bellasio and Griffiths, 2014; Wang *et al.*, 2014). Our classifications were therefore based upon published evidence about the primary decarboxylase in each case, recognizing that PCK operates in parallel with the other pathways (Wang *et al.*, 2014). As annual and perennial species might have different ecophysiological traits, we selected only perennial species, but one annual PCK species was retained because only PCK species existed in both subfamilies for interaction tests, and few PCK species were available. Nearly 60 candidate species were germinated and finally 33 species were grown on and used in the experiment (Supplementary Table S1). C₄ subtype was assigned for each species following Sage *et al.* (1999).

Habitat water availability classification

Three indices were used to measure the habitat water availability for each species: (i) Average regional mean annual temperature (MAT) and mean annual precipitation (MAP). To estimate the realized precipitation niche of each species, geo-referenced species records from the Global Biodiversity Information Facility (GBIF) were collected through GrassPortal (www.grassportal.org). MAT and MAP values from 1961 to 1990 for places that all the samples of

one species occurred were averaged as the MAT and MAP value for this species. (ii) Three habitat categories, “wet”, “intermediate”, and “arid”, were assigned based on floras, journal papers, and online herbaria (van Oudtshoorn, 1999; Visser *et al.*, 2012; <http://www.efloras.org>). The “wet” category included all species described as occurring in wetlands, bogs, swamps, or in/along rivers or other water bodies. The “arid” category included species not belonging to the “wet” category and occurring in well-drained soils described as sandy, stony, gravelly, or rocky. The “intermediate” habitat included all the remaining species, which generally grow in open grasslands or woodlands. (iii) Water score, a value based on the habitat description in floras to quantify habitat water conditions. A numerical score was assigned to each of the habitat types describing water availability, and giving equal weighting to the extremes (Hydrophyte=5, Helophyte=4, Mesophyte=3, Xerophyte=1). This resulted in a continuous sequence of values for each species, which were summarized as a range “water range” and a mean “water score” for each species (Osborne and Freckleton, 2009).

There was a positive relationship between MAP and water score, with Panicoideae in wetter habitats (Supplementary Fig. S1a). The three habitat types were also correlated with MAP, although species of Chloridoideae could also live in wet habitats (e.g. *Spartina pectinata*) and species of Panicoideae in arid habitats (e.g. *Alloteropsis semialata*). Overall, 5, 5, and 2 species of Chloridoideae and 2, 14, and 5 species of Panicoideae were classified into arid, intermediate, and wet habitats, respectively (Supplementary Fig. S1b).

Plant material, leaf longevity, and glasshouse environment

In May 2009, seeds were surface-sterilized before germination on agar plates in an incubator, and seedlings were then established in small pots of compost (M3, John Innes Seed Compost). Two weeks later, larger seedlings were transferred into 4-litre pots with 50% compost plus 50% silica sand. In total 165 pots, i.e. 5 replicates for each of the 33 species, were arranged in a glasshouse bay in the Arthur Willis Environment Centre (University of Sheffield, UK), following a randomized block design. Plants were watered every day to provide enough water. In the middle of September, supplementary nutrients (Osmocote controlled release plant food) were added to pots. After being transferred into the large pots, one leaf of each plant was tagged and observed every week from 21 July till it died. The process of leaf senescence was recorded as area percentage of dead parts.

From May to October 2009, the growth environment was controlled and recorded via a glasshouse monitoring system and a weather station (DL2e data logger with RHT2nl and QS2 sensors, Delta-T Devices Ltd, Cambridge, UK) at 30 min intervals. Day length was set as 14 h from June to August, and 17 h from August to October. Air temperature was 30/25 °C (day/night), relative humidity was 70%, and the light source was natural light plus four lamps which together delivered 400–1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in daytime.

Leaf morphology and stomatal traits

Two mature leaves were taken from each plant: one was saturated with water; the other was used for the curling experiment. Leaves were cut through the sheath under water, transferred into labelled test tubes full of water then placed in a lab sink. The sink was covered by a wet cloth with tap water dripping on it to retain moisture for leaf saturation overnight. The next morning, saturated leaves were cut again at the ligule. Leaf length and width, saturated weight, and leaf volumes (displacement method in a burette) were measured. Next, leaves were scanned on a flatbed scanner (Scanjet 4500c; HP, Berkshire, UK) to obtain the leaf area (ImageJ 1.41, Abramoff, 2004), and then oven-dried (70 °C, 24 h) for dry weight. Finally specific leaf area (SLA), LDMC, leaf density, and thickness were calculated.

Imprints with dental putty (President Plus-light body, Coltène/Whaledent Ltd., Burgess Hill, West Sussex, UK) were firstly taken

from the lamina of the adaxial and abaxial leaf surfaces, and nail polish impressions were taken from these imprints to make microscope slides. Slides were observed under an inverted microscope equipped with a digital camera (Leica Laborlux S, Wetzlar, Germany) and a computerized image analysis system (Leica Quantimet 500 Q win software). From each peel, we chose three random images as replicates and measured guard cell length (GL), width of the closed guard cell pair (SW), stomatal density (SD) and the stomatal pore area index (SPI), a dimensionless index of stomata pore area per lamina area, where $\text{SPI} = \text{SD} \cdot \text{GL}^2$ (Sack *et al.*, 2003).

The maximum diffusive conductance to water vapour (g_{max}) is used to estimate transpiration potential at the anatomical level (Brown and Escombe, 1900; Franks and Beerling, 2009).

$$g_{\text{max}} = \frac{d}{v} \cdot D \cdot a_{\text{max}} / \left(l + \frac{\pi}{2} \sqrt{a_{\text{max}} / \pi} \right)$$

where d is the diffusivity of water vapour in air at 25 °C ($\text{m}^2 \text{s}^{-1}$); v is the molar volume of air at 25 °C ($\text{m}^3 \text{mol}^{-1}$); D is stomatal density (stomata number mm^{-2}); a_{max} is the maximum area of the open stomatal pore, estimated as $\pi(p/2)^2$ where p is stomata pore length [although in Franks and Beerling (2009) p was approximated as half of the guard cell length, p was measured directly here]; l is stomata depth for fully open stomata, approximated as $W/2$; π is the geometric constant. We calculated g_{max} for both leaf sides, and summed the values to obtain a total leaf value.

Leaf curling experiment

Leaves were weighed on a four-point balance (AE163; Mettler Toledo Ltd, Leicester, UK) and leaf widths were measured at the widest part. Both weight and width of each leaf were repeatedly recorded for 2 h at 15 min intervals in a cabinet with constant environment (23–24 °C, RH 60%). Later leaf curling characteristics were recorded and oven-dried leaves were weighed. Owing to the different starting and ending points for different species, all 165 leaf curling models were fitted manually. Leaf width loss rates were slopes of curves of the relative leaf width ($\% \text{hr}^{-1}$) and absolute leaf width (mm hr^{-1}) against time. Leaf weight loss rate ($\% \text{hr}^{-1}$) was the slope of relative water content against time. During the first hour, leaf stomata were assumed to be still open, but in the next hour most leaves had already curled with stomata closed, and cuticular conductance could therefore be calculated without stomatal transpiration (Boyer *et al.*, 1997). In this study, leaf cuticular conductance ($\text{mmol m}^{-2} \text{s}^{-1}$) was the slope of the weight loss curve normalized by original leaf area during the second hour. Relative width/weight loss ($\% \text{hr}^{-1}$) was also calculated as width/weight ratio against time.

