

Variation in climatic tolerance, but not stomatal traits, partially explains Pooideae grass species distributions

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- **Background and Aims** Grasses in subfamily Pooideae live in some of the world's harshest terrestrial environments, from frigid boreal zones to the arid windswept steppe. It is hypothesized that the climate distribution of species within this group is driven by differences in climatic tolerance, and that tolerance can be partially explained by variation in stomatal traits.
- **Methods** We determined the aridity index (AI) and minimum temperature of the coldest month (MTCM) for 22 diverse Pooideae accessions and one outgroup, and used comparative methods to assess predicted relationships for climate traits versus fitness traits, stomatal diffusive conductance to water (g_w) and speed of stomatal closure following drought and/or cold.
- **Key Results** Results demonstrate that AI and MTCM predict variation in survival/regreening following drought/cold, and g_w under drought/cold is positively correlated with $\delta^{13}\text{C}$ -measured water use efficiency (WUE). However, the relationship between climate traits and fitness under drought/cold was not explained by g_w or speed of stomatal closure.
- **Conclusions** These findings suggest that Pooideae distributions are at least partly determined by tolerance to aridity and above-freezing cold, but that variation in tolerance is not uniformly explained by variation in stomatal traits.

Key words: Climatic distribution, cold tolerance, drought tolerance, grass adaptation, guard cells, Pooideae, stomata.

INTRODUCTION

Pooideae is the largest subfamily of grasses, comprising around 4000 species, and containing some of the world's most important temperate crops, including wheat (*Triticum* sp.), barley (*Hordeum vulgare*), oats (*Avena sativa*) and ryegrass (*Lolium perenne*) (GPWG, 2012). Similar to the grasses in general, Pooideae species are inferred to have originated as forest understorey herbs, followed by one to several transitions to open habitats in all but the Brachyelytreae, which is sister to the remaining tribes (Kellogg, 2001; Saarela *et al.*, 2015). In line with this observation, many Pooideae crops, such as durum wheat (*Triticum turgidum* subsp. *durum*), have high tolerance (i.e. maintain high fitness) in response to drought, allowing them to be grown in desert regions where precipitation is as low as 250 mm per year (Monneveux *et al.*, 2012). Pooideae are also unique among the grass subfamilies in that they dominate temperate to boreal latitudes in the northern hemisphere, which experience seasonal to constitutive frost (McKeown *et al.*, 2016). Like aridification, the major cooling event that gave rise to expansion of the modern temperate–continental–boreal zones occurred at the Eocene–Oligocene boundary ~35 million years ago, around the time when the major Pooideae tribes were diversifying (Zachos *et al.*, 2005; Pimentel *et al.*, 2017; Schubert *et al.*, 2019a).

Climate change has a potentially important role to play in shaping species' range limits (Franklin *et al.*, 2016). During

their expansion into novel climate zones, it is hypothesized that Pooideae species distributions were strongly influenced by climatic tolerance, as has been found for several tropical plants (Wen *et al.*, 2018), and some members of the other, primarily southern, temperate grass subfamily Danthoniodieae (Humphreys and Linder, 2013). However, in many plant clades, native range strongly over- or underestimates climatic tolerance, suggesting that other factors, such as resource competition, biotic interactions and dispersal limitation, play equally or even more important roles in shaping distributions (Humphreys and Linder, 2013; Pellissier *et al.*, 2013; Boci *et al.*, 2016; Feeley *et al.*, 2020; Pagel *et al.*, 2020). In order to predict the response of Pooideae to future climate change, and to target breeding efforts for increased agricultural yields, it is vital to understand the relative contribution of drought and extreme thermal tolerance in explaining variation in native aridity and thermal distributions, respectively.

In addition to directly exploring the role of the abiotic environment in defining plant distributions, trait-based approaches can be useful in that they provide accessible targets for assessing adaptation and for manipulation of crops under climate-changing scenarios (Aubin *et al.*, 2016; Abdala-Roberts *et al.*, 2018; Kassout *et al.*, 2019). Adaptation to drought and low temperatures (collectively climatic tolerance) likely involves selection on a combination of traits, including those that foster escape (e.g. surviving the dry/cold season as seed), avoidance [e.g. minimizing metabolism to increase water use efficiency

(WUE)], adjustment (e.g. increasing cell wall plasticity) and resistance (e.g. increasing antioxidant metabolism) (Kemp and Culvenor, 1994; Gilbert and Medina, 2016; Abid *et al.*, 2018; Gupta *et al.*, 2020). However, despite this complexity, at any given time selection might be stronger on one trait than on another, particularly in clades where the ancestor was already adapted to some amount of drought or cold (see Schubert *et al.*, 2019b for Pooideae grasses), and/or when environmental factors place opposing pressures on particular traits or trait combinations (Šimová *et al.*, 2017).

Traits that increase WUE in particular have received quite a bit of attention among agronomists, and can manifest their effects either by plants maintaining high photosynthesis and net carbon gain with diminishing water input, or by reducing growth in order to minimize water use (Leakey *et al.*, 2019). In C₃ plants, WUE is affected by several parameters, including the efficiency of enzymes involved in carboxylation, the ability to reduce photorespiration, and the structure and function of stomata [pairs of guard cells in the plant epidermis that fill with water to open pores (Leakey *et al.*, 2019)]. Each stomatal pore acts as a conduit to facilitate gas exchange for photosynthesis, and also allows root nutrient uptake and thermoregulation through evapotranspiration (water loss) (Zeiger *et al.*, 1987; Lawson and Blatt, 2014; Rui *et al.*, 2019). These positive aspects of pore opening are counterbalanced by the need to maintain an internal water supply for normal cellular functioning, growth and development (Farquhar and Sharkey, 1982; Chater *et al.*, 2017; Harrison *et al.*, 2020).

Stomatal diffusive conductance is defined as the rate of passage of CO₂ into (g_c) or water vapour out of (g_w) aerial plant organs, and is determined by the combined effects of stomatal density (D), aperture and size (S) (Franks and Farquhar, 2007; Franks and Beerling, 2009). When water availability is low or root water uptake is hampered by low temperatures, isohydric-tolerant plants such as the monocot *Commelina communis* (Commelinaceae) close their stomata before leaf wilting, resulting in low stomatal conductance and a high WUE (Wilkinson *et al.*, 2001). By contrast, anisohydric species such as the eudicot white oak (*Quercus alba*, Fagaceae) keep their stomata open for longer, allowing leaf water potential to drop to the point of leaf wilting (Yi *et al.*, 2019). Differences in stomatal closure times between species appear to be regulated by a combination of hormone exposure and sensitivity, the major hormones being salicylic and abscisic acid, both of which can affect calcium sensitivity and induce the production of reactive oxygen species (ROS) (Wilkinson *et al.*, 2001; Miura and Tada, 2014). Evidence also suggests that rapid closure is enhanced by having small stomata, with a higher surface-to-volume ratio (Aasamaa *et al.*, 2001; Drake *et al.*, 2013; Durand *et al.*, 2019), a trend that is not entirely mitigated by the broad-scale negative correlation between S and D (Hetherington and Woodward, 2003; Lammertsma *et al.*, 2011).

