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Research

Evolutionary lineage explains trait variation among 75 coexisting grass species

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Summary

- Evolutionary history plays a key role driving patterns of trait variation across plant species. For scaling and modeling purposes, grass species are typically organized into C_3 vs C_4 plant functional types (PFTs). Plant functional type groupings may obscure important functional differences among species. Rather, grouping grasses by evolutionary lineage may better represent grass functional diversity.
- We measured 11 structural and physiological traits *in situ* from 75 grass species within the North American tallgrass prairie. We tested whether traits differed significantly among photosynthetic pathways or lineages (tribe) in annual and perennial grass species.
- Critically, we found evidence that grass traits varied among lineages, including independent origins of C_4 photosynthesis. Using a rigorous model selection approach, tribe was included in the top models for five of nine traits for perennial species. Tribes were separable in a multivariate and phylogenetically controlled analysis of traits, owing to coordination of important structural and ecophysiological characteristics.
- Our findings suggest grouping grass species by photosynthetic pathway overlooks variation in several functional traits, particularly for C_4 species. These results indicate that further assessment of lineage-based differences at other sites and across other grass species distributions may improve representation of C_4 species in trait comparison analyses and modeling investigations.

Introduction

Over the past 55–60 million years (Myr), the grass family Poaceae has become one of the world's most diverse and dominant plant families. Currently, there are over 11 500 described species spread across 768 genera and 52 tribes (Soreng *et al.*, 2017). Furthermore, biomes with significant grassy layers (grasslands and savannas) cover over one-quarter of the earth's terrestrial surface (Asner *et al.*, 2004) and sequester nearly 40 Pg C yr^{-1} (Beer *et al.*, 2010). The global-scale emergence of grass-inhabited biomes and the more local-scale grass lineages that came to populate these early biomes led to lineage-specific environmental selection, and clear examples of both divergent and convergent evolution within Poaceae. For example, the Pooideae lineage evolved to be cold-climate specialists (Edwards & Smith, 2010), and the warm-climate Chloridoideae species are arid-land specialists distinct from the warm-climate Andropogoneae species that inhabit mesic sites (Lehmann *et al.*, 2019). One of the clearest examples of convergent evolution in the grasses – and one of the most critical aspects of Poaceae success globally – has been the evolution of the C_4 photosynthetic pathway. Despite accounting for *c.* 1% of all species of plants and fewer than half of all grass species (Osborne *et al.*, 2014; Christenhusz & Byng, 2016), C_4 grasses, including crops, cover *c.* 19 million km^2, and have been estimated to account for nearly a quarter of total terrestrial gross productivity (Still *et al.*, 2003).

In Poaceae, the C_4 pathway has evolved independently more than 20 times over the past *c.* 30 Myr (Grass Phylogeny Working Group II, 2012). The evolution of the C_4 pathway is thought to have been selected for by a combination of low atmospheric CO_2 and high temperature, but additional selective agents include water limitation and increased aridity in the mid-to-late Miocene (Ehleringer *et al.*, 1997; Sage *et al.*, 2018; Zhou *et al.*, 2018). So even within this oft-cited example of convergent evolution, C_4 physiology within the grasses should not be viewed as a single functional type because of the evolutionary momentum of previous divergences and changing selective agents through time. Such considerations extend even more broadly to the whole of Poaceae, where physiological and structural diversity has been selected for and preserved within separate taxonomic lineages across millions of years.

Due to the inherent physiological differences between the C_3 and C_4 photosynthetic pathways, C_4 grasses are often grouped into a single plant functional type (PFT) in macroecological analyses to simplify community structure and diversity (Griffith *et al.*, 2020). These highly abstracted PFTs are also typically used to represent grass functional biodiversity in most land models (Still *et al.*, 2003). These land models integrate a large (but incomplete) array of physical, biological, and chemical processes and are crucial for climate-related decision-making and policy (Lawrence *et al.*, 2019; Burrows *et al.*, 2020). Plant functional types are popular for capturing first-order ecosystem properties because it becomes nearly impossible to account for species-specific structure and function when creating large-scale models representing ecosystem processes (Woodward & Cramer, 1996). While PFTs may simplify the modeling process, important aspects of C_4 grass diversity resulting from their independent evolutionary origins may be overlooked (Edwards *et al.*, 2007, 2010; Liu *et al.*, 2012; Zhou *et al.*, 2023). We note that observational studies have found that differences in C_4 grass traits and growth responses are strongly linked to their lineages (Kellogg, 1999; Taylor *et al.*, 2010; Liu & Osborne, 2015).

One approach to capture this type of functional diversity is to categorize grasses into lineage-based functional types (LFTs), whereby grasses are grouped based on their phylogenetic relatedness (Griffith *et al.*, 2020). The importance of distinct lineages within the grass family and their relationships to traits has long been recognized (Hartley, 1950, 1958a,b; Hartley & Slater, 1960). While recent studies have utilized lineage-based classification to investigate trait–environment relationships within Poaceae (He *et al.*, 2009; Forrestel *et al.*, 2017), this approach is still not commonly incorporated into most macroecological analyses. Grouping grass species by evolutionary lineage, as in LFTs, rather than by photosynthetic type, as in PFTs, presents a promising method to capture the broad diversity of species' traits in an evolutionarily consistent framework since many important plant traits are known to be phylogenetically conserved (Edwards *et al.*, 2007; Liu *et al.*, 2012; Coelho de Souza *et al.*, 2016). This approach may help prevent crude oversimplifications inherent to C_3 or C_4 PFTs, where a single PFT may include species in distantly related lineages, each potentially having evolved traits unrelated to C_3 or C_4 pathways (Edwards *et al.*, 2007, 2010). Grouping traits by lineage can also explain trait variation, as seen in Edwards *et al.*'s (2007) study, where traits of *Echinochloa* appeared to be outliers in its C_4 PFT group, suggesting that the genus may have traits unique to its independent C_4 lineage. Furthermore, LFT approaches have the potential to be broadly applicable in other ecosystem types and for remote sensing and scaling applications (Anderegg *et al.*, 2022; Griffith *et al.*, 2023; R. Slapikas *et al.*, unpublished).

However, not all traits are phylogenetically conserved (Cadotte *et al.*, 2017), and factors such as photosynthetic type and life history (i.e. whether a species is annual or perennial) also explain a large proportion of trait variation. For example, Liu *et al.* (2019) showed that life history explained more of the variation in structural traits than photosynthetic pathway in grasses, while the C_3–C_4 contrast in species explained most of the variation in physiological traits. Thus, an assessment of trait variation within Poaceae is incomplete, and recent work has called for increased collection of grass traits across lineages (Griffith *et al.*, 2020).