Pressure–volume (PV) curves

Leaves were cut and rehydrated overnight as described above. After measuring the Ψ with a pressure chamber (Model 1000 Pressure Chamber Instrument, PMS Instrument Company, USA), the corresponding leaf weight was recorded immediately. The leaves were initially allowed to dry slowly to an equilibrium mass in an airtight box, and then allowed to dry further on the bench, with measurements every 15 min in each case. At the end of the experiment, oven-dried leaves were weighed to determine RWC and LDMC, and then PV curves were drawn and analysed. First a straight line was fitted via the stepwise addition of points from the linear portion of the curve at low RWC, where water potential changes only with osmotic pressure. This was used to estimate the response of osmotic potential (Ψ_o) to RWC. The fitted Ψ_o values were then extrapolated across all RWC values, and subtracted from Ψ to obtain the pressure potential (Ψ_p). Secondly, a turgor curve of Ψ_p against RWC was built by fitting a modified exponential equation (Schulte and Hinckley, 1985), from which the zero intercept is the estimated TLP. Young's modulus of elasticity (ϵ) was derived from the slope of the moisture release curve between saturation and the TLP (Lenz *et al.*, 2006).

Leaf hydraulic conductance (K_{leaf})

We followed the method of Franks (2006). One mature leaf was cut at the ligule from a well-watered plant. After being weighed on an analytical balance (W_1), it was placed immediately into the pressure chamber. Chamber pressure was increased to the balance pressure of the leaf (Ψ_1) and equilibrated for about 5 min. Then chamber pressure was increased rapidly to Ψ_2 , with $\Delta\Psi$ ($\Psi_2 - \Psi_1$) of around 0.5 MPa. The sap expressed in the first 10 s was removed, using a timer to record the duration, and the final weight of the leaf (W_2) was quickly measured to calculate $\Delta W = W_1 - W_2$. Leaves were scanned and oven-dried. K_{leaf} was calculated as (Franks, 2006):

$$K_{leaf} = \Delta W \times \text{time}^{-1} \times \text{leaf area}^{-1} \times \Delta\Psi^{-1}$$

Leaf gas exchange

On sunny days, the youngest mature leaf from a tiller of each plant was chosen and put under high light ($1800 \mu\text{mol m}^{-2} \text{s}^{-1}$) to pre-adapt for around 10 min. We used an open leaf gas exchange system (LI-6400, LI-COR, Lincoln, NE, USA), setting the conditions of the leaf chamber at: CO_2 concentration, $400 \mu\text{mol mol}^{-1}$ (provided by CO_2 cylinders); leaf block temperature, 30°C ; photosynthetically active radiation (PAR), $2000 \mu\text{mol m}^{-2} \text{s}^{-1}$; flow rate, $400 \mu\text{mol s}^{-1}$; RH in the sample cell, 70–85 %, to keep the vapour pressure deficit (VPD) in the leaf chamber around 1–1.5 kPa. Maximum net CO_2 assimilation rate (A), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), and transpiration rate (E) were recorded. Instantaneous water use efficiency (WUE_i) was calculated as A/g_s .

Statistical methods

The distribution of three C_4 subtypes in the two subfamilies was unbalanced (no NADP-ME species in Chloridoideae, only two NAD-ME species in Panicoideae, more unbalanced at the tribe level). Therefore, to distinguish phylogeny from C_4 subtype in affecting ecophysiological traits, PCK and non-PCK species were grouped to form a two-factor, complete block design (7 PCK and 5 non-PCK species in Chloridoideae; 7 PCK and 17 non-PCK species in Panicoideae). Generalized linear mixed-effects models (GLMM) with phylogeny (subfamily) and photosynthetic type (PCK and non-PCK) were fitted as two fixed factors and species as a random factor; interaction effects were detectable as there were replicates in each unit.

We also tested the effects of phylogeny and habitat water availability on ecophysiological traits using GLMMs. MAP, water score, and habitat type were tested separately with phylogeny as two fixed factors, and species as a random factor. Owing to the covariance of MAP, water score and habitat type (Supplementary Fig. S1a), the three models showed the same pattern; therefore only the model with MAP was reported because it had the lowest Akaike information criterion (AIC) value.

Phylogenetic tree and analysis

As most species were chosen based on published phylogenetic trees of C_4 lineages, the phylogeny and branch lengths among species were extracted based on the background tree in Christin *et al.* (2008). Six congeners, *Alloteropsis*, *Brachiaria*, *Melinis*, *Urochloa*, *Chloris*, and *Dactyloctenium*, were used when species sampled in the tree (Christin *et al.*, 2008) were not the same species in this study. However, three species from the genera *Heteropogon*, *Enneapogon*, and *Fingerhuthia* were not included, so their phylogenetic places were located by their closest genera *Hyparrhenia*, *Uniola*, and *Eragrostis* (Liu *et al.*, 2012). The final phylogenetic tree clearly represented six lineages (Supplementary Fig. S2).

Phylogenetic analysis reveals the degree of phylogenetic dependence for plant traits. Pagel's λ is based on a Brownian model of trait evolution (Pagel, 1999). The extent to which traits evolve from

random drift gives a λ value between 0 and 1. The value $\lambda=1$ implies strong phylogenetic dependence, whereas $\lambda=0$ indicates no phylogenetic dependence (Freckleton *et al.*, 2002).

Principal component analysis (PCA) and phylogenetic PCA (PPCA)

Both conventional PCA and PPCA were employed to investigate which traits were most important in distinguishing species. Data were log-transformed (if the original trait values were negative, such as TLP, absolute values were used) to meet the requirement of normal distribution. Conventional PCA was carried out using the R function *princomp*. The same dataset was tested again by phylogenetic PCA (PPCA) such that phylogeny was taken into account (Felsenstein, 1985). A variance-covariance matrix among species was generated by the R function *vcv* in package *ape* and PPCA was carried out using the *phyl.pca* function in package *phytools*.

Results

Subfamily comparisons

In the following discussion, we use the term “structural traits” to describe traits that are fixed by development, including morphology, leaf size/mass, and stomatal patterning, and the term “physiological traits” to include those which have dynamic responses to environmental changes, including leaf curling, leaf hydraulics, and leaf gas exchange (Table 1). We use the term “ecophysiological traits” to represent both structural and physiological traits.

The two taxonomic groups differed significantly in their structural traits. Chloridoideae species were shorter plants with smaller seeds, but with longer and much narrower leaves than Panicoideae, which led to a smaller area and volume for individual leaves (Table 1, all $P < 0.05$). The dry weight of individual leaves was similar in the two subfamilies, which lead to indistinguishable leaf dry matter content (LDMC), leaf density and thickness, but 11% greater specific leaf area (SLA) in Chloridoideae than Panicoideae. Chloridoideae showed distinctively smaller stomatal length and width, but higher stomatal density than Panicoideae. Stomatal size was similar between the two leaf sides, but stomatal density and SPI were much higher for the abaxial than adaxial surface ($P < 0.05$). Chloridoideae had smaller abaxial SPI ($P < 0.01$) than Panicoideae (Table 1). The two subfamilies also had similar leaf longevity of around 55 days; although each species had a different leaf life span and mortality rate, the overall duration of senescence was around 40% of the leaf lifespan.