Climate predictions for the Anthropocene include increasingly harsh and frequent droughts, shorter winters punctuated by severe freezing events, and rapid onset or ‘flash’ droughts (Zhang *et al.*, 2019), the latter being driven by abnormally high temperatures, winds and/or radiation, which in 2012 negatively impacted major agricultural areas across the central USA (Otkin *et al.*, 2016). Together, these heretofore aberrant

weather systems have fostered strong interest in engineering cereal crops for increased drought and low-temperature tolerance, in large part by increasing WUE through the engineering of decreased stomatal density and/or increased rates of stomatal closure (Buckley, 2005; Franks *et al.*, 2015; Martin-StPaul *et al.*, 2017; Leakey *et al.*, 2019). For example, overexpression of genes like EPIDERMAL PATTERNING FACTOR 1 (EPF1) in rice and barley simultaneously reduces stomatal density, increases WUE and increases survival when water is limiting (Hughes *et al.*, 2017; Caine *et al.*, 2019). Likewise, MITOGEN-ACTIVATED PROTEIN KINASE 12 minimizes stomatal sensitivity to abscisic acid and increases overall S , leading to higher stomatal conductance and improved WUE (Des Marais *et al.*, 2014; Campitelli *et al.*, 2016). Although stomatal traits are only one component of the plant phenotype that affects WUE, a strong negative correlation has been found between stomatal conductance and WUE in grasses such as wheat, rice, sorghum and switchgrass (Sinclair *et al.*, 2005).

Drought and cold tolerance are clearly defined by multiple interacting traits associated with individual organs and the plant as a whole. Whether stomata in particular are promising targets for improving drought and thermal tolerance of crop plants through their potential effects on WUE can be indirectly determined by natural experiments, i.e. by assessing the role of stomatal form and function in explaining natural variation in stress resistance and/or species climate distributions (Hughes *et al.*, 2017). Several such studies support significant correlations between climate variables and stomatal behaviour (Heroult *et al.*, 2013; Bourne *et al.*, 2015, 2017), stomatal area (Dittberner *et al.*, 2018; Liu *et al.*, 2018; Kassout *et al.*, 2019) and stomatal density (Kassout *et al.*, 2019) in non-grass taxa. However, few studies have tested these relationships in grasses, with a previous focus on PACMAD grasses showing little evidence for a direct relationship between precipitation and stomatal S or D (Taylor *et al.*, 2012). Here, we test predictions of the non-exclusive hypotheses that (1) tolerance to drought and low temperatures has been important for defining Pooideae species’ climate distributions, and (2) drought and low-temperature tolerances in Pooideae have been consistently, but not necessarily exclusively, shaped by stomatal form and function through their effects on WUE.

MATERIALS AND METHODS

Plant material and growth conditions

Twenty-three accessions from the US Department of Agriculture National Plant Germplasm System’s Germplasm Resources Information Network (USDA NPGS GRIN), representing seven Pooideae tribes, were chosen to maximize niche variation within each tribe (Supplementary Data Table S1). Beyond niche variation, specific accessions were chosen based on their availability in the GRIN seedbank, the existence of accession-based locality data, and germination rates >50 %. For each accession except *Lygeum spartum*, 50–60 seeds were surface-sterilized with 70 % ethanol followed by 20 % bleach:0.05 % Triton X-100. Each seed was then plated on 1 % agar and stratified for 5 d at 4 °C in the dark to promote germination (McKeown *et al.*, 2016). To warm-acclimate seeds,

the agar plates were moved to room temperature in the dark for 7 d before planting in standard potting soil. Germinating seeds in soil were initially incubated in a Conviron model A1000 Growth Chamber (Conviron, Winnipeg, MB, Canada) at 20 °C in long days (16 h light:8 h dark) with maximum light intensity (325 µmol of photons m⁻² s⁻¹), 50 % relative humidity, and ample water for 2–3 weeks depending on their leaf number. Seedlings were then thinned out to one individual per pot, and an equal number of pots per species was randomly assigned to each treatment.

Prior to specific treatment conditions, all plants were grown in four identical Conviron chambers under neutral daylengths (12 h light:12 h dark) with the lowest light intensity (125 µmol of photons m⁻² s⁻¹) for 1 week to allow conditions that would facilitate our cold chamber working efficiently at 4 °C. Individuals assigned to the two control plus two drought chambers were maintained in a common 20 °C chamber with neutral days, 50 % relative humidity, and the lowest light intensity (125 µmol of photons m⁻² s⁻¹), whereas the cold treatment was conducted under the same conditions at 4 °C in two separate chambers for 2 weeks. To control for water availability, soil moisture was assessed three times a week in the afternoon using a Fieldscout Soil Sensor Reader and Waterscout SMEC 300 Soil Moisture Temperature Sensor (Spectrum Technologies). Control and cold treatment soils were kept around 25–30 % volumetric water content, whereas drought treatment soils were not watered, and allowed to dry down to around 4–10 % relative water content. After 2 weeks of all treatments, which translated to 9 d in the drought zone for the drought treatment, individuals were transferred to a common 18–22 °C long-day (16 h light:8 h dark) climate-controlled greenhouse until flowering, death or termination of the experiment. Three weeks after each treatment, new leaf growth for each individual was scored as regrowth, and survival was subsequently determined based on the presence or absence of green material (Kasuga *et al.*, 1999). Results were converted to percentage regreening and survival for each accession.

For the rapid drought experiment, designed to mimic flash drought conditions, whereby sudden wind, solar radiation or high temperatures result in highly elevated evapotranspiration, seeds were treated as previously described, and each individually potted seedling was transferred to the greenhouse under long-day conditions. After 10 weeks, leaves from a subset of accessions were cut at the base and placed on a laboratory bench at 25 °C to induce instantaneous drought for 0, 15 or 90 min (Umezawa *et al.*, 2004; Cominelli *et al.*, 2005).

Stomatal trait measurements

To determine qualitative and quantitative features of stomata, clear tape was applied to the lower surface of attached, fully emerged young leaves for at least five individuals per accession after 2 weeks of each treatment. A thin coat of clear nail polish was then applied to the sections of leaf, which were stabilized by the tape (Hilu and Randall, 1984). Leaves were excised between 3 and 4 h after dawn and the sections separated and left to dry in a fume hood for 4–5 h. After drying, the dried cast of the epidermal surface was lifted with clear tape and fixed

to a microscope slide (Fetter *et al.*, 2019). Slides were examined using an Olympus BX60 Upright Compound Microscope at ×200 magnification for stomatal density measurements and ×1000 magnification for the remaining stomatal trait measurements. Images were captured using a SPOT Flex Camera, and total stomatal area (guard cell plus subsidiary cells), stomata subsidiary plus guard cell length and width, guard cell length (*L*), combined width of two guard cells (*W*) and pore area were assessed using the Fiji image processing package with a constant 50 µm scale (Schindelin *et al.*, 2012). For each individual plant, stomatal measurements were estimated using the mean of three or four leaf impressions, with at least ten fields being imaged for each impression. Stomata measurements for six plants per accession were taken for the control treatment and five for the drought/cold treatments. We determined *D* using the mean number of stomata visible in a 0.45-mm² field area and *S* was measured as *L* × *W* (Taylor *et al.*, 2012).

Calculation of *g_w*

Maximum stomatal conductance (*g_{wmax}*) to water vapour was calculated (Franks and Beerling, 2009) as:

$$g_{w\max} = \frac{d}{v} \cdot D \cdot a_{\max} / \left(l + \frac{\pi}{2} \sqrt{\frac{a_{\max}}{\pi}} \right) \quad (1)$$

where *d* is the diffusivity of water vapour in air (24.9 × 10⁻⁶ m² s⁻¹ at 25 °C), *v* is the molar volume of air (24.4 × 10⁻³ m³ mol⁻¹ at 25 °C, 101.3 kPa) (Franks and Farquhar, 2001) and *l* is stomatal pore length in µm; *a_{max}* is maximum area of the open stomatal pore in µm² as defined by $\pi(L/2)^2$, and *L* is stomatal pore length in µm (equal to guard cell width). Actual stomatal conductance (referred to as *g_{wCON}* without drought or cold, *g_{wCL}* after cold and *g_{wDR}* after drought) was then measured using eqn (2), a modification of eqn (1):

$$g_w = \frac{d}{v} \cdot D \cdot a_w / \left(l + \frac{\pi}{2} \sqrt{\frac{a_w}{\pi}} \right) \quad (2)$$

where actual pore area after drought or cold treatments (*a_w*, µm²) was measured manually based on ×1000 stomata images imported into Fiji using the freehand selection tool and measure functions (Schindelin *et al.*, 2012).

