Increased above-ground resource allocation is a likely precursor for independent evolutionary origins of annuality in the Pooideae grass subfamily

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Summary

- Semelparous annual plants flower a single time during their 1-yr life cycle, investing much of their energy into rapid reproduction. By contrast, iteroparous perennial plants flower multiple times over several years, and partition their resources between reproduction and persistence. To which extent evolutionary transitions between life-cycle strategies are internally constrained at the developmental, genetic and phylogenetic level is unknown. Here we study the evolution of life-cycle strategies in the grass subfamily Pooideae and test if transitions between them are facilitated by evolutionary precursors.
- We integrate ecological, life-cycle strategy and growth data in a phylogenetic framework. We investigate if growth traits are candidates for a precursor.
- Species in certain Pooideae clades are predisposed to evolve annuality from perenniality, potentially due to the shared inheritance of specific evolutionary precursors. Seasonal dry climates, which have been linked to annuality, were only able to select for transitions to annuality when the precursor was present. Allocation of more resources to above-ground rather than below-ground growth is a candidate for the precursor.
- Our findings support the hypothesis that only certain lineages can respond quickly to changing external conditions by switching their life-cycle strategy, likely due to the presence of evolutionary precursors.

Introduction

As sessile organisms, plants are unable to quickly move away from stressful environments that can adversely affect fitness. Many plants avoid seasonal stressors like drought and frost by adopting a semelparous annual (hereafter simply annual) life-cycle strategy. Annual plants flower only once during their 1-yr life cycle and persist through suboptimal environmental conditions in the form of seeds. An alternative strategy is to survive stressful conditions by adopting coping mechanisms to live through many seasons, referred to as the perennial life-cycle strategy. Iteroparous perennial (hereafter simply perennial) plants flower more than once and avoid limiting conditions through the induction of stress-resistant organs such as rhizomes and stolons to escape limiting conditions via a period of quiescence or to tolerate them (Palmer *et al.*, 2017). Although both life history strategies can be effective at maximising fitness in harsh environments, annuals tend to be more abundant than perennials in environments characterised by seasonally high temperatures, drought, frost and

grazing (Whyte, 1977; Pettit *et al.*, 1995; Tofts, 2004; Evans *et al.*, 2005; Kadereit *et al.*, 2006; Cruz-Mazo *et al.*, 2009). The latter relationship suggests that annuals may be better adapted than perennials to maximise fitness in extreme, but variable, environments.

Annual and perennial plants are characterised by differences in traits connected to resource use and reproductive timing. In general, annuals maximise their reproductive effort through high and often rapid resource acquisition during the growing season, while perennials maximise persistence, defence, and stress tolerance through conservation of resources in vegetative structures (Grime & Hunt, 1975; Bazzaz *et al.*, 1987; De Souza & Da Silva, 1987; Garnier, 1992; Atkinson *et al.*, 2016). Most studies have suggested that annuals have higher production parameters than perennials, exemplified by a high relative growth rate (RGR), large leaf area, large allocation of resources to reproductive structures and high biomass production and specific root length (Grime & Hunt, 1975; Bazzaz *et al.*, 1987; De Souza & Da Silva, 1987; Garnier, 1992; Roumet *et al.*, 2006; Atkinson *et al.*,

2016). Perennials, by contrast, are characterised by traits allowing for persistence and defence, such as high above-ground and below-ground tissue density and allocation of a higher proportion of biomass into roots (Grime & Hunt, 1975; Bazzaz et al., 1987; De Souza & Da Silva, 1987; Garnier, 1992; Roumet et al., 2006; Atkinson et al., 2016). Strikingly, even with these fundamental differences between growth strategies, the evolutionary distance between annual and perennial species may be very small (van Kleunen, 2007), and transitions between annuality and perenniality are common throughout flowering plants (Friedman & Rubin, 2015).

Repeated and rapid evolutionary transitions between complex trait states are likely to require simultaneous evolutionary changes in a suite of characters controlled by complex molecular modules. If truly convergent, this would require independent, recurrent recruitment of a coordinated set of genes or entire molecular modules. Evolution of complex traits is often nonrandomly concentrated in parts of phylogenetic trees (Marazzi et al., 2012), and it has been suggested that evolutionary 'precursors' that evolved in the common ancestor of some clades facilitated these recurrent and clustered trait origins (see e.g. Sanderson, 1991; Donoghue, 2005; Shubin et al., 2009; Aliscioni et al., 2012; Christin et al., 2013b). An evolutionary precursor is a feature (single trait or suite of traits) enabling recurring evolution of noticeable innovations, and it is detected statistically in a phylogeny (Marazzi et al., 2012). These precursors may act as developmental enablers or exaptations (Gould & Vrba, 1982; Donoghue, 2005). The solution to exactly which trait(s) or gene(s) that constitute(s) a precursor may be difficult to determine and requires large scale phylogenetic analyses of candidate traits or genes. There exist, however, a few studies of precursor traits and parallel recruitment of genes to fulfil specific functions. A well described example is the large bundle sheaths in the predominantly tropical PACMAD clade of grasses (Christin et al., 2013b), which enabled repeated, independent transitions from C_3 to C_4 photosynthesis. Furthermore, Christin et al. (2013a) has shown that the same enzymes were independently recruited to C_4 photosynthesis from C_3 ancestors in lineages of tropical grasses, and Aubry et al. (2014) used a comparative transcriptome approach to identify the parallel recruitment of trans-factors in independent origins of C_4 photosynthesis across plant families.

In this study we investigated the evolutionary transitions between life-cycle strategies in the grass subfamily Pooideae. Pooideae comprises about one-third (c. 4000) of the species richness within the grass family (Soreng et al., 2015), dominates the northern temperate and Arctic grass flora (Hartley, 1973), and includes some of the world's most important crop species, such as wheat (Triticum L. spp.), barley (Hordeum vulgare L.), rye (Secale cereale L.) and oats (Avena sativa L.). Growing demand for increased, yet sustainable, food production has prompted much interest in engineering perennial persistence traits into the largely annual crop cultivars (Crews et al., 2018). However, although much information is known about the control of flowering and yield within this group, lack of knowledge about the mechanisms conferring differences between life-cycle strategies has hampered progress in annual crop perennialisation.

