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**Article:**

Watcharamongkol, T., Christin, P.-A. [orcid.org/0000-0001-6292-8734](https://orcid.org/0000-0001-6292-8734) and Osborne, C.P. [orcid.org/0000-0002-7423-3718](https://orcid.org/0000-0002-7423-3718) (2018) C4 photosynthesis evolved in warm climates but promoted migration to cooler ones. *Ecology Letters*, 21 (3). pp. 376-383. ISSN 1461-023X

<https://doi.org/10.1111/ele.12905>

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This is the peer reviewed version of the following article: Watcharamongkol, T., Christin, P.-A. and Osborne, C.P. (2018), C4 photosynthesis evolved in warm climates but promoted migration to cooler ones. *Ecol Lett*, 21: 376-383, which has been published in final form at <https://doi.org/10.1111/ele.12905>. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.

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1 *Letter*

2 **C<sub>4</sub> photosynthesis evolved in warm climates but promoted migration**  
3 **to cooler ones**

4  
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11  
12 **Author contribution:** TW, CPO and PAC designed the study. TW generated and analysed the data.  
13 TW, CPO and PAC wrote the paper.

14  
15 **Running title:** Temperature adaptation of C<sub>4</sub> plants

16  
17 **Keywords:** evolution, C<sub>4</sub> photosynthesis, adaptation, climate, temperature niche, phylogeny

18  
19 **Count:** abstract 150 words, main text 3,790 words, 62 references, 3 figures and 2 tables.

20  
21 **Data accessibility statement:** the primary data supporting these results is archived in Dryad doi:  
22 10.5061/dryad.g8f18.

23

24

1 **Abstract**

2 C<sub>4</sub> photosynthesis is considered an adaptation to warm climates, where its functional benefits are  
3 greatest and C<sub>4</sub> plants achieve their highest diversity and dominance. However, whether inherent  
4 physiological barriers impede the persistence of C<sub>4</sub> species in cool environments remains debated.  
5 Here, we use large grass phylogenetic and geographic distribution datasets to test whether (i)  
6 temperature influences the rate of C<sub>4</sub> origins, (ii) photosynthetic types affect the rate of migration  
7 among climatic zones, and (iii) C<sub>4</sub> evolution changes the breadth of the temperature niche. Our  
8 analyses show that C<sub>4</sub> photosynthesis in grasses originated in tropical climates, and that C<sub>3</sub> grasses  
9 were more likely to colonize cold climates. However, migration rates among tropical and temperate  
10 climates were higher in C<sub>4</sub> grasses. Therefore, while the origins of C<sub>4</sub> photosynthesis were  
11 concentrated in tropical climates, its physiological benefits across a broad temperature range  
12 expanded the niche into warmer and enabled diversification into cooler environments.

13 [150 words]

14

15

# 1 INTRODUCTION

2 Temperature varies significantly over the surface of the Earth and through time, and is considered  
3 the primary factor determining the global distributions of plant species (Woodward 1990; Larcher  
4 2003). During evolution, plants have colonized almost all possible temperature niches (Kier *et al.*  
5 2005; Araújo *et al.* 2013) via a variety of biochemical, physiological and growth adaptations to  
6 either cool or warm temperatures (Sakai & Larcher 1987; Larcher 2003). One particular dimension  
7 of the temperature niche is the capacity to gain net benefits from photosynthesis under the ambient  
8 temperature conditions. Net photosynthetic gains are damped at higher temperatures because CO<sub>2</sub>  
9 fixation by Rubisco is offset by competition with O<sub>2</sub> fixation, boosting the costly photorespiratory  
10 cycle (Ehleringer & Björkman 1977; Sage & Kubien 2007). Some plants have evolved CO<sub>2</sub>-  
11 concentrating mechanisms that minimize photorespiration and, in land plants, one of the two  
12 successful mechanisms for doing this is C<sub>4</sub> photosynthesis (Ehleringer & Björkman 1977; Sage &  
13 Monson 1999; Still *et al.* 2003; Sage *et al.* 2012).

14 C<sub>4</sub> photosynthesis is a physiological process resulting from a series of biochemical and  
15 anatomical modifications over the ancestral C<sub>3</sub> photosynthetic type (Hatch 1987). Together, these  
16 concentrate CO<sub>2</sub> around Rubisco, thereby increasing its efficiency and reducing photorespiration  
17 (von Caemmerer & Furbank 2003; Sage *et al.* 2012). However, the extra biochemical reactions  
18 required for C<sub>4</sub> photosynthesis increase the energetic cost of carbon fixation, so that C<sub>4</sub>  
19 photosynthesis is predicted to outperform C<sub>3</sub> only when photorespiration is significant, especially  
20 under high temperatures (Ehleringer & Björkman 1977; Osborne & Beerling 2006). The improved  
21 performance of C<sub>4</sub> plants at high temperatures predicted from physiological measurements and  
22 theory is supported by global distribution patterns (Ehleringer & Björkman 1977; Ehleringer 1978;  
23 Griffith *et al.* 2015). Indeed, while trees are almost all C<sub>3</sub>, open biomes are predominantly occupied  
24 by C<sub>4</sub> species in hot to warm climates, and cooler open biomes are dominated by C<sub>3</sub> species (Sage  
25 *et al.* 1999b; Edwards *et al.* 2010).