Estimation of δ13C

With the exception of the rapid drought experiment, ~10 mg of fresh leaf tissue was harvested from five individuals per accession at the end of the drought and cold treatments, dried in a 105 °C oven for 24 h (Zhao *et al.*, 2016) and cut into small pieces with scissors. Since growth was minimal in the cold treatment and somewhat delayed in the drought treatment, the time point for leaf collection in control and drought plants varied according to the time at which they were developmentally matched (i.e. had the same number of leaves) to the cold-treated plants. Tissues were placed in collection microtubes (Qiagen, USA) with 3-mm tungsten carbide beads (Qiagen, Germany), put into 96-well plate racks, and lysed by shaking in a Tissue Lyser II (Qiagen, Hilden, Germany) at a frequency

of 26 shakes per second for 15 min. As a proxy for WUE, the ratio of leaf $^{13}\text{C}/^{12}\text{C}$ relative to a Pee Dee Belemnite (PDB) standard ($\delta^{13}\text{C}$) was determined at the University of Maryland Central Appalachians Stable Isotope Facility. Solid samples were analysed using a Carlo Erba NC2500 elemental analyser interfaced with a Thermo Delta V+ isotope ratio mass spectrometer (IRMS). Samples were compositionally matched with a quality-controlled in-house standard, which was interspersed among the unknown samples. The final isotopic values were expressed relative to international standards by V-PDB (Vienna Pee Dee Belemnite) and air for carbon.

Phylogenetic reconstruction

A Pooideae phylogeny of 22 species, using *Ehrharta calycina* (Oryzoideae) as an outgroup, was estimated using the chloroplast markers *matK* and *ndhF* (Supplementary Data Table S2). Gene sequences were amplified under standard PCR conditions, cleaned with ExoSAP-IT (ThermoFisher Scientific) and submitted to the Vermont Integrative Genomics Resource at the University of Vermont for Sanger sequencing. High-quality reads were aligned in MAFFT (Katoh and Standley, 2013), followed by manual alignment in Mesquite (Maddison, 2008), and subjected to Bayesian analyses in MrBayes (version 3.2; Huelsenbeck and Ronquist, 2001; Ronquist *et al.*, 2012) on the Cipres Science Gateway (Miller *et al.*, 2010) with default parameters and 10 000 000 generations. Ancestral state reconstruction was performed with continuous traits in a maximum likelihood framework using the ‘contmap’ function of the *phytools* package (Revell, 2013). In order to test whether significant phylogenetic signal was present in the continuous traits, Pagel’s λ was estimated using the *phytools* package in R (Pagel, 1999; Revell, 2012). All sequences were submitted to the GenBank database under the accession numbers MN983254–MT040770.

Climate characterization

Georeferenced coordinates for all Pooideae accessions were extracted either from GRIN (<https://www.ars-grin.gov/>) or from the Global Biodiversity Information Facility (www.gbif.org; GBIF Secretariat) (Supplementary Data Table S1). To clean the data, we used the ‘predictor’ function in R version 3.1.2, and then manually removed any remaining duplicate records, as well as those suspected of being inverted in their latitude–longitude coordinates or falling in the sea. Clean coordinates were then used to extract minimum temperature of the coldest month (MTCM; bio6) from the Worldclim database (<http://www.worldclim.org>) with the *raster* package in R version 3.1.2, and aridity index (AI) data from the Global Aridity Index and Global Potential Evapo-Transpiration Geospatial Database (<http://www.cgiar-csi.org>) (Zomer *et al.*, 2008; Trabucco and Zomer, 2019). Since most of our focal taxa are distributed in temperate, and therefore thermally seasonal, environments, MTCM was selected to describe the low-temperature extremes that each accession would incur in nature. The AI, on the other hand, is an integrated measure of water availability based on long-term

average water supply and potential evapotranspiration. Low AI refers to relatively dry climatic conditions, whereas high AI refers to relatively wet climatic conditions.

Comparative methods and statistical analyses

To test predictions for correlations among climate variables, stomatal traits and/or survival/regreening, linear regression models were run with correction for phylogeny. For one-to-one dependent/independent comparisons, we used phylogenetic independent contrasts to calculate adjusted R^2 values in R version 3.6.3 using the *phytools* package (Revell, 2012). To correct for phylogenetic signal, phylogenetic generalized least squares (GLS) models were run using the ‘*gls*’ function in the R version 3.6.3 packages *ape*, *geiger*, *nlme* and *lme4* (Paradis *et al.*, 2004), starting with the inclusion of all explanatory variables and their interaction terms, followed by model simplification. In cases where the response variable could be different for each biological replicate within an accession, all datapoints were included, and the number of individuals per species was added as a random variable. Model fit was then assessed by inspecting the Akaike information criterion (AIC) values (Sakamoto *et al.*, 1986). A full or pruned version of our chloroplast phylogeny was used to estimate Pagel’s λ for the covariances between traits (Pagel, 1999) using the *phytools* package (Revell, 2012). Plots for visualization of results were created using the *ggplot2* package (Wickham, 2016).

RESULTS

Pooideae drought and cold tolerance correlates with species climate distributions

Species representing the seven Pooideae tribes and one outgroup (*Ehrharta calycina*) included in our study overlapped substantially in their aridity and low-temperature climate space based on AI and MTCM (Fig. 1A). Consistent with this overlap, there was little phylogenetic signal in either variable that could potentially confound downstream analyses (Pagel’s $\lambda = 7.555\text{e}^{-05}$ and 0.306; $P = 1.000$ and 0.198, respectively). Based on our predictions that grasses from increasingly arid (i.e. low AI) or seasonally cold environments will have increasing levels of drought or cold tolerance, respectively, we fitted models testing the relationship between fitness traits and climate and/or stomatal variables (Table 1 and Supplementary Data Table S3). For both drought regrowth (Table 1) and drought survival (Supplementary Data Table S3), GLS model (iii) with log AI as the sole explanatory variable was better fitting than models (i) and (ii), which included both g_{wDR} and log AI, based on AIC values. For these best-fitting models, Pooideae regrowth 3 weeks after drought ($P < 0.001$; Fig. 1B, Table 1) and drought survival ($P = 0.016$, Supplementary Data Fig. S1A and Table S3) were strongly negatively correlated with log AI, respectively. In the case of cold regrowth (Table 1) and cold survival (Supplementary Data Table S3), GLS model (vii) with MTCM as the sole explanatory variable was also better fitting than models (v) and (vi), which included both g_{wCL} and MTCM, based on AIC values. Again, these best-fitting models supported