Transitions between life-cycle strategies are rapid and frequent in Pooideae, and we hypothesise that one or more evolutionary precursors may have enabled these transitions. Furthermore, in a series of papers Garnier (1992); Garnier & Laurent (1994); Garnier et al. (1997) reported consistent differences in growth-related traits between pairs of annual and perennial core Pooideae species, indicating that independent transitions occur through similar physiological changes. This suggests that growth traits are candidates for a potential precursor. To test the hypothesis that transitions between life history strategies are enabled by one or more evolutionary precursors, we modelled evolutionary transitions between perennial and annual life-cycle strategies in a phylogeny of 388 Pooideae species, and found that transitions from perenniality to annuality were clustered nonrandomly, consistent with them being facilitated by one or more precursors. We investigated if the lack of annual species in parts of the Pooideae phylogeny is due to lack of availability of suitable ecological niches. We then modelled the evolution of RGR and allocation of resources to above-ground and below-ground structures to test if these traits are candidates for evolutionary precursors and hence enable transitions between perennial and annual life-cycle strategies. Our data support high above-ground vs below-ground resource allocation during seedling growth as a strong candidate for an annuality precursor. This finding suggests that resource allocation will be an excellent target for the perennialisation of annual cereal crops.

Materials and Methods

Reconstruction of life-cycle strategy evolution and identification of precursor nodes

Life-cycle strategy data were gathered and scored as annual or perennial for 388 Pooideae species based on the Kew GrassBase (Clayton et al., 2006), Tropicos (Soreng et al., 2000), FloraBase (Florabase, 1998) and USDA (USDA NRCS, 2019) databases (Supporting Information Table S1). Using a chi-squared test, we tested if there were significantly more annuals in the core Pooideae than in the early diverging lineages. Following the approach of Marazzi et al. (2012), the fit of four different trait evolution models was tested in r8s v.1.8 (Sanderson, 2003), two including an unknown precursor state for annuality (precursor one rate and precursor two rates) and two binary state models (binary one rate and binary two rates). Under the precursor models, past and extant taxa can assume one of three character states at each node: perennial, perennial with precursor for annuality, and annual. Model fit was determined based on AIC and log likelihood. Trees based on cpDNA regions (matK, ndhF, and rbcL) were obtained from the study of Schubert et al. (2019). A sample of 1000 trees from the posterior distribution of a BEAST Markov Chain Monte Carlo (MCMC) analysis was used to test model adequacy for all four models in r8s (Sanderson, 2003). Model fit parameters were summarised across the 1000 r8s runs for all models. From the same study of Schubert et al. (2019), the time-calibrated ultrametric maximum clade credibility (MCC) tree was used to reconstruct ancestral states of annual life-cycle strategy. The precursor trait analyses in r8s are sensitive to tree size

and trait distribution along its tips (Marazzi *et al.*, 2012). To measure the robustness of our results, we tested if r8s preferred the precursor models under scenarios with random trait distributions. This was achieved by running 3000 permutations of the r8s analyses on the MCC tree, randomly assigning life-cycle strategy traits to the tree tips in each permutation.

Estimation of the ecological niche

To test if perennial lineages without the inferred precursor trait encounter environments where evolving annuality would be adaptive, we downloaded and filtered geo-referenced records for all species in the 388-species phylogeny as described in Schubert *et al.* (2019). Geo-referenced records were downloaded from the Global Biodiversity Information Facility (GBIF, 2016) using the RGBIF package in R (Chamberlain *et al.*, 2020). To exclude unreliable records we removed all coordinates that had fewer than three decimal places and employed additional filtering implemented in the SPECIESGEOCODER package (Töpel *et al.*, 2016) in R. Of the original 388 taxa, 19 had no records on GBIF and, for an additional nine taxa, none of the GBIF records passed filtering. For each of the filtered geo-referenced records, the Bioclim variables mean temperature of coldest quarter (BIO11) and mean precipitation of driest quarter (BIO17) were downloaded from the WORLDCLIM v2.0 database (Fick & Hijmans, 2017) in a 2.5 arc-minutes resolution (*c.* 21 km^2 grid) using the RASTER package in R (Hijmans, 2020). BIO11 was chosen as a proxy for seasonal cold and BIO17 as a proxy for seasonal dry conditions. To reduce the impact of outliers, the lower and upper 2.5% of each Bioclim variable and taxon were excluded, except for taxa with fewer than 11 records. Means, standard deviations and standard errors were estimated for each variable and taxon and used to evaluate whether a species' mean value is representative for the climatic variation encountered in its geographical niche. We tested whether mean values were correlated with the upper and lower 5th percentile using linear regression with the percentiles as response and the mean of each taxon as the predictor (Fig. S1). Moreover, we ranked taxa according to their mean and visualised them together with one standard deviation and standard error of the mean to evaluate the mean–variance relationship qualitatively (Fig. S2).

Few annual species have a mean temperature of the coldest quarter (BIO11) below 0°C, indicating that annual species are generally not occurring in areas with long, cold winters (see the 'Results' section). Thus, annuality does not seem to be an escape strategy from seasonal cold, leading us to discard mean temperature of the coldest quarter from further analyses. To determine if annuals and perennials with and without inferred precursor differ in their received amount of precipitation in the driest quarter (BIO17), we performed an ANOVA with BIO17 as the response and life history as the categorical explanatory variable (annual, perennial without precursor, perennial with precursor). BIO17 was square root transformed to ensure homoscedasticity across the different groups. Homogeneity of variance was further evaluated with Levene's test as implemented in the function *leveneTest* in the R package CAR (Fox & Weisberg, 2011). Post hoc tests

were performed using the function Tukey honest significant difference (HSD) from the R package STATS (RCoreTeam, 2016).