1           The majority of C<sub>4</sub> species belong to the grass family (Poaceae) (Sage *et al.* 1999a). At large  
2 scales, C<sub>3</sub> and C<sub>4</sub> grasses sort largely according to temperature (Ehleringer & Björkman 1977;  
3 Ehleringer 1978; Sage *et al.* 1999a; Osborne *et al.* 2014), and C<sub>4</sub> grasses dominate most open  
4 biomes in tropical and subtropical areas, where they achieve greater biomass and higher growth rate  
5 (Still *et al.* 2003; Edwards *et al.* 2010; Atkinson *et al.* 2016). Despite these general patterns, low  
6 temperatures do not completely exclude C<sub>4</sub> grasses. A number of C<sub>4</sub> grass species are found in  
7 alpine, steppe or high latitude habitats where they survive cold conditions during the growing  
8 season, with some species developing physiological cold acclimation to tolerate freezing (Long *et*  
9 *al.* 1975; Beale & Long 1995; Sage & Sage 2002; Márquez *et al.* 2006; Liu & Osborne 2008, 2013;  
10 Sage *et al.* 2010). These examples demonstrate that C<sub>4</sub> species can survive and compete with C<sub>3</sub>  
11 species in cold environments, at least under some circumstances.

12           The rarity of C<sub>4</sub> plants in cold climates might be explained by inherent physiological  
13 constraints on the amounts or activities of key photosynthetic proteins (Sage & Kubien 2007).  
14 However, it is also possible that the current geographical distributions reflect historical processes,  
15 with C<sub>4</sub> plants evolving in tropical and subtropical climates and inheriting traits that are poorly  
16 suited for cooler climates (Long 1999). Differentiating these scenarios requires large-scale  
17 comparisons of C<sub>3</sub> and C<sub>4</sub> lineages, while considering their evolutionary history. Past comparative  
18 work has shown that C<sub>4</sub> grasses emerged from tropical C<sub>3</sub> lineages (Edwards & Still 2008; Edwards  
19 & Smith 2010), and suggested that C<sub>4</sub> evolution in specific clades enabled a niche expansion into  
20 both cooler and warmer climates (Christin & Osborne 2014; Lundgren *et al.* 2015; Aagesen *et al.*  
21 2016). However, systematic tests for an effect of photosynthetic types on transition rates among  
22 temperature biomes are lacking.

23           In this study, we use phylogenetic and geographic distribution data for 2,133 grass species (a  
24 fifth of all grass species), including 948 C<sub>4</sub> species (a fifth of all C<sub>4</sub> grass species) representing 18  
25 independent C<sub>4</sub> lineages (most of the 24 C<sub>4</sub> groups), to assess the relationships between

1 photosynthetic types and temperature niches. Using comparative analyses, we first test for an effect  
2 of temperature on evolutionary transitions between the C<sub>3</sub> and C<sub>4</sub> photosynthetic types as well as  
3 the influence of these photosynthetic types on the migration of plant lineages among climatic zones.  
4 We then evaluate quantitatively the effect of the photosynthetic type on temperature niche breadth.  
5 Our investigations shed new light on the interplay between physiology and evolutionary history in  
6 determining the sorting of plants across the ecological space.

7

## 8 **METHODS**

### 9 **Climate dataset**

10 All available geo-referenced occurrence records (~14.3M) for the grass family (Poaceae) were  
11 extracted from the Global Biodiversity Information Facility (GBIF) web portal  
12 (<http://www.gbif.org>, accessed 10<sup>th</sup> December 2015). Records were cleaned to filter out unreliable  
13 location data using the following steps. First, duplicate records from the same location were  
14 removed. Potentially incorrect geographical data were then excluded, including those with  
15 coordinates outside the map, where the country did not match the coordinates, within 20 km of the  
16 GBIF headquarters, where longitude and latitude were exactly the same because these may  
17 represent a typo or default value, or where the georeference was recorded to a precision fewer than  
18 three decimal places. The species names were then checked against the Kew grass synonymy  
19 database (Clayton *et al.* 2006) via the software package Taxonome (Kluyver & Osborne 2013), and  
20 records without a valid name were discarded. The species with less than ten occurrences were also  
21 excluded to increase accuracy of the temperature range estimated for each species. For each set of  
22 coordinates, we extracted the mean and minimum temperatures of the coldest month, and the mean  
23 temperature of the warmest month from WorldClim version 2, 30-arc seconds resolution data (Fick  
24 & Hijmans 2017). The median and 5<sup>th</sup> and 95<sup>th</sup> percentiles from each species were considered in  
25 further analyses, to investigate range limits while avoiding extreme outliers. The temperature range

1 occupied by each species was estimated as the difference between the 5<sup>th</sup> percentile of the coldest  
2 month and 95<sup>th</sup> percentile of the warmest month (Quintero & Wiens 2013).