Here, we measured 75 species of Poaceae (Fig. 1) and analyzed physiological and structural traits (Table 1) from *in situ* tallgrass prairie populations located at the Konza Prairie Biological Station (KPBS, Manhattan, KS, USA). We present a full suite of traits that span gas-exchange, hydraulic, and key functional traits. Traits were selected based on those that are currently utilized or show promise for future incorporation into large-scale ecosystem models. Our objective was to understand how commonly studied traits vary across lineages and photosynthetic pathways in species that are growing within a single site. We assessed the ability of evolutionary lineage (tribe and C_4 lineage) and photosynthetic pathway to explain trait variability among annual and perennial species. Measuring a suite of traits from a large diversity of species growing at the same site allowed us to categorize trait variation and illustrate the broad diversity of traits that can occur within a single grassland site. We hypothesized that tribe would be the best predictor of traits, more so than photosynthetic pathway. We further hypothesized that there would be substantial variation of traits in species among the seven C_4 lineages represented at our site. We expected this variation because the evolution of C_4 lineages varies biogeographically, and it is expected that these lineages will conserve traits from the climate of the biomes in which they evolved (Edwards *et al.*, 2007; Crisp *et al.*, 2009).

Materials and Methods

Site description

Field work and data collection were conducted at the Konza Prairie Biological Station (KPBS), a historically unplowed 3487 ha tallgrass prairie located in the Flint Hills region of northeastern Kansas, USA (39°05′, 96°35′ W). Konza Prairie Biological Station is divided into 52 different watersheds, each experimentally manipulated in terms of fire frequency (burned once every 1, 2, 4, or 20 yr) and grazing (cattle, bison, or ungrazed). This management of grazing and burning creates a mosaic of microsite conditions that contribute to the high plant species diversity that is representative of the broader tallgrass prairie ecosystem. Mean annual precipitation was 812 mm (1983–2020), the majority (*c.* 70%) of which was received during the growing season (April–September). Mean growing season temperature was 21°C (1983–2020), during which the mean daytime temperature from 9:00 h to 15:00 h was 24°C.

Data collection

The flora of KPBS includes 99 grass species comprising annuals, perennials, native, and non-native species (Nippert *et al.*, 2019). In 2020, we sampled 75 grass species that exist within the boundaries of KPBS (Fig. 1). Of the 12 subfamilies and 52 tribes that are currently recognized in Poaceae (Soreng *et al.*, 2017), our study measured species representing five subfamilies (Aristidoideae, Chloridoideae, Oryzoideae, Panicoideae, and Pooideae)

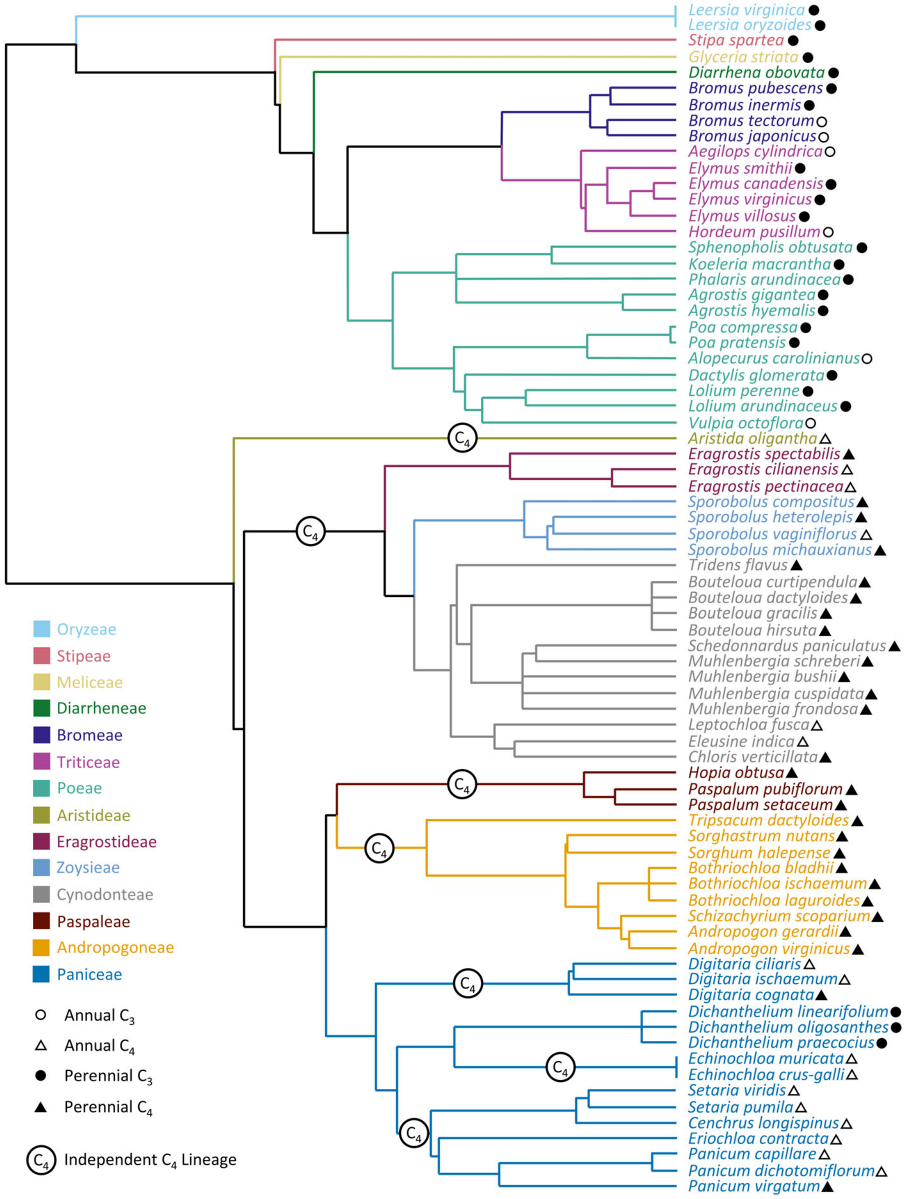

Fig. 1 Phylogeny of all 75 grass species sampled. The tree was generated using V.PHYLOMAKER (Jin & Qian, 2019). Colors indicate tribe and C_4 markings indicate independent evolution of the C_4 pathway in each lineage.

Table 1 List of all traits measured in our study.