Growth experiment

Growth trait analyses were conducted on 64 populations from 53 species (Table S2) chosen to represent most Pooideae tribes identified by Soreng *et al.* (2015). To avoid systematic errors associated with generally higher growth rates in annual vs perennial grasses, we confined our growth rate measurements to include solely perennial taxa. To synchronise germination, seeds were stratified in humid soil in darkness for 5 d at 4°C, followed by 24 h at 25°C. The pots were then transferred to a long-day glasshouse (20 h : 4 h, light : dark) at 20°C at Vollebekk, Ås, Norway (59°39′44.5″N, 10°45′3.7″E) for germination. Supplemental lighting was supplied by metal halide lamps with Master HPI-T Plus light bulbs from Philips (400W/645 E40 1SL). Following germination in March 2015, seedlings were washed and pricked out individually in 8-cm square pots containing sand. Plants were randomly rotated within and among tables weekly. Each plant was individually watered by hand every day and fertilised after 10 d with a mix of 800 g 10 l^{-1} Kristalon 9-11-30 nitrogen, phosphorus, and potassium plus 600 g 10 l^{-1} calcium nitrate 15.5 : 0 : 0 19% calcium (Yara, Norway). During the sampling period of 6 wk, we destructively harvested whole plants from each taxon at 12 time points. Roots were carefully washed by hand over a 0.5 mm pore mesh in running water, and the plants were dried in an oven (TS 8136; Termaks, Bergen, Norway) at 70°C for 48 h. Dry weight was measured for above-ground (shoots) and below-ground (roots) structures separately using a Mettler Toledo UMX2 scale (Greifensee, Switzerland) with an accuracy of 0.1 µg.

Growth traits modelling

The RGR was estimated as the slope of the linear regression of log-normal transformed total biomass against days postgermination for the exponential phase of seedling growth, in which a linear relationship of the log-normal transformed data can be expected (Paine *et al.*, 2012). We evaluated the model fit and visually inspected the fits and the raw data, and we found no indication that more complex models were needed (Rees *et al.*, 2010). RGR may however, be related to plant size (Turnbull *et al.*, 2012). To remove possible size effects, we also estimated the size standardised growth rate (SGR) as the slope of the exponential growth curve at the day the biomass reached the global median following eqn 5 from Turnbull *et al.* (2012). To investigate strategies for relative biomass allocation to above-ground and below-ground structures, we modelled the slope of the allometric relationship between above-ground and below-ground biomass for each taxon (Weiner, 2004). Biomass data were fitted to a standardised reduced major axis regression to model a scaling relationship for the log-normal transformed root and shoot biomass. A binary state for RGR and SGR was scored as above or below the mean for all taxa. For the allometric slope we scored the binary state as above or below one, which is the point above

which more resources are allocated to above-ground than below-ground growth. All traits were modelled in R v.3.3.2 (R Core Team, 2016) using the packages DOBY v.4.5.2 (Halekoh & Højsgaard, 2014) and RMISC v.1.5 (Hope, 2013).

Growth traits ancestral state reconstruction

To obtain DNA sequences for phylogenetic reconstruction of growth traits, DNA was extracted from plant material using the DNeasy Plant MiniKit (Qiagen, Valencia, CA, USA), following the manufacturer's protocol. Three DNA plastid markers (*matK*, *ndhF* and *rbcL*) were amplified using the polymerase chain reaction (PCR) on a Tetrad 2 Thermal Cycler (Bio-Rad, Hercules, CA, USA) and a Mastercycler Gradient Thermal Cycler (Eppendorf, Hamburg, Germany) using JumpStart™ REDTaq ReadyMix (Sigma-Aldrich) and following the manufacturer's protocol. Primer sequences are listed in Table S3. An annealing temperature of 58°C was found to be optimum in a gradient PCR, and products were purified using Montage SEQ plates (Millipore, Bedford, MA, USA) following the manufacturer's manual. Individual reactants were then sequenced using the TaqBigDye Terminator Cycle Sequencing kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA) and processed on a 310 ABI DNA sequencer (Perkin Elmer). Sequences were assembled and edited in SEQUENCHER v.4.10.1 (GeneCodes Corp., Ann Arbor, MI, USA) and aligned using CLUSTALX followed by manual refinement in BIOEDIT (Hall, 1999). The final alignment for the three chloroplast regions was 4305 bp long.

A phylogeny was inferred for the dataset comprising three chloroplast regions from the 53 Pooideae species and one outgroup (rice; *Oryza sativa* L., subfamily Oryzoideae) in BEAST v.1.7.4 (Drummond *et al.*, 2012). The input file to BEAST was created in BEAUTI, a part of the BEAST package, and the sequence data were partitioned according to marker regions *matK*, *ndhF* and *rbcL*. Among the partitions, the substitution models were unlinked, and the clock model was linked. The best fit nucleotide substitution model was selected to be GTR + Γ, as proposed by AICc obtained from modelTest in the R package PHANGORN v.1.99.14. (Schliep, 2010). The Γ distribution was approximated with four discrete rate categories. The speciation pattern was set to a Yule process. In BEAST, two MCMC chains were run for 20 million generations, with trees sampled every 10 000 generations. To verify that the analyses had converged and reached acceptable effective sample sizes (ESS > 200) the log files were checked in TRACER v.1.6.1 (Rambaut *et al.*, 2013). The two tree files were combined, discarding the first 10% as burn-in, using LOGCOMBINER v.1.7.4 in BEAST (Drummond *et al.*, 2012). The combined tree file was summarised into a MCC tree with mean branch lengths using TREEANNOTATOR v.1.7.4 in BEAST (Drummond *et al.*, 2012).

From previous BEAST analysis we randomly selected 1000 trees from the posterior distribution to test model adequacy for six models of continuous trait evolution for growth rates. For each tree we ran model tests using the R package GEIGER v.2.0.6.1 (Pennell *et al.*, 2014) and summarised model fit parameters across all runs. Model fit was evaluated by identifying the model with the lowest AICc score. For the best model we ran ancestral state reconstructions on the 1000 trees using the R package PHYTOOLS v.0.6.60 (Revell, 2012), summarised the ancestral state estimates for each branch and plotted them on the MCC tree. For species that had more than one accession in the growth experiment we used the average trait values of the accessions for ancestral state reconstructions. Missing trait values of the rice outgroup were imputed using the R package RPHYLOPARS v.0.2.9 (Goolsby *et al.*, 2017). Ancestral state reconstructions of discrete growth rate states were conducted performing BEAST analyses as described above. The discrete trait data were included as separate partition, evolving under an asymmetrical evolution model. The final phylogenies were visualised and edited in FIGTREE v.1.4 (Rambaut, 2012).

Differences in trait values for RGR, SGR and the allometric slope of biomass allocation to above vs below-ground structures between perennials with and without precursor were tested with two-tailed *t*-tests.

Data accessibility

Data from the growth experiment have been deposited in the DRYAD repository (doi: 10.5061/dryad.cvdncjt1j). Chloroplast sequences are published in the GenBank database under accession nos. MT433127–MT433176 (rbcL), MT433177–MT433225 (matK) and MT433226–MT433274 (ndhF).