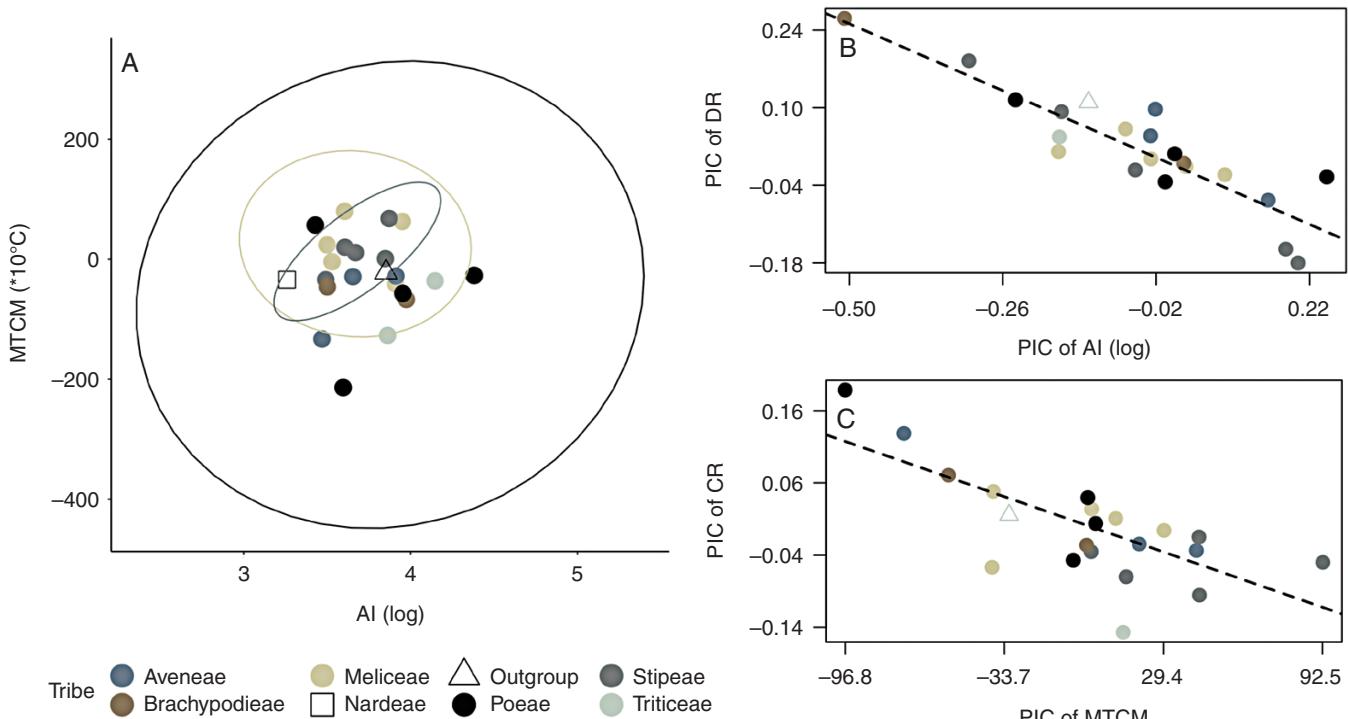


FIG. 1. Pooideae niche space and its correlation with regrowth after drought or cold. (A) Overlapping niche space for focal Pooideae accessions and *Ehrharta calycina* (Oryzoideae) as the outgroup based on a biplot of AI and MTCM. Each tribe is represented by a different colour or symbol. Ellipses represent a bivariate equivalent of the 95 % confidence interval for each tribe where sampling allows. (B) The percentage of individuals per accession that developed new leaves 3 weeks after drought (DR) is negatively correlated with log AI ($r^2 = 0.791, P < 0.001$). (C) The percentage of individuals per accession that developed new leaves 3 weeks after above-freezing cold (CR) is negatively correlated with MTMC ($r^2 = 0.483, P < 0.001$). (B) and (C) are based on fitted GLS models with phylogenetic independent contrasts (PIC) (dashed black lines, Table 1).

a significant negative relationship between MTCM and cold regrowth ($P = 0.004$, Fig. 1C, Table 1) and survival ($P < 0.001$, Supplementary Data Fig. S1B and Table S3).

Pooideae S and D are strongly negatively correlated

Stomatal conductance is regulated primarily by *S* and *D* (Huang and Xu, 2015). However, many studies have shown a trade-off between making larger stomata and increasing their relative numbers. Consistent with these findings, we determined that *S* and *D* are strongly negatively correlated across Pooideae based on a linear regression analysis without ($r^2 = 0.522, P < 0.001$) (Fig. 2A) and with correction for phylogeny ($r^2 = 0.372, P = 0.001$) (Fig. 2B). *Lygeum spartum* (Nardeae) had the largest ($1104 \mu\text{m}^2$) and least dense ($8.5 \text{ stomata mm}^{-2}$) stomata, whereas *Brachypodium sylvaticum* (Brachypodieae) had the smallest ($117 \mu\text{m}^2$) and most dense ($81.7 \text{ stomata mm}^{-2}$) stomata (Fig. 2A). Maximum likelihood-based ancestral state reconstruction of both traits suggested that the last common ancestor of Pooideae had relatively intermediate *S* and *D*, with a shift to smaller, more dense stomata in the ancestors of Meliceae (represented by *Melica*) plus Stipeae (represented by *Nassella*), and Brachypodieae, and to larger, less dense stomata in the Triticeae (e.g. *Triticum aestivum*) (Fig. 2C). Despite this general pattern, there was no significant phylogenetic signal for *S* ($\lambda = 1.10; P = 0.45$) or *D* ($\lambda = 0.68; P = 0.37$) based on Pagel's λ .

g_w is strongly correlated with $\delta^{13}\text{C}$

As predicted, g_{wDR} and g_{wCL} were both significantly lower than g_{wCON} and g_{wmax} (Fig. 3A), suggesting that the focal grasses of this study partially closed their stomata under drought and cold, thus conserving water. To determine to what extent g_w values limit $\delta^{13}\text{C}$ (a time-integrated estimate of intrinsic WUE), we regressed g_{wCON} , g_{wDR} and g_{wCL} individually on $\delta^{13}\text{C}$ measured under control, drought and cold conditions, respectively (Fig. 3B–D). Whereas both g_{wCON} (Fig. 3B; $r^2 = 0.355; P = 0.006$) and g_{wDR} (Fig. 3C; $r^2 = 0.265; P = 0.028$) showed a significant negative correlation with $\delta^{13}\text{C}$, g_{wCL} (Fig. 3D) did not show any significant association with $\delta^{13}\text{C}$ after correction for phylogeny. However, when two species (*Agrostis stolonifera* and wheat) were removed from the cold analysis, we then found a significant negative correlation between g_{wCL} and $\delta^{13}\text{C}$ ($r^2 = 0.398; P = 0.020$) (Supplementary Data Fig. S2A). The fact that there was also a significant negative correlation between $\delta^{13}\text{C}$ and *S* indicates that Pooideae grasses with smaller stomata generally have higher intrinsic WUE, independent of closing behaviour ($r^2 = 0.336; P < 0.001$) (Supplementary Data Fig. S2B).

g_w after drought or cold does not explain survival or regreening

We predicted that low stomatal conductance, as measured by g_w after drought or cold, would offer improved drought and cold survival and regreening. However, this was not supported

TABLE I. GLS models assessing support for predicted relationships between drought regrowth (DR) or cold regrowth (CR) and aridity index (AI), minimum temperature of the coldest month (MTCM), stomatal diffusive conductance after drought (g_{wDR}) and/or stomatal diffusive conductance after cold (g_{wCL}), with correction for phylogeny