Trait	Type
Maximum flowering height (cm)	Structural
Maximum vegetative height (cm)	
Specific leaf area (SLA; $cm^2\ g^{-1}$)	
Leaf dry matter content (LDMC; $g\ g^{-1}$)	
Leaf thickness (mm)	
Foliar C : N	
Osmotic potential (MPa)	Physiological
$\delta^{13}C$ (‰)	
Vc_{max} ($\mu mol\ m^{-2}\ s^{-1}$)	
J_{max} ($\mu mol\ m^{-2}\ s^{-1}$)	
Vp_{max} ($\mu mol\ m^{-2}\ s^{-1}$)	

and 14 tribes (Fig. 1; Andropogoneae, Aristideae, Bromeae, Cynodonteae, Diarrheneae, Eragrostideae, Meliceae, Oryzeae, Paniceae, Paspaleae, Poeae, Stipeae, Triticeae, and Zoysieae). Importantly, this represented species from seven of the independent C_4 origins in grasses (Fig. 1; Andropogoneae, *Aristida*, Chloridoideae, *Digitaria*, *Echinochloa*, *Paspalum*, and the MPC Clade – the clade which constitutes the subtribes Cenchrinae, Melinidinae, and Panicinae), including the two most dominant lineages globally (Lehmann *et al.*, 2019). Sampling occurred throughout the entirety of the site – there was no specific location for measuring all the species given that we measured naturally established individuals growing within their own viable habitat. Five replicate populations were marked for each species. The replicates varied at distances ranging from a few meters to a few kilometers depending on the abundance of the species to ensure independence. For rare species, it was often difficult to find more than a few individuals across all of KPBS, so replicates of rare species tended to be in closer proximity than the replicates of dominant species. For the dominant species (e.g. *Andropogon gerardii* Vitman) that number in the hundreds of millions of individuals on-site, capturing the breadth of the population differences was beyond the scope of this project. We also recognize that many of our species were collected in different habitats, such as varying fire regimes, grazing treatments, proximity to water sources, soil type, and canopy cover. However, it is precisely because of this landscape mosaic that KPBS supports such a great diversity of grass species; linking trait differences to specific microsite environmental conditions was not the goal of this project. Instead, our goal was to understand the sympatric trait variability occurring within landscape patterns which many ecosystem models aim to summarize.

A suite of plant traits was collected for each replicate in coordination with the initiation of flowering for each species (Table 1). We used flowering as a key phenological event to synchronize trait measurements. Traits measured include photosynthetic A–C_i response curves (to derive Vc_{max}, J_{max}, and Vp_{max} (for C_4 species)), leaf osmotic potential at full turgor (MPa), specific leaf area (SLA; $cm^2\ g^{-1}$), leaf dry matter content (LDMC; $g\ g^{-1}$), leaf thickness (mm), maximum plant flowering and vegetative heights (cm), foliar C : N, and $\delta^{13}C$ leaf composition. Vc_{max} is the maximum rate of carboxylation of Rubisco ($\mu mol\ m^{-2}\ s^{-1}$), J_{max} is the maximum rate of electron transport (μmol electrons $m^{-2}\ s^{-1}$), and Vp_{max} is the maximum rate of carboxylation of PEPc ($\mu mol\ m^{-2}\ s^{-1}$).

Leaf gas exchange was measured with a LI-6400XT Portable Photosynthesis System (Li-COR Inc., Lincoln, NE, USA) on leaves for two to three replicates of each species. Measurements were taken between 9:00 h and 15:00 h on healthy, fully expanded leaves and were not affected by diel increases in temperatures or midday depression in assimilation (Supporting Information Fig. S1). Gas exchange measurements for A–C_i response curves were collected by taking measurements at eight concentrations of CO_2 in the following order: 400, 300, 200, 100, 50, 500, 800, and 1000 $\mu mol\ CO_2\ mol^{-1}$. The in-chamber PAR intensity was fixed at 2000 $\mu mol\ m^{-2}\ s^{-1}$, relative humidity was maintained between 40% and 60%, and the leaf was allowed a minimum of 90 s to a maximum of 450 s to equilibrate between changes in $[CO_2]$. Measurements were taken in optimal ambient light conditions with little-to-no cloud cover. Electron transport rate (J_{max}) and maximum carboxylation (Vc_{max}) for C_3 species were determined using the curve-fitting procedures described in the 'PLANTECOPHYS' R package (Duursma, 2015). For C_4 species, we used the Excel curve fitting procedure described by Zhou *et al.* (2019). A–C_i parameters were corrected to a common leaf temperature (25°C).

Osmotic potential, a trait linked to drought tolerances of species and related to the turgor loss point (Bartlett *et al.*, 2012a,b), was measured using a Vapro® Vapor Pressure Osmometer (Model 5600; Logan, UT, USA) for three leaf replicates of each species. For each replicate, one individual grass tiller was harvested and subsequently clipped with the stem underwater. The stems remained underwater overnight before osmotic potential measurements were taken the next day. Our measurement protocol followed Griffin-Nolan *et al.* (2019). In brief, a single, fully hydrated leaf was removed from the grass tiller, punched with a 5-mm leaf tissue punch, and quickly wrapped in aluminum foil before being submerged in liquid nitrogen for 1 min. Once removed, the frozen tissue was immediately pierced to lyse cell contents. This tissue was then equilibrated in the osmometer's tissue chamber for 10 min before measurement.

For leaf measurements, one leaf was taken from each replicate. Leaf area was measured in the field using Leafscan, a mobile app for measuring the surface area of individual leaves (Anderson & Rosas-Anderson, 2017). Leaf wet mass was measured after leaf rehydration, and leaf dry mass was measured after the leaf had been dried for at least 48 h at 60°C. Rehydration was performed by submerging the leaf in water for 24–72 h. Leaf thickness was derived from $(SLA \times LDMC)^{-1}$ and multiplied by 10 to convert to mm (Vile *et al.*, 2005). SLA ($cm^2\ g^{-1}$) was calculated as the leaf area divided by the leaf dry mass and LDMC ($g\ g^{-1}$) was calculated as the leaf dry mass divided by the leaf wet mass.

Maximum flowering height and maximum vegetative height (cm) were measured from the ground to the highest point of the inflorescence or the uppermost leaf, respectively. At each replicate, five randomly selected individuals were measured; the tallest inflorescence and tallest leaf were used for maximum heights.

Leaf C and N content and stable C isotopic composition were measured at the Stable Isotope Mass Spectrometry Laboratory at Kansas State University. For each replicate, five leaves were dried at 60°C for a minimum of 48 h and then homogenized using an amalgamator. Total C and N of homogenized leaf samples were measured following combustion using an Elementar vario Pyro cube coupled to an Elementar Vision mass spectrometer for isotope analysis. Isotopic abundance ratios were converted to δ notation using:

$$\delta = \left[\frac{R_{sample}}{R_{standard}} - 1\right] \times 1000,$$

where R is the ratio of heavy (^{13}C) to light (^{12}C) isotopes for the sample and standard, respectively. Working laboratory standards were annually calibrated against the internationally accepted standard, Vienna Pee-Dee Belemnite for $\delta^{13}C$. Within-run and across-run variability of the laboratory working standard (apple leaves) was $< 0.05‰$.