Results

Evidence for evolutionary precursors of annuality

Transitions from perennial to annual life-cycle strategy within the 'core' Pooideae lineage (Brachypodieae–Triticeae–Aveneae–Poeae) have happened more frequently than in the early diverging lineages (Fig. 1). The scoring of annual and perennial life-cycle strategies in the 388 Pooideae species included in the phylogeny from Schubert *et al.* (2019) revealed that 3% of species in the noncore Pooideae were annuals, whereas within core Pooideae 36% of species are annuals. Results from a chi-squared test confirmed that the number of annuals was significantly higher in the core Pooideae ($\chi^2 = 50.1$, df = 1, $P \ll 0.0001$). Among the four tested Markov models of trait evolution along the MCC tree, we found the precursor two-rate model (AIC = 293.90; log likelihood = −177.95) to be superior to the three alternative models: precursor one-rate model (AIC = 298.36; log likelihood = −148.18), binary one-rate model (AIC = 313.53; log likelihood = −155.77) and binary two-rate model (AIC = 296.68; log likelihood = −146.34) (Table S4). The posterior distribution among the 1000 tested trees also showed that the precursor two-rate model was the best model (Table S5). According to the precursor two-rate model, there were at least 51 trait shifts between perenniality and annuality in the Pooideae (Table S6; Fig. 1), with a precursor being inferred in the most recent common ancestor (MRCA) of the core Pooideae, and more recently in the Stipeae clade containing the lineages *Nassella* (Trin) E. Desv./ *Jarava* Ruiz & Pav. and *Austrostipa* S.W.L. Jacobs & J. Everett (Figs 1, S3). The rate of precursor state gain/loss was modelled as
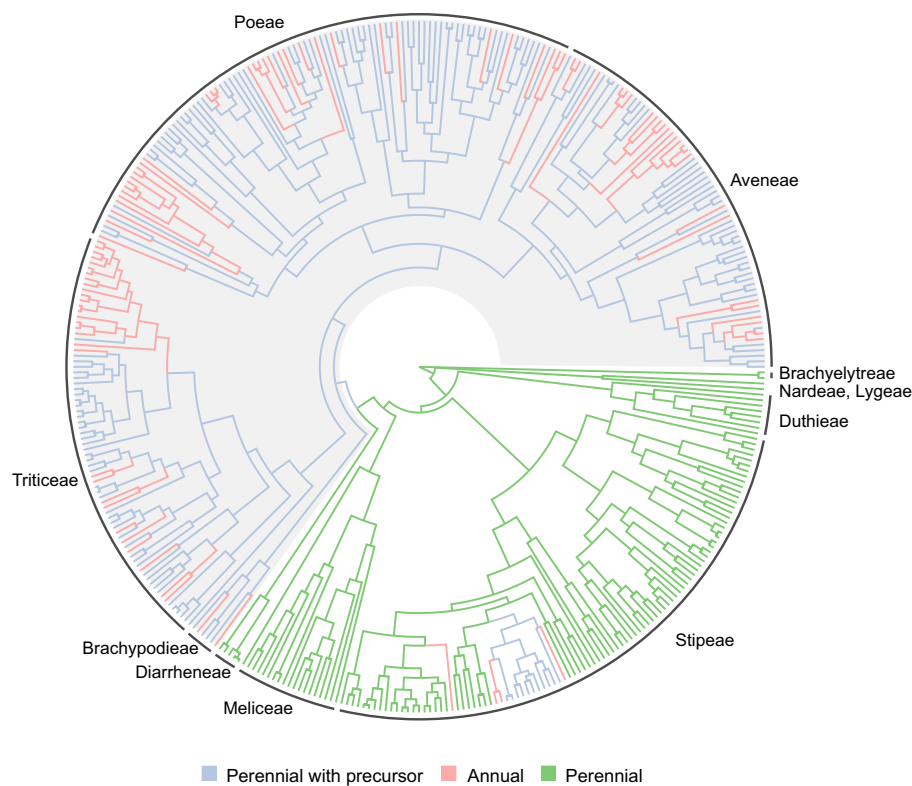
Fig. 1 Repeated evolution of annuality was driven by one or more precursor traits. Maximum clade credibility (MCC) tree based on three chloroplast markers (Schubert *et al.*, 2019) showing multiple independent origins of annuality (red branches) from perenniality (blue and green branches). Precursor traits that enabled transitions from perennial (blue branches) to annual life-cycle strategies were inferred to evolve at the base of core Pooideae (indicated with grey shading) and within Stipeae, using the best-fitting precursor two-rate model in r8s (Marazzi *et al.*, 2012).

relatively slow (0.00535), while the rate of annuality gain/loss appeared to be relatively fast (0.0233) (Table S4). The results of the r8s permutation test showed that in only 1 out of 3000 permutations, the precursor model with two rates was preferred over the 'binary two-rates' model (Table S7). These results indicated that r8s does not favour the precursor model in the Pooideae due to the rate of observed annual to perennial species, but due to the concentration of independent emergences of annual species in the core Pooideae.

Ecological opportunity for evolving annuality

We found a significant relationship between the mean values for both bioclimatic variables and the corresponding upper and lower 5th percentiles (Fig. S1a–d), meaning that the means follow the same trend as the entire species range. With a few exceptions, variance is constant across the different life history classes (Fig. S2a,c), yielding confident estimates for the mean in most taxa (Fig. S2b,d) that can be used as adequate approximations of a species' bioclimatic niche.