Drought regrowth	
(i) DR ~ log AI + g_{wDR} + (log AI × g_{wDR})	AIC = -32.529, $\lambda = -0.179$, d.f. = 22, log-likelihood = 22.264, intercept $t = 3.588$, intercept $P = \mathbf{0.002}$ log AI: $t = -2.449$, $P = \mathbf{0.024}$ g_{wDR} : $t = 0.462$, $P = 0.649$ log AI × g_{wDR} : $t = -0.486$, $P = 0.632$
(ii) DR ~ log AI + g_{wDR}	AIC = -33.034, $\lambda = -0.099$, d.f. = 22, log-likelihood = 21.517, intercept $t = 11.186$, intercept $P = \mathbf{0.000}$ log AI: $t = 11.186$, $P = \mathbf{0.000}$ g_{wDR} : $t = -0.763$, $P = 0.454$
(iii) DR ~ log AI	AIC = -38.249, $\lambda = -0.161$, d.f. = 22, log-likelihood = 23.124, intercept $t = -11.911$, intercept $P = \mathbf{0.000}$ log AI: $t = -8.464$, $P = \mathbf{0.000}$
(iv) DR ~ g_{wDR}	AIC = -10.920, $\lambda = -0.527$, d.f. = 22, log-likelihood = 9.460, intercept $t = -8.480$, intercept $P = \mathbf{0.000}$ g_{wDR} : $t = 0.254$, $P = 0.801$
Cold regrowth	
(v) CR ~ MTCM + g_{wCL} + (MTCM × g_{wCL})	AIC = -8.179, $\lambda = 0.304$, d.f. = 22, log-likelihood = 10.089, intercept $t = 12.484$, intercept $P = \mathbf{0.000}$ MTCM: $t = 0.722$, $P = 0.479$ g_{wCL} : $t = -1.101$, $P = 0.285$ MTCM × g_{wCL} : $t = -1.636$, $P = 0.119$
(vi) CR ~ MTCM + g_{wCL}	AIC = -18.238, $\lambda = 0.332$, d.f. = 22, log-likelihood = 14.119, intercept $t = 12.973$, intercept $P = \mathbf{0.000}$ MTCM: $t = -3.057$, $P = \mathbf{0.006}$ g_{wCL} : $t = -0.293$, $P = 0.772$
(vii) CR ~ MTCM	AIC = -22.544, $\lambda = 0.293$, d.f. = 22, log-likelihood = 15.272, intercept $t = 25.349$, intercept $P = \mathbf{0.000}$ MTCM: $t = -3.243$, $P = \mathbf{0.004}$
(viii) CR ~ g_{wCL}	AIC = -27.323, $\lambda = 0.560$, d.f. = 22, log-likelihood = 17.661, intercept $t = 10.962$, intercept $P = \mathbf{0.000}$ g_{wCL} : $t = -0.568$, $P = 0.576$

Grey shading indicates models of best fit based on lowest AIC value; P -values in bold are significant at the <0.05 level.

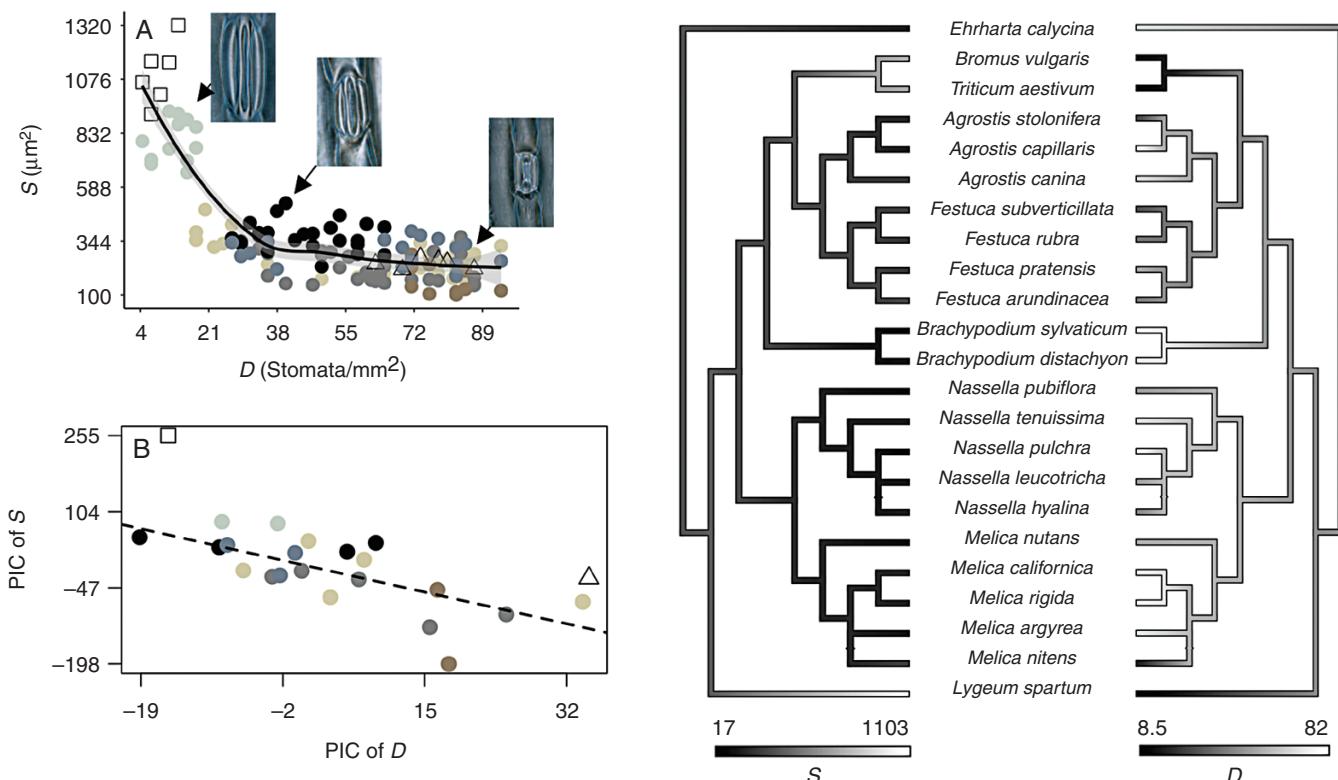


FIG. 2. Correlation between stomatal size (S) and density (D) across Pooideae. (A) S and D are negatively correlated based on all sampled individuals. The fitted curve is an estimation of S at a given D and vice versa. Equally scaled stomata images are shown for *Triticum aestivum* (left), *Festuca arundinacea* (middle) and *Brachypodium sylvaticum* (right) as examples. Each tribe is represented by a different colour or symbol corresponding to those in Fig. 1. (B) Average S and D per taxon remain significantly negatively correlated (dashed black line; $r^2 = 0.56$, $P < 0.001$) after implementation of phylogenetic independent contrasts (PIC) based on a fitted GLS model. (C) Ancestral state reconstruction of S and D across Pooideae based on a chloroplast (*matK-ndhF*) phylogeny. The grey scale bar reflects the trait value; black signifies low and light grey high.