Several physiological traits differed among the taxonomic groups. Leaves of Chloridoideae curled and lost water at a similar absolute rate to those of Panicoideae after excision. However, owing to narrower absolute leaf widths, relative width loss of Chloridoideae was much faster, and rolling was completed in a shorter time. After stomata closed, leaf cuticular conductance was slightly smaller for Chloridoideae than Panicoideae (Table 1). Chloridoideae also operated at a more negative leaf water potential in ambient conditions (Ψ) and at saturation (Ψ_{sat}) than Panicoideae. The K_{leaf} of Chloridoideae was lower than that of Panicoideae. For leaf gas exchange, the two subfamilies showed no significant differences. Finally,

Table 1. Comparisons of 40 traits between Chloridoideae and Panicoideae, for both total and PCK-only species in the glasshouse experiment

Data are means±SEM, sample sizes are shown in the heading. Level of significance for t-tests: * P<0.05; ** P<0.01; *** P<0.001; ns, not significant. Abbreviations: LDMC, leaf dry matter content; SLA, specific leaf area; SD, stomatal density; SPI, stomatal pore index; g_{max} , maximum stomatal conductance to water vapour; Ψ_{sat} , saturated leaf water potential; Ψ_{osat} , saturated leaf osmotic potential; TLP, turgor loss point; ϵ , Young's modulus of elasticity; K_{leaf} , leaf hydraulic conductance; A, photosynthetic rate; g_s , stomatal conductance; C_i , intercellular CO₂ concentration; E, transpiration rate; WUE_i, instantaneous water use efficiency; MAT, mean annual temperature; MAP, mean annual precipitation.

	Chloridoideae (total 12 species 7 PCK species)	Panicoideae (total 21 species 7 PCK species)	P (total)	P (PCK only)
Morphology and leaf longevity				
Culm height (cm)	55±5.5	91±6.2	***	ns
Leaf length (cm)	41±2.4	34±1.4	*	***
Leaf width (mm)	6±0.3	11±0.4	***	***
Leaf area (cm ²)	22±1.6	28±1.7	**	ns
Leaf volume (cm ³)	0.5±0.04	0.6±0.04	*	*
Leaf dry weight (mg)	69±8.0	78±5.3	ns	*
Seed size (mm ²)	.88±.15	2.11±.12	***	**
Leaf longevity (days)	53±2.1	57±2.4	ns	ns
Leaf structure				
LDMC (%)	17±0.6	17±0.4	ns	ns
SLA (m ² kg ⁻¹)	45±2.1	40±1.0	*	ns
Leaf density (g cm ⁻³)	0.14±0.006	0.14±0.005	ns	ns
Leaf thickness (mm)	0.19±0.011	0.21±0.005	ns	ns
Stomatal traits				
Guard cell length (µm)	24±1.0	32±0.9	***	***
Stomatal width (µm)	15±0.4	18±0.4	***	ns
Stomatal pore length (µm)	12±0.5	18±0.7	***	***
Abaxial SD (mm ⁻²)	223±18.3	136±6.9	***	***
Adaxial SD (mm ⁻²)	147±16.3	105±7.7	*	ns
Abaxial SPI (dimensionless)	10.9±0.56	13.4±0.54	**	ns
Adaxial SPI (dimensionless)	7.5±0.74	9.1±0.52	ns	ns
g_{max} (mol m ⁻² s ⁻¹)	5.0±0.24	4.5±0.16	ns	ns
Leaf curling				
Leaf curling rate				
Relative width loss (% hr ⁻¹)	77±10	37±3	***	*
Absolute width loss (mm hr ⁻¹)	2.6±0.26	2.5±0.21	ns	ns
Leaf weight loss rate				
Relative weight loss (% hr ⁻¹)	14±0.4	14±0.5	ns	*
Leaf cuticular conductance (mmol m ⁻² s ⁻¹)	0.27±0.019	0.29±0.015	*	ns
Relative width/weight ratio loss (% hr ⁻¹)	4.88±0.51	2.53±0.24	**	ns
Leaf hydraulic traits				
Ψ_{sat} (MPa)	-0.20±0.02	-0.16±0.01	*	ns
Ψ_{osat} (MPa)	-1.1±0.04	-1.0±0.02	ns	**
TLP (MPa)	-1.2±0.04	-1.1±0.03	ns	***
ϵ (MPa)	0.12±0.009	0.11±0.004	ns	*
Ψ (MPa)	-0.54±0.034	-0.45±0.020	*	ns
K_{leaf} (mmol m ⁻² s ⁻¹ MPa ⁻¹)	17±0.9	19±0.7	*	**
Leaf gas exchange				
A (µmol CO ₂ m ⁻² s ⁻¹)	18±1.1	17±0.6	ns	ns
g_s (mol H ₂ O m ⁻² s ⁻¹)	0.16±0.012	0.17±0.008	ns	*
C_i (µmol CO ₂ mol ⁻¹)	190±5.5	196±4.2	ns	ns
E (mmol H ₂ O m ⁻² s ⁻¹)	3±0.2	3±0.1	ns	ns
WUE _i (A/g _s)	117±3.3	111±2.5	ns	ns
Habitat traits				
Water range	2.0±0.6	2.1±0.8	ns	ns
Water score	1.95±0.56	2.83±0.56	***	**
MAT (°C)	17.8±1.6	19.9±0.8	ns	ns
MAP (mm)	764±98	1090±75	*	ns

habitat water score and MAP of Chloridoideae species were lower than those of Panicoideae (Table 1).

When only PCK species were included, results of *t*-tests on some traits between the two subfamilies changed (Table 1). Culm height, leaf area, and SLA were no longer significantly different, and nor were stomatal width, adaxial stomatal density, SPI, leaf cuticular conductance, Ψ , Ψ_{sat} , and MAP. However, leaf dry weight, relative weight loss, Ψ_{osat} , TLP, ϵ , and g_s now differed significantly (Table 1).

Phylogenetic signals

Most traits did not show phylogenetic dependence (Table 2A). None of the structural traits, except leaf width and seed size, showed phylogenetic signals, and neither did any other leaf structural traits including LDMC (all λ values < 0.06, with $P > 0.05$ for $\lambda = 0$). However, most of the stomatal traits showed high λ values, with the exception of adaxial stomatal density. The derived stomatal indices SPI and g_{max} had no phylogenetic signals. None of the λ values of parameters involved in the leaf curling, leaf longevity, hydraulic measurements and leaf gas exchange, were significantly different from zero, except leaf relative width and width/weight loss. For habitat traits, strong phylogenetic signals were found for water score and MAT, but there were no signals for water range and MAP (Table 2A).