To test if perennial lineages without the inferred precursor are present in environments where we initially predicted that evolving annuality would be adaptive (i.e. seasonally freezing or arid) and thus would have the ecological opportunity to evolve annuality, we plotted niche space of the 388 Pooideae species based on mean temperature of the coldest quarter and precipitation of the driest quarter (Fig. 2). Compared with the niche space as a whole, the 95 annual species (one from a 'no precursor' clade; 94 from a 'precursor' clade) (Figs 1, S3) clustered in relatively warm and dry environments. As few annual species were found in areas with strong seasonal cold, we concluded that annuality is not an escape

strategy from seasonal cold. We thus focused on precipitation in the driest quarter as a possible selective pressure driving the evolution of annuality. To determine whether perennial lineages without the ancestral precursor state lacked annuality simply due to the absence of low precipitation as a selection pressure, we used ANOVA to compare the precipitation received by species of the three life-cycle strategy classes. Perennials with the precursor, perennials without the precursor, and annuals differed significantly in how much precipitation they receive in the driest quarter ($F_{2,344} = 17.64$, $P = 5.1e^{-8}$). Subsequent post hoc tests further revealed that, whereas annuals receive significantly ($P = 3e^{-5}$) lower precipitation in the driest quarter than perennials with the precursor trait, perennials lacking the precursor occupy the similarly low precipitation niche space as annuals ($P = 0.588$) (Fig. 2). Together, these data indicated that perennials without precursor traits occur in environments where annuality might be advantageous (i.e. arid or seasonally dry environments for example; Goldblatt, 1978; Ludwig *et al.*, 1988; Evans *et al.*, 2005), but that they have less potential to evolve annuality.

Increased biomass allocation to above-ground vs below-ground structures, but not growth rates, coincides with the inferred predisposition for evolving annuality

Based on earlier studies (described previously in this paper), we reasoned that traits acting as important prerequisites for the evolution of annuality might include growth rates and biomass allocation patterns. To determine if major changes in these traits coincided with the precursor nodes inferred from our reconstruction of life-cycle strategy evolution (Fig. 1), time series growth

Fig. 2 Perennial species that lack the precursor for annuality have nonetheless experienced the environmental selective pressures linked to evolution of this trait. Niche space, based on mean temperature of the coldest quarter and precipitation of the driest quarter, was estimated for 360 Pooideae species based on the species mean derived from GBIF locality records. Shapes outside the box show the distributions for species in the three life-cycle strategy categories: perennials with the precursor (blue), perennials without the precursor (green), and annuals (red).
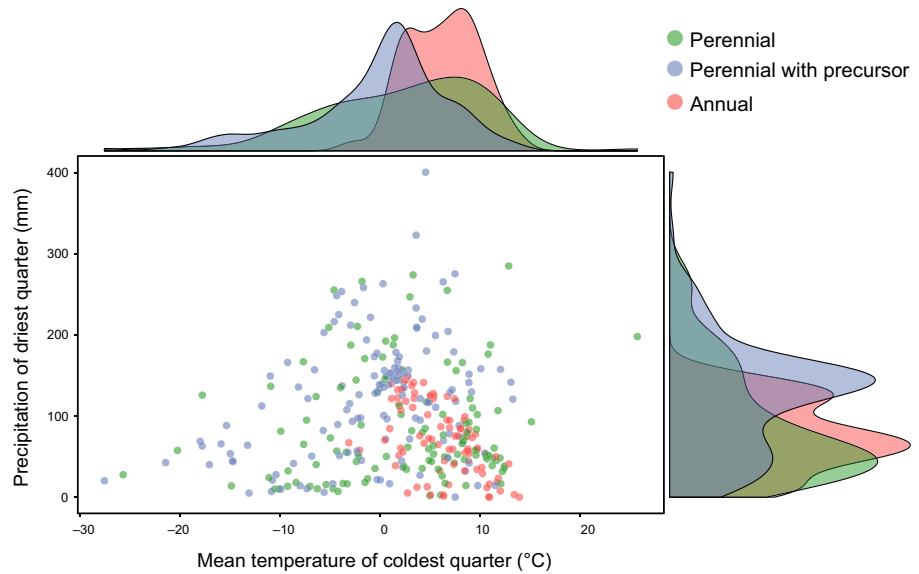
data for 53 representative Pooideae perennials were used to infer the ancestral state of RGR, SGR and allometric slope of the allocation of biomass to above-ground vs below-ground structures (Table S8; Figs S4, S5). No conclusions could be drawn about growth traits and evolution of the Stipeae precursor as only one species (*Oloptum miliaceum* (L.) Röser & Hamasha) from this clade was included in the growth experiment. Thus, only the Pooideae precursor node was considered.

Contrary to predictions, reconstructions with continuous growth rate data (RGR and SGR) did not indicate major changes in growth rates before, or concomitant with, the inferred precursor (Fig. S5a,b). To further investigate if high growth rates coincided with the evolution of the precursor, we also reconstructed ancestral states for high or low growth rates (scored as RGR or SGR above or below the grand mean; Figs 3, S6). These models showed that neither an above mean RGR nor SGR evolved within core Pooideae until significantly later than the inferred precursor node, increasing at least nine times independently in core Pooideae for both RGR and SGR (Figs 3, S6). The results indicated that major increases in RGR and SGR did not coincide with the Pooideae precursor node inferred from our reconstruction of life-cycle strategy evolution (Fig. 1). Nevertheless, RGR was significantly ($P = 0.009$) lower in perennial Pooideae species lacking the inferred precursor relative to perennial core Pooideae species with the precursor (Fig. 4a) suggesting that RGR evolution might actually have been shaped by the same precursor as annuality. There was no difference in SGR between perennials with and without the inferred precursor ($P = 0.905$).

Ancestral state reconstruction of the allometric slope of biomass allocation to above-ground vs below-ground structures (Fig. S5c) showed an increase in above-ground relative to below-ground biomass within perennial core Pooideae branches leading to the core Pooideae. Furthermore, the allometric slope of biomass allocation to above-ground vs below-ground structures in perennial Pooideae lacking the precursor was significantly lower ($P = 0.021$) than in perennials with the precursor (Fig. 4b). To investigate if a shift in resource allocation from more resources to below-ground

than above-ground ground structures (allometric slope < 1) to more resources to above-ground than below-ground structures (allometric slope > 1) we reconstructed ancestral states of discrete allometric slope (above or below 1; Fig. 5). The allometric slope of the allocation of biomass to above-ground vs below-ground structures is inferred to have been below 1 early in the history of Pooideae, increasing above 1 at four nodes, of which 1 corresponded to the precursor node at the base of core Pooideae. In the core Pooideae clade, the single increase above 1 in the allometric slope of biomass allocation to above-ground vs below-ground structures was then followed by at least 10 decreases to below 1. Based on ancestral state reconstruction of discrete data above or below 1, the allometric slope of the allocation of biomass to above-ground vs below-ground structures represented a good candidate for the precursor for annuality in perennial Pooideae species (Fig. 5).