Statistical analysis

All statistical analyses were performed in R v.4.2.2 (R Core Team, 2022). For each trait, we used linear mixed effects models with photosynthetic pathway, tribe, or C_4 lineage as predictors in separate models and species as a random effect using the 'NLME' package (Pinheiro *et al.*, 2017):

$$\text{Trait} \sim \text{Predictor} + (1|\text{species}).$$

We analyzed annuals and perennials separately, given the important influence of life history on species traits. Annuals have unique life history trade-offs and comprise a small proportion of vegetation abundance at both the local and regional scales in temperate perennial grasslands. However, we included annuals in this study because they add to the diversity of perennial grasslands,

Table 2 Akaike information criterion (AICc) comparisons of models with tribe or photosynthetic pathway as predictors and species as a random effect.

Trait	Life History	Factor	AICc
Maximum flowering height (cm)	Annual	Tribe	142.05
		Photosynthetic Pathway	144.02
	Perennial	**Tribe**	**126.38**
		Photosynthetic Pathway	129.46
		Tribe + Photosynthetic Pathway	**124.49**
Maximum vegetative height (cm)	Annual	Tribe	138.41
		Photosynthetic Pathway	140.24
	Perennial	**Tribe**	**165.17**
		Photosynthetic Pathway	168.60
		Tribe + Photosynthetic Pathway	**163.75**
Specific leaf area (SLA; cm^2 g^{-1})	Annual	Tribe	44.022
		Photosynthetic Pathway	**40.46**
	Perennial	Tribe	45.79
		Photosynthetic Pathway	**38.82**
		Tribe + Photosynthetic Pathway	48.20
Leaf dry matter content (LDMC; g g^{-1})	Annual	Tribe	−321.71
		Photosynthetic Pathway	**−340.57**
	Perennial	Tribe	−872.26
		Photosynthetic Pathway	**−914.95**
		Tribe + Photosynthetic Pathway	−870.71
Leaf thickness (mm)	Annual	Tribe	−346.75
		Photosynthetic Pathway	**−386.32**
	Perennial	Tribe	−831.47
		Photosynthetic Pathway	**−878.35**
		Tribe + Photosynthetic Pathway	−826.65
Foliar C : N	Annual	**Tribe**	**687.31**
		Photosynthetic Pathway	722.04
	Perennial	Tribe	1699.90
		Photosynthetic Pathway	1746.69
		Tribe + Photosynthetic Pathway	**1694.32**
Osmotic Potential (MPa)	Annual	Tribe	50.51
		Photosynthetic Pathway	**42.11**
	Perennial	**Tribe**	**87.83**
		Photosynthetic Pathway	90.47
		Tribe+Photosynthetic Pathway	91.05
$Vc_{\max}$ (μmol m^{-2} s^{-1})	Annual	Tribe	60.71
		Photosynthetic Pathway	**53.44**
	Perennial	Tribe	170.89
		Photosynthetic Pathway	**145.42**
		Tribe + Photosynthetic Pathway	158.74
$J_{\max}$ (μmol m^{-2} s^{-1})	Annual	**Tribe**	**347.88**
		Photosynthetic Pathway	389.86
	Perennial	Tribe	870.73
		Photosynthetic Pathway	953.61
		Tribe + Photosynthetic Pathway	**862.29**

Annual and perennial species were analyzed separately. For perennial species, we also included an additive model with tribe and photosynthetic pathway as predictors. Bolded values indicate lowest AICc value (±2).

have unique traits, and can dominate disturbed microsites. For annuals and perennials separately, we compared models with tribe or photosynthetic pathway as predictors with species as a random effect using Akaike information criterion (AICc):

$$\text{Trait} \sim \text{Tribe} + (1|\text{species}),$$

$$\text{Trait} \sim \text{Photosynthetic Pathway} + (1|\text{species}).$$

We did not test whether interactions were present between tribe and photosynthetic pathway in this study given that these factors are confounded with one another. For instance, only one of the 14 tribes sampled in this study included both C_3 and C_4 species (Paniceae).

For perennial species, we also included an additive model with tribe and photosynthetic pathway as predictors because Paniceae included both C_3 and C_4 perennial species (Table 2):

$$\text{Trait} \sim \text{Tribe} + \text{Photosynthetic Pathway} + (1|\text{species}),$$

We used Principal Component Analysis (PCA) to visually assess whether certain traits were associated with grass photosynthetic pathway or tribe. Trait values were averaged across the five replicates for each species. Data were log-transformed and standardized to linearize relationships among traits and ensure each variable held equal weight in the analysis. We imputed $J_{\max}$ and $Vc_{\max}$ for species that had poor or missing A–C_i curves (9% of perennial species and 13% of annual species) using the 'PCAMETHODS' package (Stacklies *et al.*, 2007; described in Fridley *et al.*, 2022). We ran a separate PCA for perennial and annual species with the imputed dataset using the princomp function. To account for the nonindependence of related species in our ordination, we also ran a phylogenetic PCA using the phyl.pca function in the 'PHYTOOLS' package (Revell, 2012). The use of phylogenetic PCA also inherently addresses the influence of different species richness values for lineages at Konza Prairie. We ran a one-way ANOVA to assess whether tribe or photosynthetic pathway primarily explained patterns in the two primary axes, PC1 and PC2.

We considered the use of phylogenetic comparative methods (e.g. PGLS; Phylogenetic Generalized Least Squares), which account for trait nonindependence due to phylogenetic distance. However, we were not interested in trait–trait comparisons and focused instead on lineage differences, which PGLS would not allow as lineage is confounded with the phylogenetic covariance structure. Instead, we were interested in major trait differences distinguishing grass lineages and if those differences would be better explained by lineage or by photosynthetic type.

Results

Principal component analyses

The conventional PCA and phylogenetic PCA explained a similar amount of variation in PFTs for perennial and annual species (Fig. 2). In the phylogenetic PCA for perennial species, the association of foliar C : N, plant height, and LDMC is somewhat tighter, and overall alignment of SLA with PC1 is strengthened compared with conventional PCA (Fig. 2a,b). Additionally, the similarity in our PCA and phylogenetic PCA provides confidence that the differing species richness levels for lineages at Konza Prairie do not significantly bias our results. For perennial species, PC1 and PC2 significantly varied by tribe and photosynthetic pathway in the conventional PCA (Table S1; Fig. 2a). A–C_i parameters, $Vc_{\max}$, and $J_{\max}$ differentiated C_3 and C_4 species along PC2 and C_4 species were correlated with high LDMC and foliar C : N. Similar patterns were found when controlling for phylogeny, and PC2 strongly varied by photosynthetic pathway ($P < 0.001$; Table S1) but PC1 did not ($P > 0.05$; Table S1; Fig. 2b). Specific leaf area and leaf thickness were negatively associated with one another and orthogonal to $Vc_{\max}$ in both the conventional and phylogenetic PCA.