PCA and PPCA results

In the conventional PCA, the first two axes explained 23% and 18% of total variation, respectively (Fig. 1A, B; Supplementary Table S2). Leaf structural traits (density, LDMC, area), culm height, and TLP were on the negative side of PC1, whereas SLA was on the positive side of PC1. Guard cell length, stomatal width, seed size, and leaf thickness and width were loaded on the negative side of PC2, whereas stomatal density and relative width loss during leaf curling were on the positive side of PC2 (Fig. 1A). PCA could not distinguish species between Chloridoideae and Panicoideae, or species from three C_4 subtypes along PC1. However, the two subfamilies were separated along PC2, showing larger stomata and slower leaf curling rates for Panicoideae species as in Fig. 1A (Fig. 1B).

After accounting for the phylogenetic background, PPCA showed different patterns and explained the total variation as 24% and 16% for PC1 and PC2, respectively (Fig. 1C, D; Supplementary Table S2b). As with the conventional PCA, leaf structural traits and TLP loaded on PC1, but stomatal traits, seed size and leaf curling traits were no longer on PC2, with the exception of leaf size traits (Fig. 1C). Instead, leaf hydraulic traits (K_{leaf} , g_s , and Ψ_{sat}) emerged along PC2 in separating the two subfamilies, although the extent of separation was weaker in PPCA (Fig. 1D).

Table 2. Results for (A) phylogenetic tests, (B) generalized linear mixed-effects models (GLMM) for phylogeny \times photosynthetic type, and (C) GLMM for phylogeny \times habitat water availability

(A) Estimated λ values for 40 indices of the 33 grass species (*N*). (B) GLMM with phylogeny (S, subfamily) and photosynthetic type (PT, PCK, and non-PCK) as two fixed factors, species as a random effect. (C) GLMM with phylogeny (S, subfamily) and habitat water availability (MAP, mean annual precipitation) as two fixed factors, species as a random effect. Sample size (*n*), λ values, P values for phylogenetic tests; Total number of individuals sampled (*n*), F values, d.f. (for each factor), and P values for GLMM are reported. Level of significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant. Significant results are in bold.

Ecophysiological trait	<i>N</i>	(A) Phylogenetic test			<i>n</i>	(B) GLMM of C_4 subtype			(C) GLMM of habitat water			
		λ	<i>P</i> ($\lambda=0$)	<i>P</i> ($\lambda=1$)		S (1, 29)	PT (1, 29)	S \times PT (1, 29)	S (1, 29)	MAP (1, 29)	S \times MAP (1, 29)	
Morphology and leaf longevity												
Culm height	33	0.03	ns	**	165	3.12*	.05	.99	2.46	.01	.38	
Leaf length	33	0.00	ns	***	224	4.65*	3.25	1.22	1.35*	.02	.71	
Leaf width	33	0.28	*	***	224	10.22**	.20	.32	7.63*	.28	.46	
Leaf area	33	0.00	ns	***	223	1.39	.04	2.16	.85	.36	.12	
Leaf volume	33	0.00	ns	***	223	1.06	.03	4.81*	.49	.43	.62	
Leaf dry weight	33	0.00	ns	***	223	.31	.25	4.38*	.14	1.23	1.56	
Seed size	33	0.25	*	***	165	56.24***	2.75	1.12	58.85***	7.73**	8.17**	
Leaf longevity (days)	33	0.00	ns	**	163	.24	.61	.82	.34	1.37	.25	
Leaf structure												
LDMC	33	0.00	ns	***	222	.00	.44	.16	.02	.63	4.67*	
SLA	33	0.00	ns	***	214	1.27	2.02	2.88	.22	.40	4.96*	
Leaf density	33	0.00	ns	***	217	.02	1.00	.66	.00	1.53	5.14*	
Leaf thickness	33	0.00	ns	***	222	.92	.06	3.96	.31	.09	.30	
Stomatal traits												
Guard cell length	33	0.71	***	**	99	18.89***	1.63	.23	16.63***	1.09	1.64	
Stomatal width	33	0.62	*	**	99	9.71**	3.37	1.13	7.29**	.31	.19	
Stomatal pore length	33	0.58	**	***	99	15.14***	.69	.22	14.18***	.91	2.20	
Abaxial SD	33	0.93	***	ns	92	13.17**	4.70*	3.89	14.01***	.26	3.10	
Adaxial SD	33	0.00	ns	***	99	8.76*	1.27	2.07	3.00	.00	2.65	

Table 2. Continued

Ecophysiological trait	N	(A) Phylogenetic test			n	(B) GLMM of C ₄ subtype			(C) GLMM of habitat water		
		λ	P (λ=0)	P (λ=1)		S (1, 29)	PT (1, 29)	S×PT (1, 29)	S (1, 29)	MAP (1, 29)	S×MAP (1, 29)
Abaxial SPI	33	0.08	ns	***	92	6.08*	1.30	8.33**	3.07*	.20	.23
Adaxial SPI	33	0.01	ns	***	99	1.07	.41	1.18	.68	.05	.23
<i>g</i> _{max}	33	0.00	ns	ns	99	1.37	4.22*	.01	3.27	.02	7.38
Leaf curling											
Leaf curling rate											
Relative width loss	33	0.52	**	***	163	14.97***	.01	.15	13.12**	1.94	4.17
Absolute width loss	33	0.00	ns	***	163	.02	1.53	.00	.21	.12	1.32
Leaf weight loss rate											
Relative weight loss	33	0.00	ns	**	163	.00	.32	2.48	.15	.00	.27
Leaf cuticular conductance	33	0.00	ns	*	163	1.42	.02	.04	.61	.28	.73
Relative width/weight ratio loss	33	0.36	ns	***	163	5.79*	.40	.01	3.21	1.58	1.53
Leaf hydraulic traits											
Ψ _{sat}	33	0.03	ns	***	162	3.46*	.01	.03	3.28	2.88	7.21*
Ψ _{osat}	33	0.00	ns	**	162	1.10	1.03	4.81*	2.31	.62	3.48
TLP	33	0.00	ns	*	162	1.01	1.28	5.06*	2.48	.56	2.91
ε	33	0.00	ns	**	162	1.11	1.55	2.66*	2.74	.95	7.82**
Ψ	33	0.01	ns	**	196	3.08*	1.32	.76	2.29	.06	.62
<i>K</i> _{leaf}	33	0.00	ns	***	165	1.37	.03	2.12*	.64	.33	1.36
Leaf gas exchange											
<i>A</i>	33	0.00	ns	***	140	.13	.27	3.26	.31	.10	.29
<i>g</i> _s	33	0.00	ns	***	140	.11	.15	1.48	.05	.02	.01
<i>C</i> _i	33	0.00	ns	***	137	.34	.00	.09	.02	.01	.46
<i>E</i>	33	0.00	ns	***	140	.05	.06	2.94	.41	.20	.09
WUE _i	33	0.00	ns	***	140	1.29	.01	.02	.23	.03	.33
Habitat traits											
Water range	33	0.00	ns	***	33	.27	.00	.00	1.06	1.68	1.54
Water score	33	0.55	*	ns	33	17.28**	.82	.21	20.53***	20.66***	11.43**
MAT	28	0.90	*	ns	28	1.92	2.96	.08	.07	23.89***	10.13**
MAP	28	0.22	ns	***	28	6.71*	.47	.60	–	–	–

Phylogeny interacted with C₄ subtype and habitat water availability

Phylogeny and C₄ subtype affected ecophysiological traits differently. Subfamily divergences were found for culm height, leaf length and width, seed size, stomatal size-related traits, leaf relative width loss, Ψ_{sat}, Ψ, water score, and MAP. Meanwhile, only *g*_{max} was affected by C₄ subtype (Table 2B). Although several indices showed significant interactions, most of them were affected by neither subfamily nor C₄ subtype (Table 2B). Compared with the exploratory two-way ANOVA results (data not shown), species as a random factor in GLMM reduced detectable effects from fixed factors or their interaction, indicating that inter-specific differences were an important source of variance. Traits with significant C₄ subtype or interaction effects were plotted in Fig. 2A–D. Within Chloridoideae, PCK species always had higher values than NAD-ME species, whereas within Panicoideae, there were no general patterns among three C₄ subtypes, but the value of PCK species was the main driver for an interaction.