Discussion

Multiple transitions to annuality in core Pooideae were enabled by an evolutionary precursor

The transition from perenniality to annuality is commonly heralded as a highly labile adaptive strategy that has evolved to mitigate seasonal stresses, and in particular to avoid drought (Sherrard & Maherali, 2006; Cruz-Mazo *et al.*, 2009; Friedman & Rubin, 2015). Consistent with this, our data evidenced at least 48 origins of annuality from the ancestral perennial state. These origins of annuality are not randomly distributed. Instead, only 3% of noncore Pooideae are annuals as opposed to 36% in the core Pooideae, and there are only three inferred origins of annuality within noncore Pooideae, but at least 45 origins within core Pooideae. These evolutionary trends, combined with the phylogenetic structure of life-cycle strategy in Pooideae, suggest the existence of internal constraints on the evolvability of annuality. As common shifts in life-cycle strategy exist across angiosperms, this finding provides novel insight into the evolution of this important trait (Friedman & Rubin, 2015).
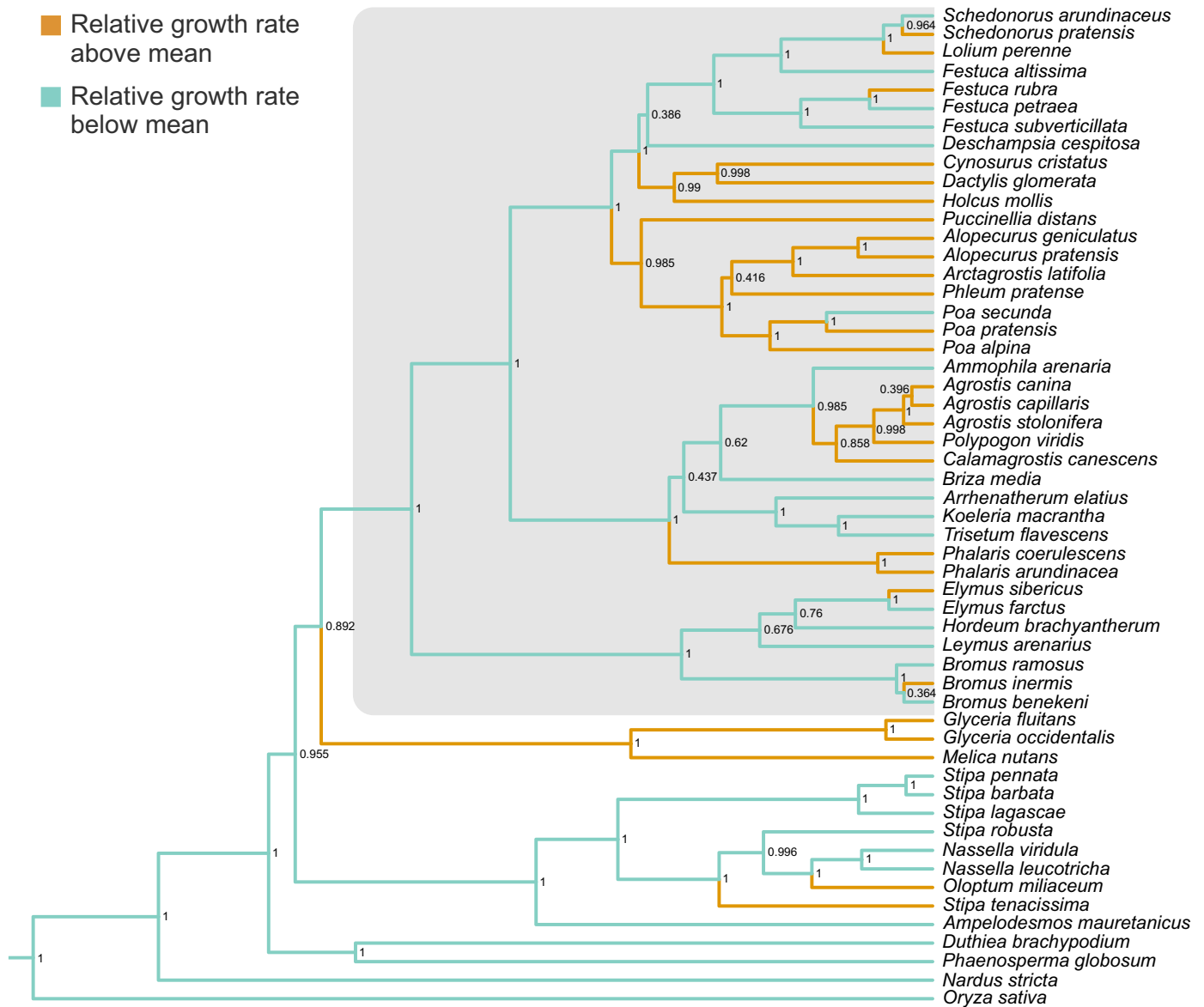
Fig. 3 Relative growth rate (RGR) is not a precursor for the evolution of Pooideae annuality. Maximum clade credibility (MCC) tree based on chloroplast markers *matK*, *ndhF* and *rbcL* showing the inferred evolution of RGR modelled in Beast. RGR was treated as a discrete variable, either being above (orange branches) or below (blue branches) the clade mean, with high values meaning faster seedling growth. Only perennial species were sampled in order not to bias the analysis. Numbers represent posterior probabilities for trait reconstructions at each branch. Core Pooideae are indicated by grey shading.

Internal constraints on evolution can be caused by developmental and genetic architecture underlying trait complexes (Friedman *et al.*, 2015). We identified two such positive developmental constraints that are likely to have evolved as precursors for annuality, once at the base of core Pooideae and again within Stipeae. These precursors may have facilitated the release of internal constraints through increased or decreased modularity, decreased antagonistic pleiotropy, phenotypic plasticity, and/or the evolution of other traits that precede key adaptations (Williams, 2008; Cacho & Strauss, 2014; Christin *et al.*, 2015; Minelli, 2016). Moreover, a long, dry season seems to be a strong driver for the evolution of the annual life-cycle strategy in the core Pooideae, as perennials with precursors and annuals diverge significantly in their dry season precipitation in line with expectations from the

literature (e.g. Goldblatt, 1978; Ludwig *et al.*, 1988; Evans *et al.*, 2005). Environments with a long, dry season where annuality is regarded as adaptive are also available for perennials without the precursor. Nevertheless, they rarely evolve the annual life-cycle strategy. This supports the hypothesis that there are genetic constraints to evolving annuality in noncore perennials, rather than absence of ecological opportunity. An alternative scenario is that the perennials without the precursor have such specialised adaptations to aridity that their adaptations to persist outweigh the annual species escape strategy. In this case the precursor would be loss of adaptations to persist in arid climates in the core Pooideae ancestor, increasing the pressure on its descendants to evolve an annual life form to inhabit arid conditions. We find this scenario unlikely, as it would also imply that all noncore Pooideae in nonarid climates