For annual species, PC1 and PC2 did not significantly vary by photosynthetic pathway but did vary by tribe ($P = 0.05$ and 0.006, respectively; Table S1; Fig. 2c). The Paniceae tribe contained the most species and was clustered and strongly associated with leaf thickness and height. Similar to perennial species, the phylogenetic PCA showed similar patterns and trait associations as the conventional PCA (Table S1; Fig. 2c,d).

Annual grasses

The following traits significantly differed among tribes for annual grasses: maximum flowering height, maximum vegetative height, SLA, LDMC, foliar C : N, and $Vc_{\max}$ (Fig. S2; Table S2). Only two traits, $Vc_{\max}$ and $J_{\max}$, differed significantly among C_3 and C_4 species, and both are photosynthetic traits (Tables S2, S3). Importantly, structural traits did not significantly differ between C_3 and C_4 annual grasses.

We used AICc to assess the best model for each trait (Table 2). Grouping species by photosynthetic pathway was the best model for SLA, LDMC, leaf thickness, osmotic potential, and $Vc_{\max}$. Grouping species by tribe was the best model for C : N and $J_{\max}$. Two traits, maximum flowering height and maximum vegetative height, did not favor models grouping species by tribe or photosynthetic pathway (AICc scores did not differ by > 2).

Perennial grasses

The following traits significantly differed among tribes for perennial grasses: maximum flowering height, maximum vegetative height, SLA, LDMC, leaf thickness, foliar C : N, and osmotic potential (Fig. 3; Table S4). Only three traits differed among C_3 and C_4 perennial grasses: LDMC, C : N, and $Vc_{\max}$ (Tables S3, S4).

We used AICc comparisons of these models to further assess the best model for each trait (Table 2). Of these nine traits, the 'best' model for five traits was either tribe or the additive model (photosynthetic pathway + tribe). In every instance where the best model was the additive model, the second-best model was tribe, indicating that tribe was the more important predictor driving these results compared with photosynthetic pathway in perennial grasses. Grouping species by tribe was the best model for maximum flowering height, maximum vegetative height, and osmotic potential. Grouping species by photosynthetic pathway was the best model for SLA, LDMC, leaf thickness, and $Vc_{\max}$. The additive model tribe + photosynthetic pathway was the best model for maximum flowering height, maximum vegetative height, C : N, and $J_{\max}$. We note that the additive model and the tribe-only model for maximum flowering height and maximum vegetative height were equal candidates (AICc did not differ by > 2).

C_4 lineages

We found that five traits significantly differed across C_4 lineages: maximum flowering height, maximum vegetative height, LDMC, $\delta^{13}C$, and osmotic potential (Fig. 4). Importantly, traits of the two dominant C_4 lineages in our region, the Andropogoneae and the Chloridoideae, varied substantially. On average, members of the Andropogoneae lineage had higher maximum flowering heights (Fig. 4a), maximum vegetative heights (Fig. 4b), $\delta^{13}C$ (Fig. 4g), and osmotic potential (Fig. 4h) compared with the Chloridoideae lineage.

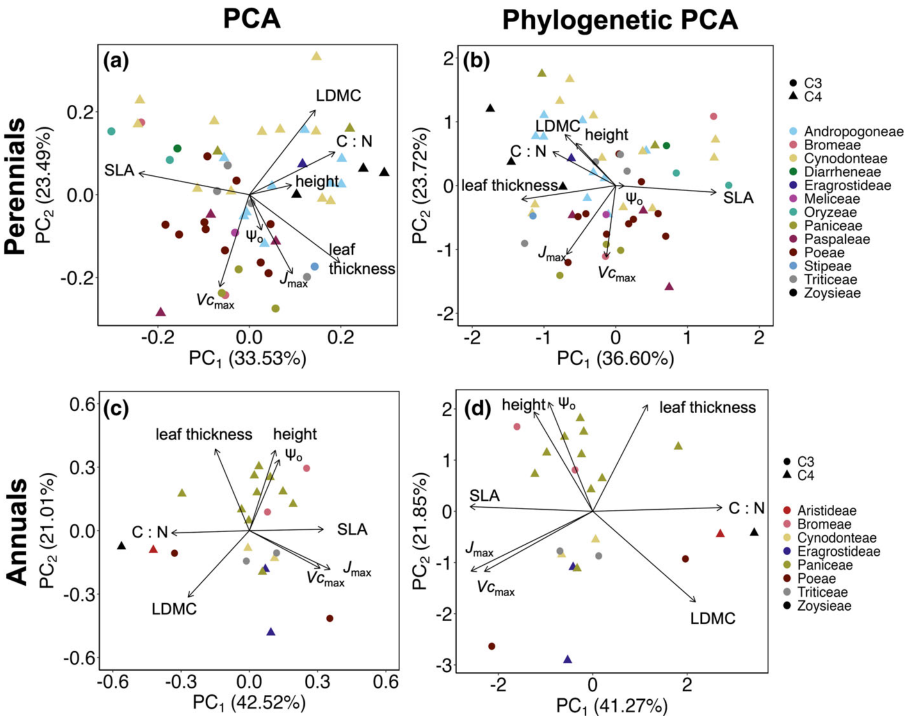

Fig. 2 Conventional Principal Component Analysis (PCA) and phylogenetic PCA of all species and eight functional traits sampled at KPBS. Species are separated by annual and perennial life histories. Points are classified by C_3 (circle) and C_4 (triangle) photosynthetic pathways. Colors represent grass tribes. Boxplots for visual representation for how variation of traits is partitioned among tribes and photosynthetic pathways for the two primary PCA axes are available in Supporting Information Figs S3–S6.

$\delta^{13}C$ and C_4 subtype

We also found significant differences in $\delta^{13}C$ by C_4 biochemical subtype (PCK, NAD-ME, and NADP-ME; $P < 0.0001$; Table S5). PCK species had the lowest $\delta^{13}C$ (-13.99 ± 0.17‰), a similar value to that of NAD-ME species (-13.84 ± 0.09‰), while NADP-ME species had the highest $\delta^{13}C$ (-12.70 ± 0.06‰). *Bouteloua curtipendula* (Michx.) Torr., a mixed NAD-ME/PCK species (Gutierrez *et al*., 1974), had a $\delta^{13}C$ value of -13.96 ± 0.11‰. Lastly, three *Sporobolus* species (*Sporobolus compositus* (Poir.) Merr., *Sporobolus heterolepis* (A. Gray) A. Gray, and *Sporobolus vaginiflorus* (Torr. ex A. Gray) Alph. Wood) had an average $\delta^{13}C$ of -13.59 ± 0.11‰. It is not currently known whether these three species utilize NAD-ME or PCK pathways.