Habitat water availability also interacted with phylogeny, although most traits were affected by neither of the two factors (Table 2C). MAP alone had no effects but it interacted with subfamily for several traits, including seed size, LDMC, SLA,

leaf density, Ψ_{sat}, ε, water score, and MAT. Traits with significant interaction effects were further investigated (Fig. 2E–H). Within Chloridoideae, particular species had extreme values; for example, *Zoysia japonica* lives in wet habitats and has large seeds. High average SLA values of Chloridoideae were driven by two species (*Sporobolus nebulosus* and *Dactyloctenium scindicum*). Two species in Chloridoideae, *Zoysia japonica* and *Chloris elata*, had extremely high ε values. The MAT of the realized niche for each species showed no clear pattern with MAP.

Leaf water relations traits

The comparison of leaf curling showed that the wider a leaf was, the faster the absolute rate of curling, with subfamily as an important factor in explaining variance (Fig. 3A). Although the two subfamilies had similar absolute width loss rates, the time to achieve curling end points was much faster for Chloridoideae species, with an extreme species, *Sporobolus nebulosus* that could quickly curl in 10 min (Fig. 3B). On the other hand, leaf cuticular conductance and relative weight loss showed no differences among species.

As subfamily and C₄ subtype interactively affected PV curve parameters (Table 2B), they were analysed separately

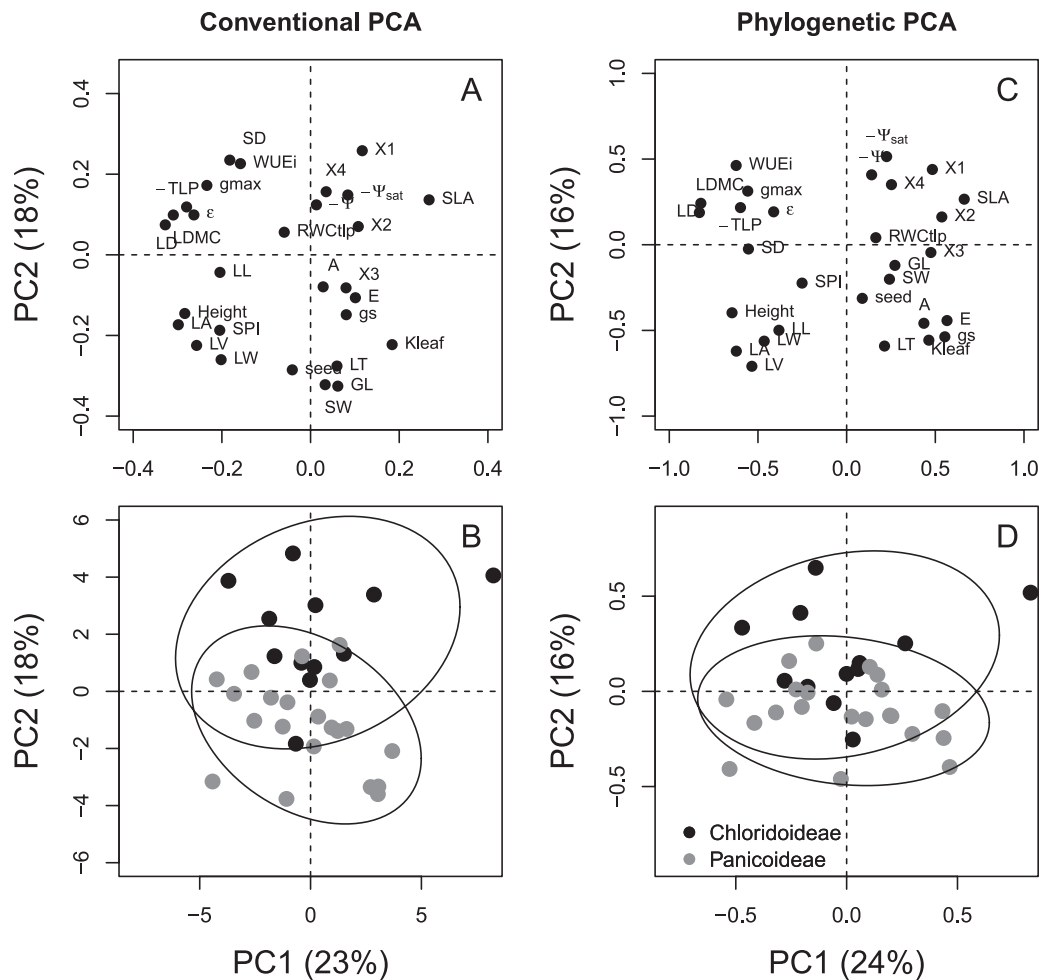


Fig. 1. Conventional principal component analysis (PCA) and phylogenetic PCA (PPCA) for the first two principal components (PC) based on 29 plant traits of 33 C_4 grasses. (A, C) PC loadings and (B, D) species scores with Chloridoideae (black) and Panicoideae (grey) circled. The percentages of variance explained by the first two PCs are in the axis labels. See [Supplementary Table S2](#) for PC loadings, [Table 1](#) for trait abbreviations, with the addition of: LD, leaf density; LA, leaf area; GL, guard cell length; SW, stomatal width; LT, leaf thickness; LW, leaf width; LV, leaf volume; X1, relative width loss; X2, relative weight loss; X3, leaf cuticular conductance; X4, relative width/weight ratio loss.

([Fig. 4A](#)). In the turgor loss phase, NAD-ME species in Chloridoideae had the most elastic leaves (the shallowest slope indicates the smallest ϵ), whereas PCK species in Chloridoideae had the least elastic leaves. These two groups of Chloridoideae species also had more negative Ψ_{sat} (i.e. lower y intercepts) than Panicoideae species ([Fig. 4B](#)). In the osmotic phase, NAD-ME species from both subfamilies showed the two steepest slopes, indicating less negative Ψ_{osat} and TLP, whereas PCK species in Chloridoideae had the most negative Ψ_{osat} and TLP ([Fig. 4C](#); [Supplementary Table S3](#)).