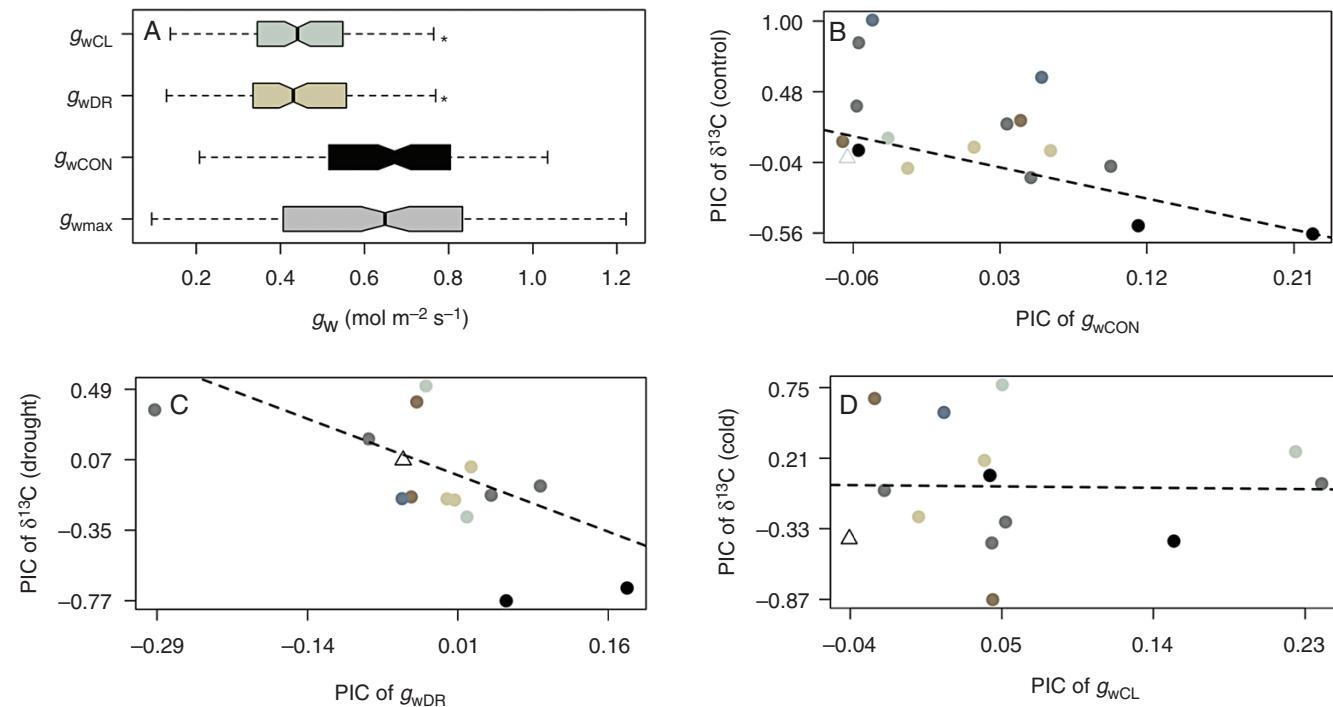


FIG. 3. Estimated stomatal conductance (g_w) under different treatments and its relationship to $\delta^{13}\text{C}$, a proxy for WUE. (A) Boxplots show estimated stomatal conductance across Pooideae tribes under control (g_{wCON} , black), drought (g_{wDR} , green-grey) and cold (g_{wCL} , cyan) conditions, compared with maximum stomatal conductance (g_{wmax}) (grey). * $P < 0.001$ relative to g_{wCON} based on Dunnett's test. (B–D) g_{wCON} (B) and g_{wDR} (C), but not g_{wCL} (D), significantly explain $\delta^{13}\text{C}$ according to fitted linear regression after implementing phylogenetic independent contrasts (PIC) (Supplementary Data Table S3). Dashed black lines show linear relationships among variables. Each tribe is represented by a different colour or symbol corresponding to those in Fig. 1.

by our data. For drought regrowth, GLS model (ii), which included both log AI and g_{wDR} as explanatory variables, had a lower AIC value than model (iv) with g_{wDR} alone (Table 1; Fig. 4A). Conversely, GLS model (iv), which included only g_{wDR} , was a slightly better fit than the more complex models (i) and (ii) to explain drought survival (Supplementary Data Table S3; Fig. 4C). In both cases, there was no significant relationship between g_{wDR} and the fitness variable ($P = 0.454$ for drought regrowth in Table 1; $P = 0.397$ for drought survival in Supplementary Data Table S3; Fig. 4A and C, respectively). In the case of cold regrowth (Table 1) and survival (Supplementary Data Table S3), GLS model (viii) with g_{wCL} as the sole explanatory variable fitted better than when both MTCM and g_{wCL} were included, based on the lowest AIC values. Similar to drought, neither cold regrowth ($P = 0.576$ in Table 1; Fig. 4B) nor cold survival ($P = 0.922$ in Supplementary Data Table S3; Fig. 4D) had any significant relationship with g_w .

Stomatal closure with rapid drought is proportionally similar across Pooideae and does not reflect species climate distributions

In addition to partially closing their stomata in response to long-term seasonal drought, plants can regulate stomatal aperture on a diurnal basis, potentially allowing them to escape excessive water loss during the time of greatest evapotranspiration, and/or during periods of flash cold/drought. The speed of this response can impact the ability of plants to avoid wilting, and in some species closure is faster with

small stomata. To test whether the speed of stomatal closure under 15- or 90-min rapid drought is correlated with log AI and/or S, we ran GLS models for these variables. Based on the lowest AIC values, the simpler models (iii) and (iv) fitted best to explain stomatal closure with 15-min drought, and (vii) and (viii) fitted best to explain stomatal closure with 90-min drought (Supplementary Data Table S4). The amount of stomatal closure from well-watered control to 15 min ($P = 0.130$; Fig. 5A; Supplementary Data Table S4) or 90 min ($P = 0.664$; Fig. 5B; Supplementary Data Table S4) drought conditions had no significant relationship with size. Similarly, no relationship was found between log AI and stomatal closure at 15 or 90 min (15 min, $P = 0.213$; 90 min, $P = 0.588$; Supplementary Data Table S4). When stomatal closure was translated to g_w in drought (g_{wD}), models (x) and (xiv), which included both log AI and g_{wCON} , were better fitting to explain g_{wD} at 15 and 90 min, respectively, than models with g_{wCON} alone (Supplementary Data Table S4). Nonetheless, there was no significant relationship between log AI and g_{wD} at either time point (15 min, $P = 0.248$; 90 min, $P = 0.453$). Finally, AIC values indicated that models (xii) and (xvi), with g_{wCON} only, explained g_{wD} better at 15 and 90 min, respectively, compared with models also including log AI (Supplementary Data Table S4). For these models, g_{wCON} did predict g_{wD} at 15 min ($P = 0.004$; Fig. 5C; Supplementary Data Table S4), and marginally at 90 min ($P = 0.055$; Fig. 5D; Supplementary Data Table S4). Since the intercept was not significantly different from zero (Supplementary Data Table S4), this correlation between