Discussion

Evolutionary lineage captures differences in trait variation

Here, we illustrate that grouping grass species based on both PFT (plant functional type) and LFT (lineage-based functional types) frameworks captures structural and ecophysiological trait variation present in a highly diverse community. While our results suggest there are advantages for both grouping frameworks, we first highlight the trait variability exhibited by different lineages of grasses despite all species inhabiting the same site. A framework based on evolutionary relatedness (LFT) reflects key trait differences that occur within a single site without oversimplifying community diversity. A PFT approach to trait measurements in grassy ecosystems, particularly for dominant C_4 grasses, misses important trait variation that exists across the landscape, and the LFT grouping may serve as a constructive framework for improving functional trait representation in grassland macroecological analyses and ecosystem models (Griffith *et al*., 2020).

By combining ecophysiological and plant functional trait variability within a multivariate analysis of broad phylogenetic extent, we found previously unobserved patterns of trait coordination among suites of grass functional traits and gas-exchange parameters. While SLA and leaf thickness load strongly onto PC1, we found that Vc_{max} was orthogonal, being associated more with PC2 and LDMC. This result expands upon previous studies that find stronger relationships between SLA and leaf thickness than with LDMC (e.g. Niinemets, 2001; Griffith *et al*., 2016). Similarly, this result complements patterns of coordination between hydraulic traits and LDMC (Griffith *et al*., 2016; Griffin-Nolan *et al*., 2023) that are critical for describing ecosystem drought responses (Blumenthal *et al*., 2020; Wilcox *et al*., 2021). In our phylogenetically controlled analysis, tall plants with higher LDMC and foliar C : N are associated with lower Vc_{max}. Expected leaf economics patterns between SLA and C : N were maintained in both annuals and perennials. Overall, these patterns contributed to a strong clustering by tribe in ordination space for both annuals and perennials.

Structural traits are commonly measured in plant surveys to draw inferences on plant life history strategies and physiology at large scales (Wright *et al*., 2004; Reich, 2014). In our grassland system, photosynthetic type did not explain any differences found in structural traits except C : N and LDMC in perennial grasses (Tables S3, S4). By contrast, between both annual and perennial grasses there were significant differences found among tribes for all structural traits except the leaf thickness of annual species (Tables S2, S4). Structural traits are typically indicative of the life history strategy utilized by a particular species (Liu *et al*., 2019). Slower growing, perennial species tend to allocate

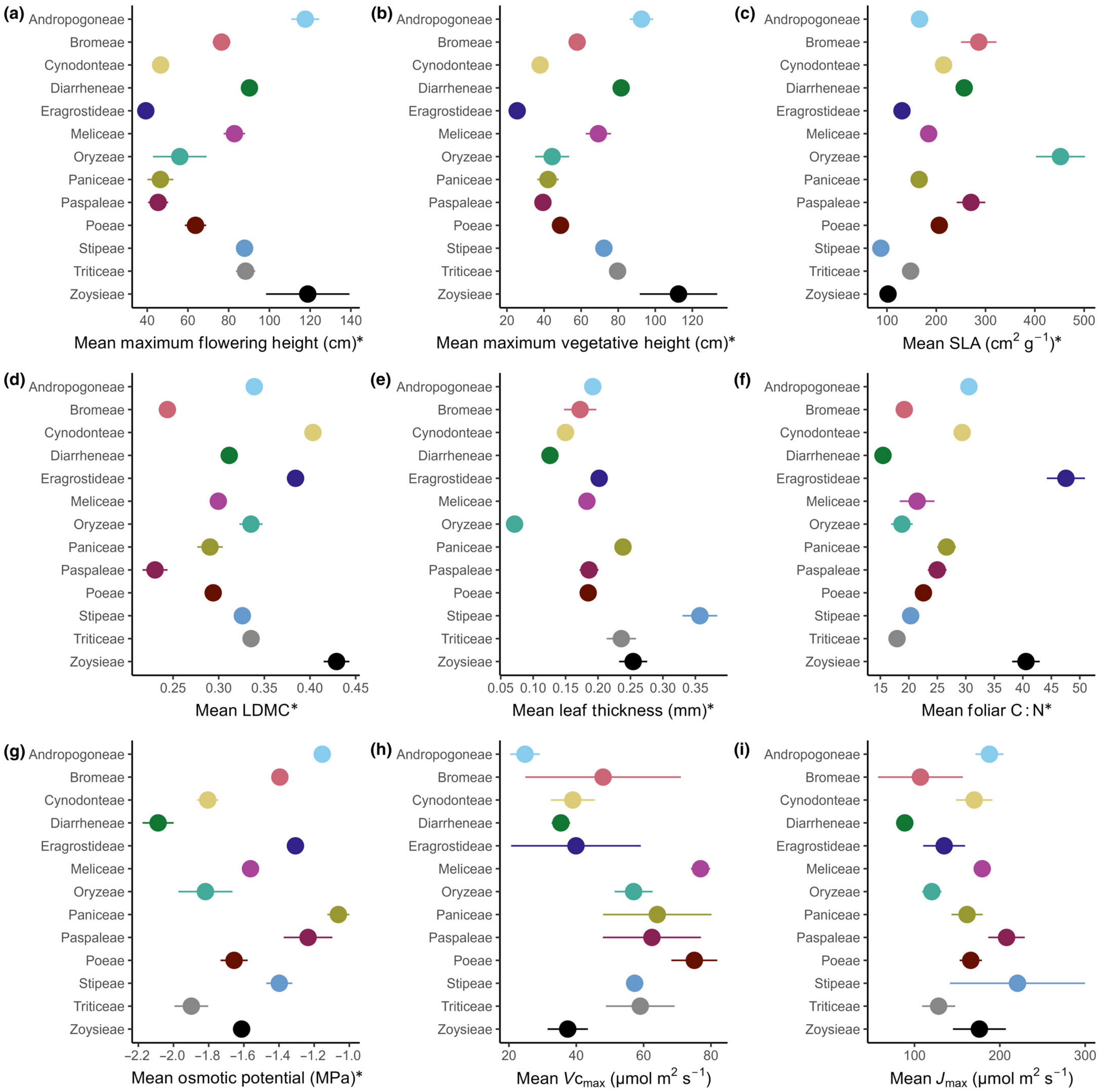

Fig. 3 Mean values for the structural and physiological traits in perennial grass species grouped by tribe. Error bars represent ±1SE. *Indicates traits that significantly differed by tribe ($P < 0.05$). LDMC, Leaf dry matter content; SLA, specific leaf area.