There were relationships among different hydraulic and structural traits. Both TLP and ϵ increased with LDMC, suggesting that greater LDMC was associated with less elasticity and a more negative TLP ([Fig. 5A, B](#)). The ϵ was positively related with RWC at the TLP, meaning that a steeper initial fall in pressure potential with RWC led to higher RWC at the TLP ([Fig. 5C](#)). ϵ was negatively related with K_{leaf} , such that that the most elastic leaves also had the highest hydraulic conductance ([Fig. 5D](#)). Other leaf traits such as SLA, leaf thickness, and volume were also explored in the tests, but only LDMC showed significant relationships with hydraulic traits.

Discussion

Phylogeny interacts with photosynthetic type and habitat water availability

Phylogenetic patterns have previously been recognized in the distributions of C_3 and C_4 photosynthetic types ([Edwards et al., 2010](#)), C_4 biochemical subtypes ([Taub, 2000](#)), and habitat water gradients ([Osborne and Freckleton, 2009](#)) among species. However, this is the first study to investigate the ecophysiological adaptations associated with these patterns. We found that phylogeny interacted with both photosynthetic type and water availability for some structural and most hydraulic traits ([Fig. 2](#); [Table 2B, 2C](#)).

Previous studies comparing ecophysiological traits between NAD-ME and NADP-ME species have implied that there may be an interaction between phylogeny and C_4 subtype, but none of them had sufficient statistical power to test it directly ([Ghannoum et al., 2002](#); [Taylor et al., 2010](#)). Our results provided solid evidence that both phylogeny and C_4 subtype are associated with ecophysiological trait variation. Specifically, phylogeny tended to have a greater influence on structural

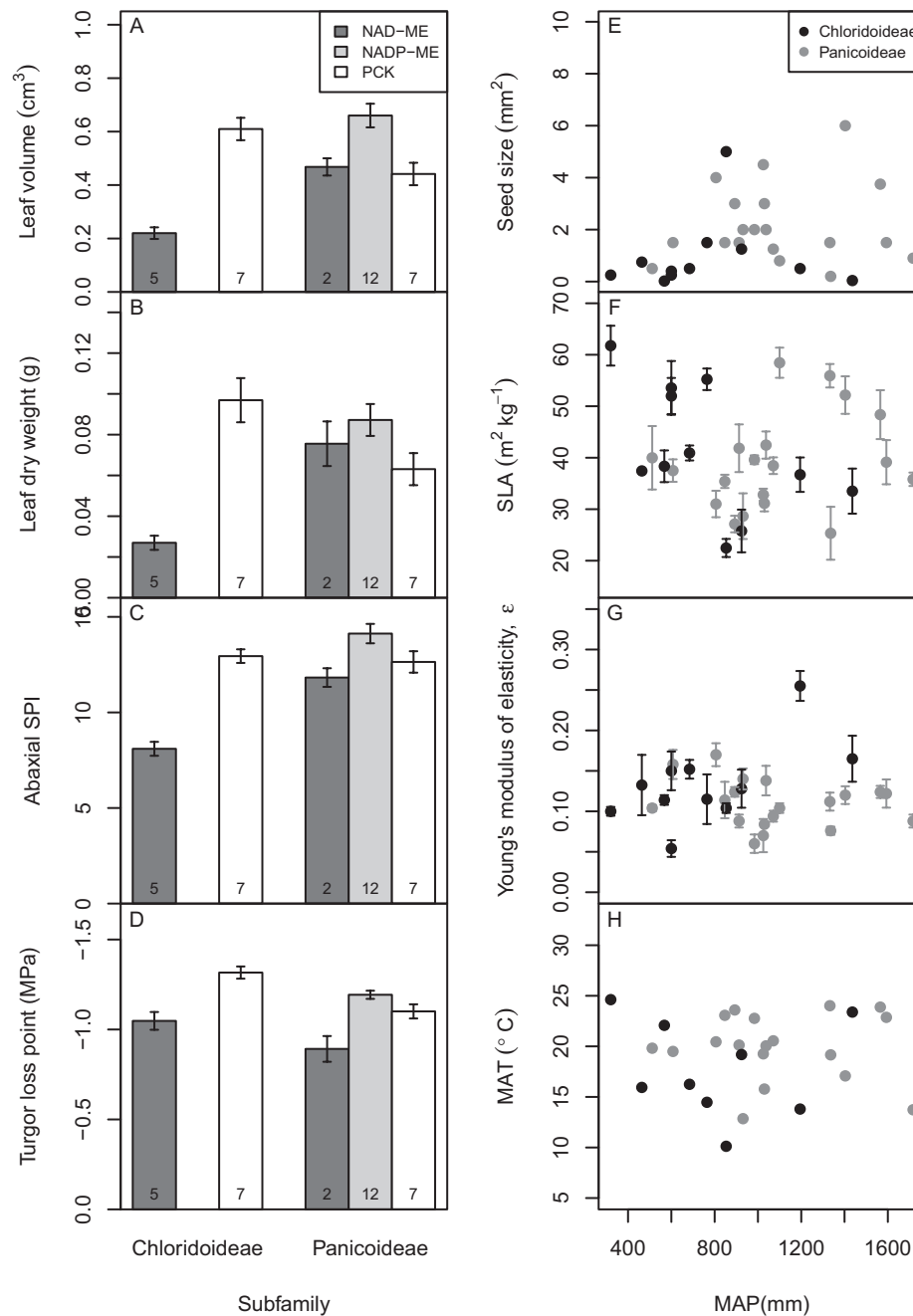


Fig. 2. Comparisons of trait values with significant two-factor interactions. (A–D) Subfamily × C₄ subtype and (E–H) subfamily × habitat type. Traits are selected according to GLMM results (Table 2b–c). Species numbers are shown under each bar.

traits (Table 2A), whereas C₄ subtype tended to be more related with physiological traits, and their interactions especially affected leaf hydraulic traits (Table 2B). One explanation for this pattern may be that structural traits from distant lineages are less labile during evolution than physiological traits (Ackerly and Donoghue, 1998). In contrast, leaf physiological traits depend more on instantaneous responses like stomatal behaviour and enzyme activities, which are likely to be influenced more by C₄ subtypes (Ghannoum, 2009). Another possible explanation for weak phylogenetic signals in physiological traits is that species diversity in this study was limited, and phylogenetic signals become harder to detect at a small phylogenetic scale (Cavender-Bares *et al.*, 2009).

In the GLMMs we classified photosynthetic type into PCK/non-PCK groups, as only PCK species occur in multiple lineages across both subfamilies. The interaction between PCK/non-PCK groups and subfamily indicated the possibility that PCK may operate plastically in otherwise NADP-ME or NAD-ME species, which corresponds to recent biochemical and molecular studies finding the co-existence of PCK activity in NADP-ME or NAD-ME species (Bräutigam *et al.*, 2014; Wang *et al.*, 2014). Therefore, whether ecophysiological traits of plants reflects biochemical differences needs more study, and care should be taken in future comparative experiments based on classical C₄ subtypes, especially when they have independent origins.