Fig. 4 Growth traits are significantly different between perennial Pooideae species with and without inferred annuality precursors. Distribution and box plots of relative growth rate (RGR) (a) and the allometric slope of biomass allocation to above-ground vs below-ground structures (b) are shown for perennials with (blue shading) and without (green shading) the inferred precursor trait. This could be due either to these traits being precursors themselves (i.e. possibly allometric slope; see Fig. 5) or the fact that evolution of these traits is affected by the precursor (i.e. possibly RGR; see Fig. 3). Boxplots show median, 25% and 75% percentiles, and whiskers indicate highest and lowest value no further away from the 25% and 75% percentile than 1.5 times the interquartile range, respectively.
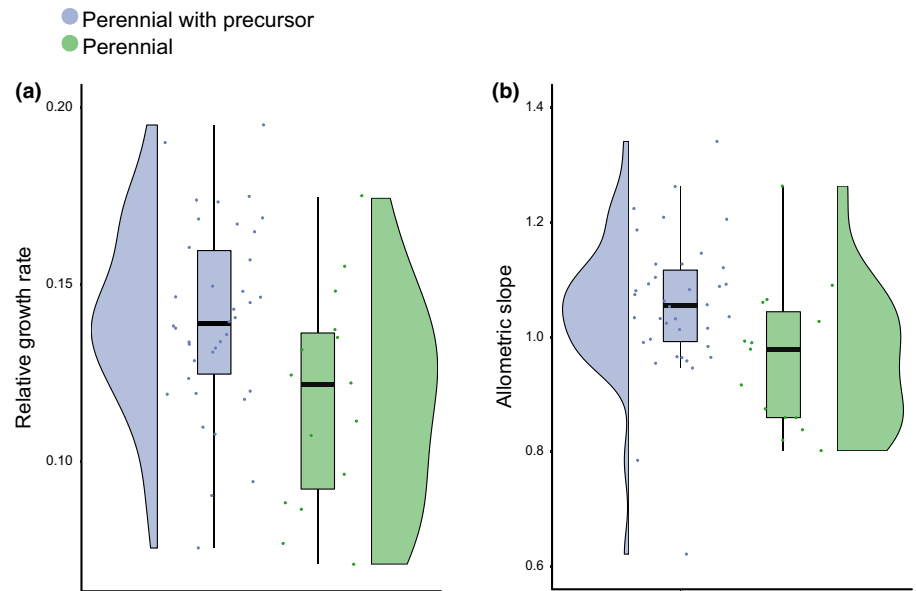
would also possess adaptations to arid climates. However, the decision to reject this scenario requires further testing.

Growth traits are candidates for the precursor

More so than perennials, the success of the annual life-cycle strategy requires individual plants to make rapid transitions from vegetative to reproductive growth following the onset of inductive cues, such as warm days. In several plant species, reproductive transition occurs only after a certain biomass is attained (Wang, 2014), thus fast growth in these species will be selected for when the period between germination and suitable flowering conditions is short (Diggle, 1999). Several studies (Garnier, 1992; Garnier & Laurent, 1994; Garnier *et al.*, 1997) previously investigated growth-related morphological and physiological differences between annual and perennial core Pooideae species pairs, and found that production parameters such as relative growth rates, leaf water content, specific leaf area, leaf production rate and cell types promoting a higher photosynthetic efficiency were higher in annuals than perennials, whereas leaf density, which is thought to be more important for longevity and persistence, was higher in perennials. The consistent differences in traits between annuals and perennials were interpreted as adaptive for the two life history strategies to promote fast growth and high productivity in annuals and slow growth and persistence in perennials (Garnier & Laurent, 1994). Based on these consistent differences, it was concluded that, if annuality was repeatedly derived from perenniality, the same morphological changes must have evolved repeatedly in disparate genera (Garnier, 1992). This conclusion is in line with our inference of one or more evolutionary precursors that facilitated parallel, and hence nonindependent, transitions to annuality in core Pooideae. A trend of higher RGR in core Pooideae annuals relative to perennials has been described previously (Grime & Hunt, 1975; Garnier, 1992). In our study, we found that perennial species with the precursor had significantly higher RGR than perennial species without precursor. However, we found no evidence for increased RGR in the

ancestor of core Pooideae that might have predisposed its descendants in core Pooideae to evolve annuality. Rather, shifts to high RGR occurred after the inferred precursor nodes for evolving annuality in core Pooideae, and therefore might themselves be shaped by precursor traits. Furthermore, we found no evidence of SGR being part of the precursor, and no differences in SGR were found between perennials with and without precursor, indicating that size standardised growth evolved independently of the precursor.

A related, but distinct, growth trait with an evolutionary switch preceding the numerous independent transitions to annuality in core Pooideae is the plants partitioning of their resources between above-ground and below-ground structures as they grow (allometric slope of biomass allocation to above-ground and below-ground structures). In the case of Pooideae, we found that perennial species within the core Pooideae, as opposed to noncore Pooideae, tended to allocate more resources to above-ground vs below-ground growth within the initial weeks of seedling development. Markedly, ancestral state reconstruction shows that this shift coincides with the evolution of the precursor and thus supports the idea that growth traits are strong candidates for the precursor.