greater resources toward nonphotosynthesizing tissues, trading off maximizing photosynthesis for resource intensive and more durable leaves and stems, along with a high investment in the root system for acquiring water, nutrients, and prolonging life span (Grime, 1977; Monaco *et al.*, 2003; Reich, 2014). By contrast, fast-growing annual species allocate most resources toward producing thin, broad leaves to quickly maximize photosynthesis, investing energy into reproductive tissue before senescence (Grime, 1977; Garnier & Laurent, 1994; Garnier *et al.*, 1997; Reich, 2014). However, results from this study demonstrate stark differences in structural traits among tribes containing annual and perennial life histories. For instance, the perennial species we measured in Andropogoneae, a globally dominant tribe in high precipitation and fire-prone grassy ecosystems, had lower SLA and LDMC and greater leaf thickness, maximum flowering height, and maximum vegetative height than perennial species in another dominant tribe, Cynodonteae, which tends to dominate drier sites than Andropogoneae (Fig. 3). We highlight these

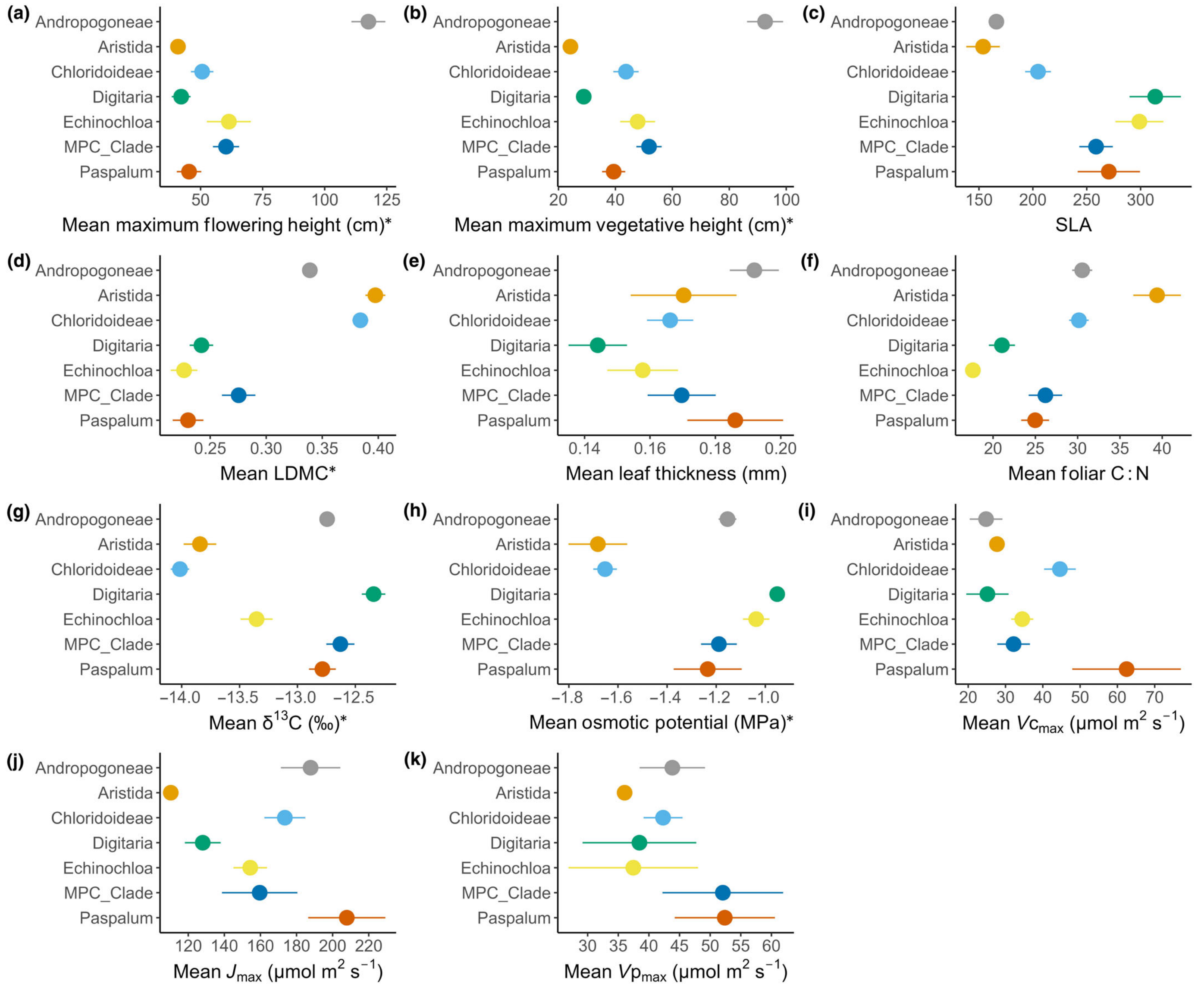

Fig. 4 Mean values for the structural and physiological traits in C_4 species grouped by C_4 lineage. Error bars represent ±1SE. Each of these lineages represents an independent origin of C_4 photosynthesis. *Indicates traits that significantly differed by C_4 lineage ($P < 0.05$). LDMC, Leaf dry matter content; SLA, specific leaf area.

differences because these dominant, perennial species in our grassland system all have the same life history strategy but differ in their structural traits. Furthermore, such trait differences may also facilitate co-occurrence of closely related, dominant species (Gray & Smith, 2022). Our results show many commonly measured structural traits do not vary by photosynthetic type in this grassland despite known growth advantages of the C_4 photosynthetic pathway in hot environments (Atkinson *et al.*, 2016; Simpson *et al.*, 2020). These important distinctions among perennial species in dominant lineages are not captured when grouping grasses by photosynthetic pathway.

We also highlight that unusual trait variation among species may be best understood when models are based on evolutionary lineage. For example, the Oryzeae tribe directly contradicts some conventional expectations for perennial species. In our study, Oryzeae consisted of two perennial C_3 species (*Leersia virginica* Willd. and *Leersia oryzoides* (L.) Sw.), both of which exhibited traits typical of annual species. For instance, Oryzeae had the highest mean SLA and the lowest mean leaf thickness among both annual and perennial grasses (Figs 3c,e, S2c,e). In studies that do not consider phylogeny, this stark outlier may appear unusual. Both species share the same genus and occupy similar niches in the tallgrass ecosystem; they are rhizomatous species that grow near streams and within wet areas (Darris & Bartow, 2004; Ritz, 2012) and bloom later in the season than almost any other C_3 grass species at KPBS. It may not be as important for these species to invest heavily in expensive stem or leaf tissues since they occupy entirely different habitats than dominant grass species at KPBS. Although *Leersia* is a dominant genus in grasslands in other regions of the world, such as in South America (Dirven, 1965; Medina & Motta, 1990; Andrade *et al.*, 2019), these two *Leersia* species are not ecologically dominant in this region and do not contribute greatly to broad ecosystem function in North American tallgrass prairie. However, they provide an excellent illustrative example of how species can have traits that differ from common predictions made based on life histories or photosynthetic pathways.