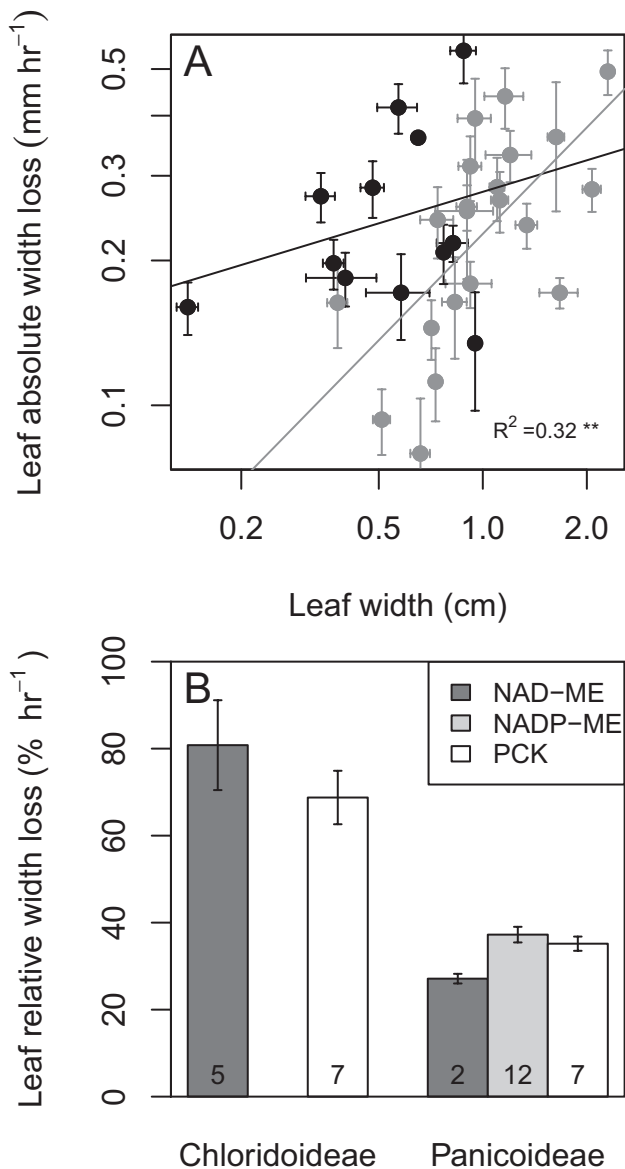


Fig. 3. Leaf rolling in response to water loss. (A) Absolute leaf width loss against leaf width. (B) Relative leaf width loss for subfamilies and C_4 subtypes. In A, subfamilies are Chloridoideae (black) and Panicoideae (grey), and each point is a species with means \pm SE ($n=6$). In B, species numbers are shown under each bar.

Habitat water availability has been an important selection force in C_4 grass evolution (Osborne and Freckleton, 2009), and is an important determinant of the phylogenetic composition of C_4 grass communities (Visser *et al.*, 2012). The interaction between water availability and phylogeny in explaining ecophysiological traits thus suggests different adaptation strategies in different lineages, particularly represented by leaf water relations traits (Table 2C). For example, within Chloridoideae, *Zoysia japonica* lives in wet habitats with large seeds and high ϵ values, and *Sporobolus nebulosus* has a high SLA (Fig. 2E–H), which indicates that not all Chloridoideae species live in dry areas with drought tolerant plant traits.

Distinct structural but subtle physiological differences

Most Chloridoideae species occur in drier habitats, and have leaf structures with more drought tolerance potential, but do not show significant hydraulic advantages over Panicoideae except in relative curling rate, Ψ , Ψ_{sat} , and K_{leaf} (Table 1). However, the difference between PCA and PPCA indicated that, after considering phylogenetic relationships, some leaf hydraulic traits (K_{leaf} , g_s , and Ψ_{sat}) did have the potential to distinguish the two subfamilies (Fig. 1B–D), despite the weak phylogenetic signals of most physiological traits (Table 2A).

Structurally, smaller but denser stomata, as in Chloridoideae compared with Panicoideae, are more rapidly controlled during short-term water stress (Franks and Farquhar, 2007). Longer and much narrower leaves of Chloridoideae are also directly related with a faster relative curling rate. Quick-to-achieve leaf curling end points are ecologically meaningful as they offer an effective means of saving water during drought, and curling is found in many species of dry habitats (Grammatikopoulos and Manetas, 1994). Chloridoideae also had higher SLA, which was linked with lower leaf cuticular conductance and K_{leaf} , implying a higher internal resistance of leaves (Sack *et al.*, 2013).

PV curves detected two strategies for species in Chloridoideae to reach a higher potential drought resistance than Panicoideae (Fig. 4; Supplementary Table S3). Within Chloridoideae, NAD-ME species had highly elastic leaves (lower ϵ), giving the largest capacity to deviate from an ideal osmotic system (lower Ψ_{sat} and RWC at TLP), which may buffer transient changes in transpiration and contribute to water storage for survival after stomata close (Sack *et al.*, 2003; Bartlett *et al.*, 2012). These traits imply a drought avoidance strategy in NAD-ME species of Chloridoideae. In contrast, PCK species in Chloridoideae achieved low osmotic potentials (lower Ψ_{osat} and TLP), implying high solute concentrations in cells for either osmo-protection or antioxidant defence (Gullo and Salleo, 1988; Carmo-Silva *et al.*, 2009), and a drought tolerance strategy. In this study, lower ϵ and more negative Ψ_{osat} did not occur together in the same species, as they contributed to drought strategies differently, i.e. Ψ_{osat} influenced the TLP, whereas ϵ was associated more with RWC at the TLP (Bartlett *et al.*, 2012).

We also uncovered several ecophysiological patterns by integrating water relations traits: (i) leaf stomatal and cuticular conductances to water vapour, and hydraulic conductance to liquid water were lower in Chloridoideae than Panicoideae owing to the linkages between hydraulics, transpiration, and stomatal control (Osborne and Sack, 2012). However, these relationships are weakened by their semi-independent responses to irradiance and dehydration (Guyot *et al.*, 2012). (ii) Among a series of leaf structural traits, only LDMC showed significant relationships with hydraulic traits such as TLP and K_{leaf} (Fig. 5), suggesting that some leaf economic spectrum traits were linked with hydraulic traits. (iii) We did not find any relationships between leaf longevity and other ecophysiological traits in C_4 grasses, although leaf longevity is typically correlated