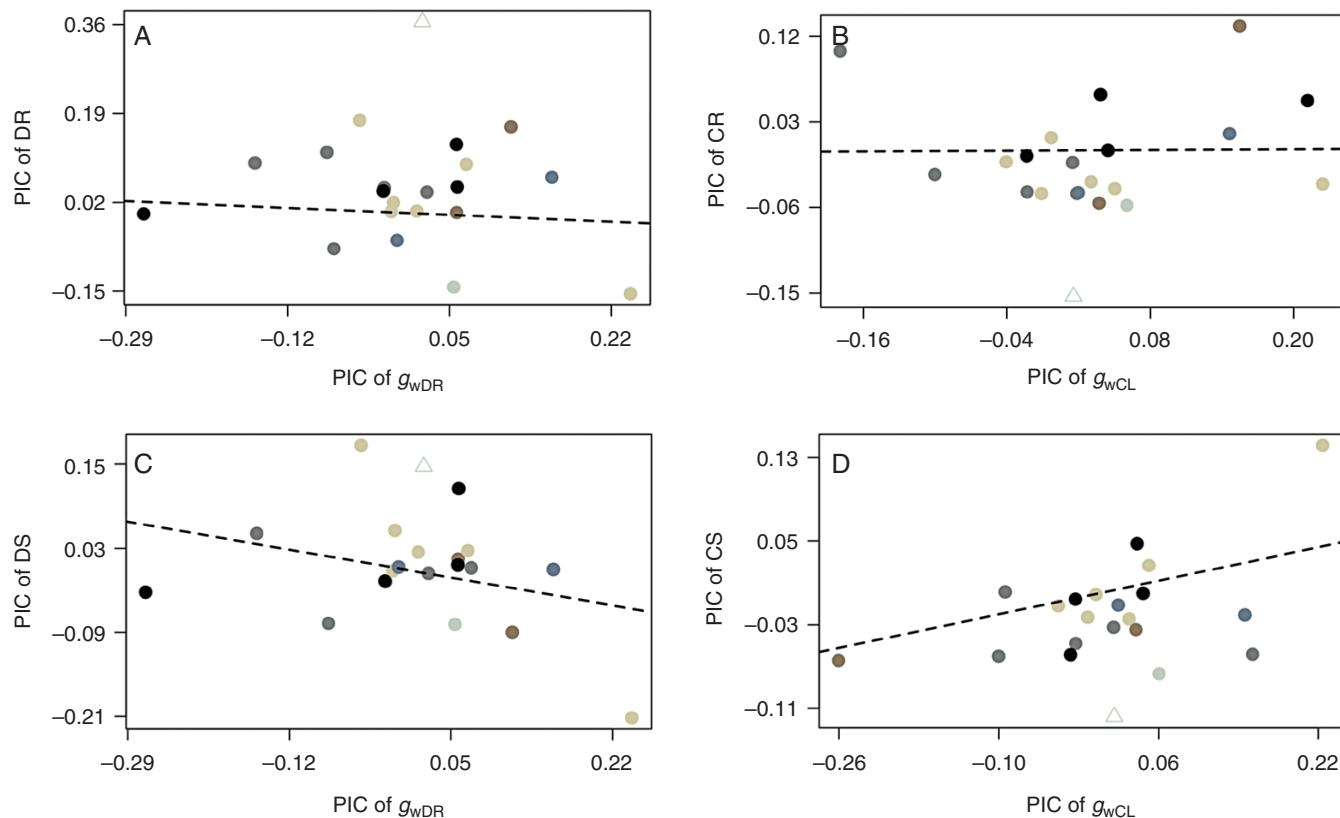


FIG. 4. Relationship between estimated stomatal conductance (g_w) with regrowth and survival after drought or cold. Fitted regression analysis after implementation of phylogenetic independent contrasts (PIC) shows that (A) g_w after drought (g_{wDR}) does not explain drought regrowth (DR), (B) g_w after cold (g_{wCL}) does not explain cold regrowth (CR), (C) g_{wDR} does not explain drought survival (DS), and (D) g_{wCL} does not explain cold survival (CS), based on taxon averages (dashed black lines) (Table 1). Each tribe is represented by a different colour or symbol corresponding to those in Fig. 1.

g_{wCON} and g_{wD} at 15 min suggests a general proportional response in stomatal closure that maintains the relative difference in water conductance across species.

DISCUSSION

Pooideae grasses are known to inhabit some of the world's most stressful environments, from arctic tundras (e.g. *Deschampsia antarctica*) that are frozen for several months of the year to the semi-arid steppe (e.g. *Stipa krylovii*), receiving precipitation below potential evapotranspiration (Chew *et al.*, 2012; Beck *et al.*, 2018; Wang *et al.*, 2019). In order to survive and reproduce in these harsh and varied environments, species employ a combination of morphological and physiological mechanisms as a means of escaping, avoiding, tolerating or resisting various strains caused by stress (Blum, 2016). Although the ancestor of Pooideae was probably already adapted to certain levels of abiotic extremes (Zhong *et al.*, 2018; Schubert *et al.*, 2019b), it has been unclear whether changes to the limits of drought or cold tolerance have been important in shaping this subfamily's distribution. Sampling across several tribes with overlapping temperature and precipitation niches, we found that the climate distributions of Pooideae can partially be explained by tolerance to drought and cold. To elucidate the most important mechanisms underlying these differences in tolerance, we started with

a subset of stomatal traits that can influence short-term WUE, g_{wmax} having previously been indirectly linked to dry habitats through the evolution of C_4 photosynthesis in largely tropical PACMAD grasses (Taylor *et al.*, 2012). Although Pooideae g_{wmax} under different conditions was a good predictor of $\delta^{13}\text{C}$, and hence likely WUE, it could not explain drought/cold survival or regreening. Taking this finding together with the fact that the speed of stomatal closure was also not related to flash drought tolerance, we conclude that variation in stomatal traits has not been a consistent driver of climatic distributions in Pooideae grasses. This highlights potential limits to modifying stomatal traits for increased plant productivity, and paves the way for future work assessing the role of other (e.g. water transport) traits involved in grass drought/cold adaptation.

Role of drought and cold tolerance in shaping organismal distributions

Despite the potential for both biotic and abiotic factors in shaping organismal distributions, a long-standing hypothesis is that abiotic factors are more important in temperate than in tropical environments, due to their relative instability (Dobzhansky, 1950; MacArthur, 1984). A recent meta-analysis focusing on local adaptation across plants, animals and bacteria lends support to this hypothesis (Briscoe Runquist *et al.*,

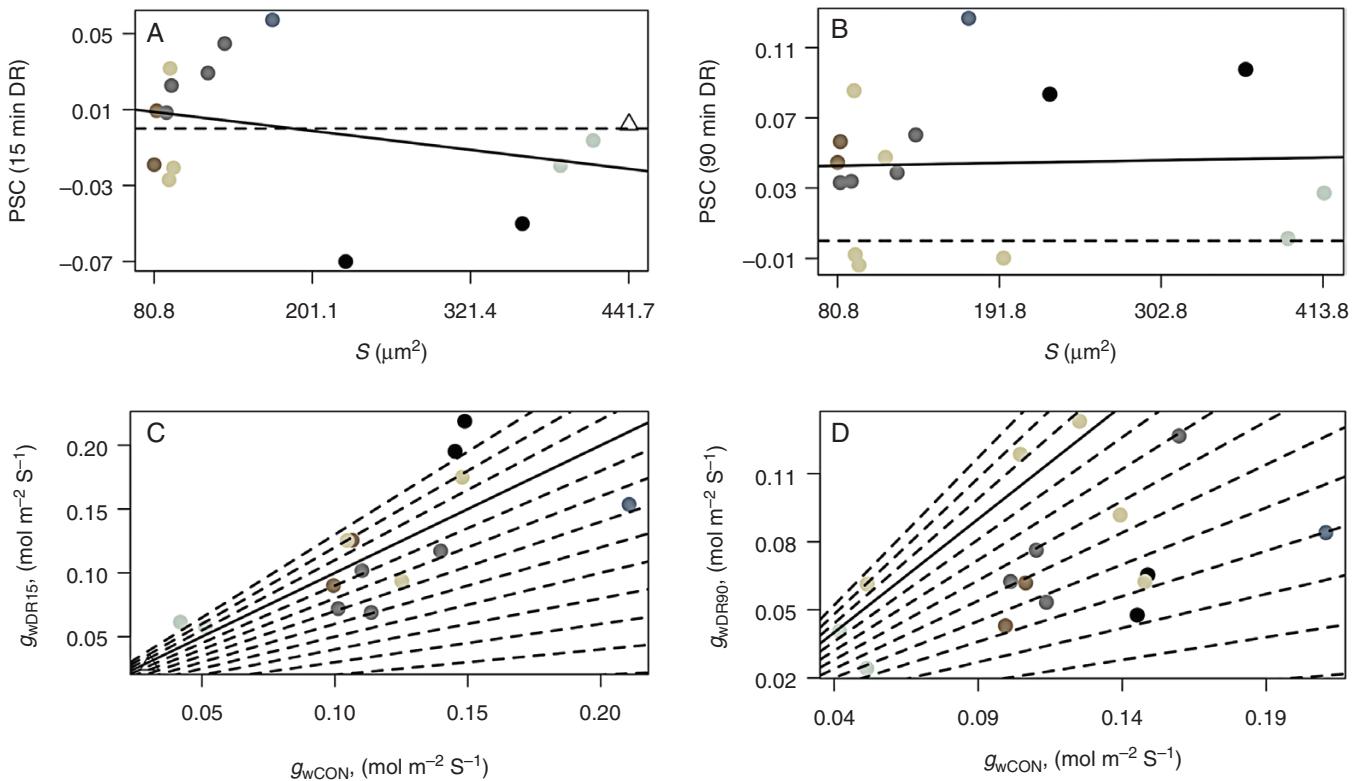


FIG. 5. Stomatal closure in Pooideae after short-term drought is not affected by stomata size (S), but is affected by estimated stomatal conductance under control conditions (g_{wCON}). (A) Amount of stomatal closure (PSC) from time 0 (no drought) to 15 min of severe drought has no significant relationship with S ($P = 0.130$) (Supplementary Data Table S4), as illustrated by the solid regression line. Approximately half the species have started to close their stomata (above the dashed line) by this time point. (B) Amount of stomatal closure from time 0 (no drought) to 90 min of severe drought is not correlated with S ($P = 0.664$) (Supplementary Data Table S4), as illustrated by the solid regression line. (C) g_w after 15 min of drought is positively correlated ($P = 0.002$; intercept ~ 0) (Supplementary Data Table S3) with g_w under control conditions (g_{wCON}). (D) g_w after 90 min of drought is marginally positively correlated ($P = 0.047$; intercept ~ 0) (Supplementary Data Table S3) with g_{wCON} . Points below the solid 1:1 line in (C) and (D) have reduced their g_w following drought; points on dashed isocline lines show a proportional response in stomatal closure from time 0 to the end of drought.