Trait variation within the C_4 photosynthetic pathway

Beyond analyzing trait variation solely from different tribes, we also analyzed how traits differed in unique lineages using the C_4 photosynthetic pathway. Critically, we found that nearly half of the traits we measured varied among C_4 lineages. If all C_4 species were grouped into a singular PFT, this variation would be overlooked. Focusing on the two dominant lineages of C_4 grass in North America (Andropogoneae and Chloridoideae) illustrates this point, as the Andropogoneae evolved to occupy wetter environments than the arid-specializing Chloridoideae (Lehmann *et al.*, 2019). Andropogoneae species measured at our site were taller (Fig. 4a,b) and had higher average $\delta^{13}C$ (Fig. 4g) and osmotic potential (Fig. 4h), which may reflect differences in water use strategies among these C_4 lineages. Systematic differences in $\delta^{13}C$ within C_4 plant lineages seem to be associated with water use differences among photosynthetic subtypes and are of sufficient magnitude to be considered when choosing end members in isotope studies (Hattersley, 1982). These trait differences suggest grouping grasses by photosynthetic type likely overlooks large differences in ecosystem water and carbon cycling among C_4 plants (Liu & Osborne, 2015). These differences may actually reflect differences in photosynthetic subtype between Chloridoideae (primarily NAD-ME) and Andropogoneae (NADP-ME) that are better captured when grouping grasses by lineage (Liu & Osborne, 2015; Griffith *et al.*, 2020).

PFTs, lineages, and grassland ecosystem modeling

Our results are consistent with other studies (Taylor *et al.*, 2010; Liu *et al.*, 2012; Griffith *et al.*, 2020) that demonstrate the importance of incorporating phylogenic history into trait comparison analyses. Grouping grasses by lineage allows studies to account for the evolutionary histories of species, where important functional traits may be conserved (Edwards *et al.*, 2007; Liu *et al.*, 2012; Coelho de Souza *et al.*, 2016). However, our work also validates the use of PFTs in certain instances. For example, several traits analyzed in this study are known to be highly sensitive parameters in commonly used land models. Differences in measurements of these influential traits disproportionally drive uncertainty in land models outputs, such as gross primary productivity, and include SLA, foliar C : N, and Vc_{max} (Göhler *et al.*, 2013; Ricciuto *et al.*, 2018; Fisher *et al.*, 2019; Huo *et al.*, 2019). Our results from AICc suggest that SLA and Vc_{max} were best represented by PFTs but C : N was best represented by tribe (Table 2). Thus, while SLA and Vc_{max} may be appropriately represented by PFTs in land models, C : N would be better represented by evolutionary lineage.

For use in land models, it may be especially important to consider how well PFTs represent existing trait variation in dominant lineages of grasses, especially in influential traits. In the CLM4.5, for example, C_4 grasses are represented by a single PFT with a mean Vc_{max} of 51.6 $\mu mol\ m^{-2}\ s^{-1}$ while C_3 grass PFTs have a mean Vc_{max} of 78.2 $\mu mol\ m^{-2}\ s^{-1}$ (Oleson *et al.*, 2013). In our study, the mean Vc_{max} of perennial species in the dominant C_3 tribe Poeae was $75.1 \pm 6.8\ \mu mol\ m^{-2}\ s^{-1}$ (Fig. 3h), indicating that the C_3 PFTs represent our dominant C_3 species well. However, the two dominant C_4 lineages in our study, Andropogoneae and Chloridoideae, have mean Vc_{max} of $24.8 \pm 4.4\ \mu mol\ m^{-2}\ s^{-1}$ and $44.6 \pm 4.2\ \mu mol\ m^{-2}\ s^{-1}$, respectively (Fig. 4i). In this case, the C_4 PFT represents Chloridoideae better than the Andropogoneae, indicating that functional trait variation within the Andropogoneae would not be accounted for in CLM4.5 in the North American tallgrass prairie ecosystem. A primary motivation of our study was to better understand and constrain trait variation at a single site that might be represented as one data point in macroecological analyses; however, we acknowledge that there are limitations to this approach. For example, many grassland ecosystems do not have multiple dominant lineages of grasses spanning different photosynthetic pathways.

The trait differences among tribes and C_4 lineages reported here come from species existing in the same local environment within a relatively small geographic site. Despite the high variability in trait values, evolutionary lineage was a strong predictor of alternative trait combinations that allow species to persist in the variable intercontinental climate of tallgrass prairie. If species were sampled at the macroecological scale – across different regions or environments – we expect to see even more distinct trait variation among lineages. Trait differences among lineages shown in this study are likely conservative, as we expect larger variation across sites. Further work comparing the traits of grass species globally is warranted to more accurately determine how traits vary across lineages.

Conclusions

We found that evolutionary lineage and photosynthetic pathway explain differences in both structural and physiological traits among the 75 species of grasses we measured. Additionally, important structural and physiological traits differed across C_4 lineages, emphasizing how dominant lineages of C_4 grasses may exhibit trait variation within a single grassland site. We conclude that while PFTs represent a valid strategy for grouping grass species for some analyses, incorporating evolutionary history is important for capturing trait diversity among C_4 lineages. This strategy will improve the representation of grass functional diversity and highlight unique trade-offs of grass species to local environmental variability.

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Competing interests

None declared.

Author contributions

RCD and JBN designed the study. RCD, JBN and JHT collected the data. RCD, ERW, DMG and JBN analyzed the data. RCD, ERW, JHT, JBN, BRH, WJR, CJS and DMG contributed to the revising and editing of the manuscript.

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Data availability

Trait data collected for this project are available from the Konza Prairie LTER Data Catalog http://lter.konza.ksu.edu/data (doi: 10.6073/pasta/f5a57d6436f931690d7f7529f87772db).

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Diurnal time series of photosynthetic rates at $CO_2 =$ 400 ppm extracted from the A–C_i curves.

Fig. S2 Mean values of each trait for annual grass species grouped by tribe.

Fig. S3 Boxplot of PC1 and PC2 scores for annual species (conventional PCA).

Fig. S4 Boxplot of PC1 and PC2 scores for annual species (phylogenetic PCA).

Fig. S5 Boxplot of PC1 and PC2 scores for perennial species (conventional PCA).

Fig. S6 Boxplot of PC1 and PC2 scores for perennial species (phylogenetic PCA).

Table S1 P-values of PC1 and PC2 for the conventional PCA and phylogenetic PCA of annual and perennial grass species.

Table S2 F, df, and P of all trait comparisons with either tribe or photosynthetic pathway as the predictor for annual grasses.

Table S3 Mean values of each trait for C_3 and C_4 species sorted by life history (annuals and perennials).

Table S4 F, df, and P of all trait comparisons with either tribe or photosynthetic pathway as the predictor for perennial grasses.

Table S5 F, df, and P of $\delta^{13}C$ comparisons among C_4 subtypes.