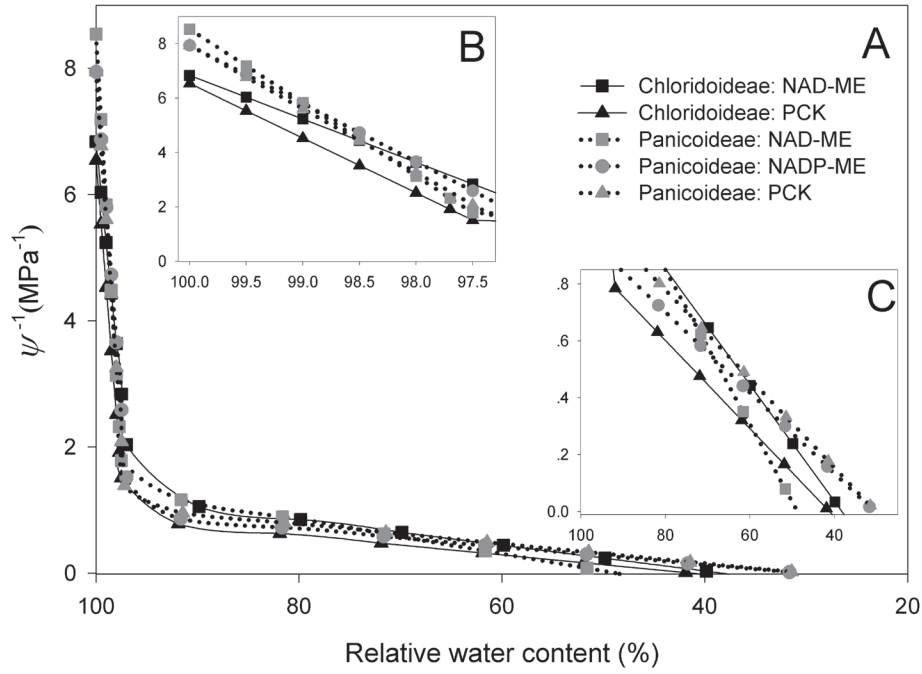


Fig. 4. Physiological responses to leaf water loss. Pressure–volume (PV) curves of different subfamily and C_4 subtype groups. (A) The entire PV curve; (B) a magnified view of turgor loss phase and (C) a magnified view of osmotic loss phase. Curves are modelled on average values of species in each group.

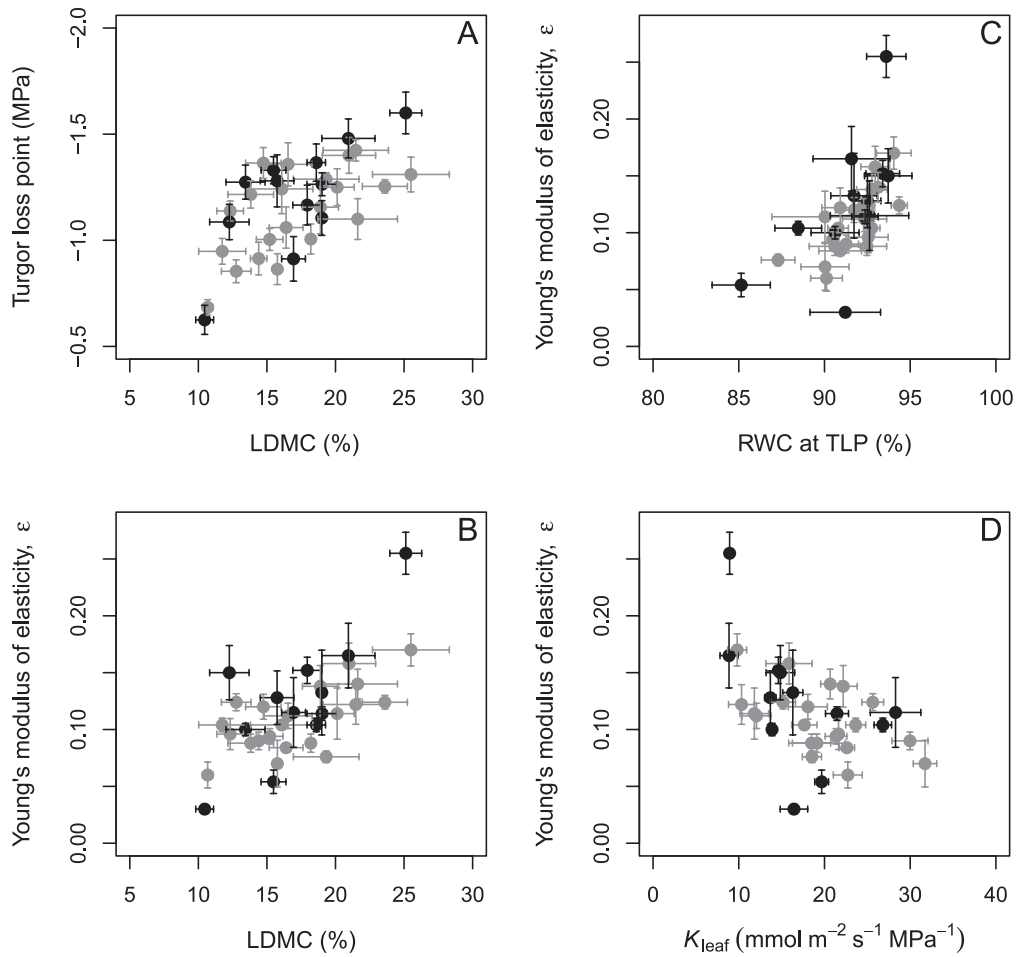


Fig. 5. Correlations among leaf hydraulic traits. (A) TLP with LDMC, Young's modulus of elasticity (ϵ) with (B) LDMC, (C) RWC at TLP, and (D) leaf hydraulic conductance (K_{leaf}).

with leaf construction and physiological traits (Wright *et al.*, 2004) and K_{leaf} (Simonin *et al.*, 2012) in most dicots. The lack of a relationship may arise because grass leaves all turn over quickly, which means there is little interspecific variation among the species investigated.

The lack of significant physiological advantages for Chloridoideae species in this study might also arise from the differences between glasshouse and field conditions. For example, a humid (RH 70%) environment and no drought stress might limit the hydraulic advantages of dumbbell shaped stomata under high humidity (Franks and Farquhar, 2007). Furthermore, some C_4 species in drier places might use a drought escape strategy by growing in the rainy and hot summer to avoid drought or frost (Lambers *et al.*, 1998; Ibrahim *et al.*, 2008), which could also confound direct comparisons obtained in a glasshouse.

Conclusions

Phylogenetic divergences within C_4 grasses have given rise to contrasting habitat type, plant size, stomatal size and number, leaf shape, and leaf relative curling rates in Chloridoideae and Panicoideae, but differences in leaf hydraulic and gas exchange traits between the two subfamilies are weak. This pattern can be explained by the interactions of subfamily \times C_4 subtype and subfamily \times habitat water availability in affecting ecophysiological traits, especially those linked to leaf water relations. Phylogeny and C_4 subtype each tend to have stronger effects on structural and physiological traits, respectively, and the interaction between subfamily and habitat type results in different adaptation strategies. All Chloridoideae species have faster relative leaf curling rates than Panicoideae, irrespective of C_4 subtype; but Chloridoideae species have two different ways to reach higher drought resistance potential than Panicoideae, NAD-ME species by drought avoidance, and PCK species by osmotic adjustment. This study elucidated the roles of C_4 subtype and habitat type in affecting ecophysiological differences between subfamilies. The work expands our understanding of water relations in major C_4 grass lineages, with implications for explaining their regional and global distributions in relation to climate.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Species list with seed source accessions, photosynthetic subtype, life form, and habitat type.

Table S2. PCA and phylogenetic PCA (PPCA) for the first two principal components based on 29 plant traits of 33 C_4 grasses.

Table S3. Parameters for PV curves in subgroups in Fig. 4.

Figure S1. The consistency among MAP, water score and habitat type.

Figure S2. Phylogenetic relationships of the 33 species with C_4 photosynthetic subtypes labelled.

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