2020). However, the phylogenetic scale at which variation in adaptations to abiotic extremes are important for shaping distributions remains unclear (Donoghue, 2008). Indeed, many studies have also demonstrated the importance of biotic factors in influencing temperate plant distributions (Edwards and Smith, 2010; Pellissier *et al.*, 2010), particularly as facilitators in potentially stressful environments (Klanderud *et al.*, 2015), or have revealed mismatches between tolerance and climate (Choat *et al.*, 2012; Craine *et al.*, 2013; Humphreys and Linder, 2013). For the largely northern temperate grass subfamily Pooideae, we have shown that climate distributions can be predicted by whole-plant responses to both drought and above-freezing cold. This finding is consistent with previous evidence that cold adaptation in this group evolved in a stepwise fashion (Zhong *et al.*, 2018) and is often the basis for local adaptation (Byars *et al.*, 2007; Vigeland *et al.*, 2013; Kreyling *et al.*, 2019; Roybal and Butterfield, 2019; Zhao *et al.*, 2019), and that drought tolerance correlates with aridity within populations of the Pooideae grass *Brachypodium distachyon* (Des Marais *et al.*, 2016). Consequently, it is likely that aridity and temperature will be key predictors of range expansion and contraction of Pooideae under predicted climate change (Jalili *et al.*, 2010; Williams *et al.*, 2015; Zarch *et al.*, 2017), and will determine

the suitability of different crop genotypes to specific geographical regions (Monneveux *et al.*, 2012; Lesk *et al.*, 2016).

Pooideae traits underlying variation in drought and cold tolerance

The strong relationship found between drought/cold tolerance and Pooideae climate distribution raises the question as to which traits have been most important for conferring variation in responses to drought/cold and whether they are consistent across the subfamily. Water use efficiency is one dimension that potentially affects drought, and possibly cold, tolerance, and is known to be shaped by a number of interacting factors, including plant hydraulics, lateral root architecture, metabolic rate and stomatal conductivity (Tron *et al.*, 2015; Leakey *et al.*, 2019). Moreover, $\delta^{13}\text{C}$ as a proxy for WUE has proven effective in breeding programmes of Australian Drysdale and Rees bread wheat varieties (Condon *et al.*, 2004), and recent studies on *Setaria* and maize have revealed that negative associations between $\delta^{13}\text{C}$ and the ratio of plant biomass to water use is partially driven by intrinsic WUE (Feldman *et al.*, 2018; Twohey *et al.*, 2019).

The specific role of stomatal trait plasticity as a response to a short-term reduction in soil water or water vapour has been in several previous grass studies, within both the BOP clade (e.g. Xu and Zhou, 2008; Zhang *et al.*, 2019) and the PACMAD clade (e.g. Williams and Black, 1994; Maricle *et al.*, 2007; Ripley *et al.*, 2010; Ocheltree *et al.*, 2014). Stomatal closure in response to cold has likewise been found in cold-sensitive cultivars of rice (Aghaee *et al.*, 2011), as well as a number of other species other than grasses (e.g. Wilkinson *et al.*, 2001), possibly due to impaired root water uptake and/or to reduce photodamage when biochemical pathways are compromised (Agurla *et al.*, 2018). Consistent with these results, we found that our focal Pooideae species closed their stomata under both drought and cold conditions, and that there was a functional relationship between g_w and $\delta^{13}\text{C}$ under all treatments. However, under severe drought conditions, designed to mimic flash drought conditions, which are becoming more prevalent under climate change (Dai *et al.*, 2013; Wang *et al.*, 2016), stomatal closure was proportional to starting conditions. This suggests that closing behaviour, at least under our laboratory conditions (Tardieu, 2012; Clauw *et al.*, 2015), is somewhat uniform across the subfamily irrespective of drought tolerance and, by extension, climate distribution. Similar results were also obtained under gradual drought and cold conditions, whereby there was no significant relationship between g_w and whole-plant fitness traits.

The lack of evidence for stomatal trait diversification driving Pooideae distributions suggests that stomatal trait variation has been shaped by other factors, such as escape from pathogens (Melotto *et al.*, 2008), and/or that abiotic extremes can affect stomatal trait evolution in different ways. On the latter point, reduced $g_{w\max}$ (or related parameters) and increased WUE have been shown to mitigate wilting and loss of turgor pressure across variable aridity zones, as in Siberian elms (*Ulmus pumila*) (Park *et al.*, 2016), *Eucalyptus* (Bourne *et al.*, 2015) and temperate grasses of north-eastern China (Guo *et al.*, 2017). On the other hand, increased $g_{w\max}$ and reduced WUE might facilitate leaf cooling where arid sites are also hot, as in *Protea repens* (Carlson *et al.*, 2016), or allow xerophytes to take maximum advantage of water availability during the wet growing season, as in several forest communities of eastern China (Liu *et al.*, 2018). Divergent strategies for dealing with temperature extremes are also apparent. For example, a study on forest species in eastern China demonstrated a positive relationship between $D \times S$ and mean annual temperatures below 10 °C, but a negative relationship between these parameters above 10 °C (Liu *et al.*, 2018). Because many temperate species are exposed to high and low temperatures in the summer and winter, respectively, The minimum temperature of the coldest month might not adequately describe the interaction of seasonal selective temperature regimes. A recent study on *Stevia rebaudiana* (Asteraceae) also showed that low temperature significantly increases, rather than decreases, D and $D \times S$ in newly emerging leaves, leading to reduced WUE (Hajihashemi *et al.*, 2018). Given our results, future studies should be done to test for differential selection on stomatal traits across geography and phylogeny; determine the relationship between further life history traits, stomata and climate distribution; and assess the importance of other traits underlying resilience to

drought and cold. On the latter point, several candidate traits are worth considering in this context: leaf morphology, shoot-to-root biomass ratio, investment in osmoprotectants, sugar accumulation, and hydraulics, to name but a few (Valliyodan and Nguyen, 2006; Kooyers, 2015; Leakey *et al.*, 2019).

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following. Figure S1: Pooideae survival after drought or cold is correlated with niche variables AI and MTCM. Figure S2: a subset of stomatal diffusive conductance after cold and stomatal size explains $\delta^{13}\text{C}$. Table S1: Pooideae accessions, collection localities and climate distributions. Table S2: summary of the primers used to amplify chloroplast markers. Table S3: GLS models assessing support for predicted relationships between drought survival or cold survival and AI, MTCM, stomatal diffusive conductance after drought and/or stomatal diffusive conductance after cold, with correction for phylogeny. Table S4: GLS models assessing support for predicted relationships between aridity index or stomatal diffusive conductance in control conditions and stomatal diffusive conductance after drought, and AI or stomatal area and proportion stomatal closure with drought, after correction for phylogeny.

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