The allocation of energy to different organs has received much attention as it is predicted that annual species would invest more energy in reproductive structures and resource acquisition, whereas perennials would invest to facilitate persistence. A higher allocation of energy to reproductive structures in annual species has indeed been identified in comparative studies (Pitelka, 1977; Bazzaz *et al.*, 1987), however a higher root : shoot ground ratio in perennials compared with annuals has been identified in some studies (Zangerl & Bazzaz, 1983; De Souza & Da Silva, 1987) but not in others (Gaines *et al.*, 1974; Turkington & Cavers, 1978). Notably, in a study of annual and perennial core Pooideae species, Garnier (1992) did not identify a difference in ratios of biomass in above-ground vs below-ground organs. However, a significant difference in allometric relationship was identified, indicating that annual species over time invest more energy in above-ground vs below-ground structures compared with

Fig. 5 Biomass allocation to shoot vs roots is a good candidate for the precursor for evolution of Pooideae annuality. Maximum clade credibility (MCC) tree based on chloroplast markers *matK*, *ndhF* and *rbcL* showing the inferred evolution of the allometric slope of biomass allocation to above-ground vs below-ground structures modelled in Beast. Allometric slope was treated as a discrete variable, either being above (orange branches) or below (blue branches) one, with values above one meaning more biomass accumulation in above-ground rather than below-ground structures during seedling development. Only perennial species were sampled in order not to bias the analysis. Numbers represent posterior probabilities for trait reconstructions at each branch. Core Pooideae are indicated by grey shading.

perennials, which in turn may lead to significant differences in above-ground vs below-ground ratios over time (Garnier, 1992). These results are in line with our results. Taken together, these data indicated that perennials with more allocation of resources to above-ground rather than below-ground structures are good candidates for evolving annuality.

We are fully aware that we sampled a small fraction of the total diversity of perennial Pooideae species in this experiment, as the nature of the material (root and shoots from small specimens) and the sampling density per species (24 samples per species) limited the number of species that can be adequately handled. Nevertheless, we have focused on random sampling across the phylogeny, while also

covering the larger tribes (Soreng *et al.*, 2017). We thus believe that these results are representative of the distribution of diversity in growth traits over the Pooideae subfamily, and thus are suitable for reconstruction of evolutionary history of growth traits.

Whether allometric relationships are available for natural selection and/or if they confer strong constraints on biological variation is much debated (Enquist & Niklas, 2002; Sack *et al.*, 2002; Pélabon *et al.*, 2014; Poorter *et al.*, 2015). Recent research has suggested that allometric relationships can be the target of selection, at least on a macroevolutionary scale (Bolstad *et al.*, 2015; Harrison, 2015; Poorter *et al.*, 2015). A change in partitioning of resources to above-ground rather than below-ground structures in perennial ancestral

core Pooideae may have been a response to falling CO_2 levels throughout the Cenozoic era, starting just before the evolution of the precursor at c. 48.4 Ma (Schubert *et al.*, 2019). Less available CO_2 (Anagnostou *et al.*, 2016) could possibly have triggered the evolution of an increased shoot : root ratio to compensate for loss of photosynthetic productivity. A positive relationship between increased atmospheric CO_2 and root : shoot ratio has been found for C_3 grain crop species of Pooideae, however it remains to be tested whether this is true for other species within this clade (Rogers *et al.*, 1995; Poorter & Nagel, 2000). Subsequently, if soil nutrients were not rate limiting due to easy sequestration through microbiota–plant associations, the relative increase in shoot biomass in the MRCA of core Pooideae, and again within Stipeae, could have been crucial to provide the necessary photosynthate to become a successful, fast growing annual (Atkinson *et al.*, 2016).

Conclusions

The fact that perenniality and annuality have evolved many times, and that perennial traits such as delayed flowering time and increased meristem indeterminacy show positive pleiotropy at least in the core eudicots (Melzer *et al.*, 2008; Friedman *et al.*, 2015), suggest that there exist few internal constraints to the evolution of annuality in angiosperms. Nevertheless, although transitions are common in Pooideae, we have found that certain clades are predisposed towards the evolution of annual life-cycle strategies and that these transitions are facilitated by one or more evolutionary precursors. Indeed, Pooideae clades that allocate more resources to above-ground rather than below-ground structures as seedlings have evolved annuality overwhelming more than others. To determine if this is a common trend across angiosperms, independent lineages need to be investigated, and further potential precursor traits investigated. It is also likely that developmental decisions at several stages throughout the plant's life can be a part of the precursor, for instance regulating the length of the juvenile phase (Bergonzi *et al.*, 2013; Hyun *et al.*, 2019) or partitioning different shoot meristems to be either generative or vegetative (Wang *et al.*, 2009; Deshpande *et al.*, 2015; Kiefer *et al.*, 2017). However, how these processes are regulated in grasses is unknown and the little that we do know comes from a few model species. Further studies are required to elucidate how annual and perennial species differentiate the spatio-temporal control of reproductive structure development. Identification of precursor traits paves the way for a new understanding of trait evolution and its underlying genetic foundation. At the same time, increasing the focus on the grass family will be critical for elucidating clade-specific genetic, developmental and environmental factors and their trade-offs, that potentially limit evolvability in the face of climate change and increasing efforts to breed perennial traits into annual cereal crops (Cox *et al.*, 2006).

Author contributions

SF designed the research. CLL performed the experiments. SF, CLL and MS analysed and interpreted the data. HMH, TM, BT and JCP gave input on analyses and interpretation of the results. SF, CLL and JCP wrote the paper with feedback from HMH, TM, MS and BT.

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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 Correlation of mean Bioclim variables and upper and lower fifth percentile.

Fig. S2 The ranked mean and standard deviation and error for Bioclim variables based on GBIF coordinates extracted for species included in estimation of the climatic niche.

Fig. S3 Detailed phylogeny of evolution of the life-cycle strategy classes annual, perennial with precursor and perennial.

Fig. S4 Distribution of model fit estimates for models of continuous trait evolution.

Fig. S5 Ancestral state reconstruction of relative growth rate (RGR), size standardised growth rate (SGR) and the allometric slope of biomass allocation to above-ground vs below-ground structures based on a Beast reconstruction using continuous trait data.

Fig. S6 Maximum clade credibility (MCC) tree based on chloroplast markers *matK, ndhF* and *rbcL* showing the inferred evolution of size standardised growth rate (SGR) modelled in Beast. SGR was treated as a discrete variable.

Table S1 Life-cycle strategy data for the 388 Pooideae species.

Table S2 Species list for the comparative growth experiment.

Table S3 Primer sequences for PCR and sequencing of chloroplast markers.

Table S4 Model selection for the r8s analysis.

Table S5 Posterior distribution for the 1000 tested trees in r8s.

Table S6 Inferred state shifts in maximum clade credibility (MCC) tree.

Table S7 Permutation test for r8s precursor analysis.

Table S8 Summary of model estimates for six different models of continuous trait evolution.

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