3

#### 4 **Phylogenetic tree**

5 We used a published time-calibrated phylogenetic tree that includes 3,595 species of grasses,  
6 covering the whole family and using a time-calibration hypothesis based upon macrofossils and  
7 microfossils (Spriggs *et al.* 2014). Taxa without occurrence data after filtering were pruned from the  
8 tree, resulting in a dataset of 2,133 species with both phylogenetic and distribution data.

9 Comparison of the proportions of C<sub>3</sub> and C<sub>4</sub> species in each climatic region between those available  
10 in GBIF and the subset included in the phylogenetic tree shows that the filtering by the phylogeny  
11 did not bias the dataset (Table S1 and Figure S1). Each analysis was first conducted on the whole  
12 grass family. However, C<sub>4</sub> origins only exist within one of the two major clades of grasses, the  
13 PACMAD clade (Grass Phylogeny Working Group II 2012). We therefore repeated the analyses  
14 considering only this clade of 1,197 species to check the robustness of results.

15

#### 16 **Modelling transition rates between photosynthetic and climatic types**

17 The aims of this analysis were to test whether climatic regions influence the rates of transitions  
18 among photosynthetic types, and whether photosynthetic types in turn influence the rates of  
19 transitions among climatic types. The photosynthetic type of each species was assigned based on  
20 the literature (Osborne *et al.* 2014). Climate was categorized using the Köppen-Geiger  
21 classification, as the analysis required binary characters, and this is a well-established and widely  
22 known climate scheme (Peel *et al.* 2007). Specifically, we used three climatic types, based on  
23 temperature: tropical (mean temperature of the coldest month above 18 °C); temperate (mean  
24 temperature of the coldest month between 0 and 18 °C); and cold (mean temperature of the coldest  
25 month below 0 °C, which includes continental, polar and alpine climates). The temperate range was

1 further divided into freezing and non-freezing conditions, since freezing represents a particular  
2 physiological challenge to plants (Sakai & Larcher 1987). Minimum temperature of the coldest  
3 month was used to identify regions that are exposed to freezing. Climate types were assigned based  
4 on species median values.

5 Transition rates among photosynthetic and climatic types were estimated for each pair of  
6 climate classes that are adjacent on the temperature gradient: (i) tropical vs temperate without  
7 freezing; (ii) temperate without freezing vs temperate with freezing; and (iii) temperate with  
8 freezing vs cold. For each independent combination of adjacent climates, Pagel's method (Pagel  
9 1994, 1999; Pagel & Meade 2006) was used to model the eight possible evolutionary transitions  
10 between the four states (two adjacent climates multiplied by two photosynthetic types). The model  
11 was fitted using a maximum likelihood method to derive point estimates of log-likelihoods in the  
12 package BayesTraits (Pagel & Meade 2006).

13 By fixing some parameters, we tested four hypotheses for each pair of adjacent climates  
14 using likelihood ratio tests on nested models. First, we tested whether the rate of transitions from  
15 warmer to cooler climates (i.e. tropical to temperate without freezing, temperate without freezing to  
16 temperate with freezing, or temperate with freezing to cold climates) differs between C<sub>3</sub> and C<sub>4</sub>  
17 lineages (by contrasting rates of 1→2 and 5→6, 2→3 and 6→7, or 3→4 and 7→8, Fig. 1). Second,  
18 we tested whether the rate of reverse transitions from cooler to warmer climates differs between C<sub>3</sub>  
19 and C<sub>4</sub> lineages (by contrasting rates of 2→1 and 6→5, 3→2 and 7→6, or 4→3 and 8→7, Fig. 1).  
20 Third, we tested whether the rate of transitions from C<sub>3</sub> to C<sub>4</sub> states differs between warmer and  
21 cooler climates (by contrasting rates of 1→5 and 2→6, 2→6 and 3→7, or 3→7 and 4→8, Fig. 1).  
22 Finally, based upon previous work suggesting that reversions from C<sub>4</sub> to C<sub>3</sub> photosynthesis are  
23 unlikely (Christin *et al.* 2010; Grass Phylogeny Working Group II 2012), we tested whether  
24 transitions from C<sub>4</sub> to C<sub>3</sub> are possible in either of the two climates (by contrasting rates of 5→1,  
25 6→2, 7→3, and 8→4 with a rate fixed to zero, Fig. 1).



1 Bias in the underlying species sampling could theoretically influence the results of these  
2 tests if either C<sub>3</sub> or C<sub>4</sub> species within a particular climate regime were under- or over-represented.  
3 The GBIF database has a known bias, with particular regions being well sampled (e.g. Europe,  
4 North America, Australia) and other regions being poorly covered, especially in the tropics (e.g.  
5 India, parts of Africa). However, this bias only creates problems for our analysis if C<sub>3</sub> or C<sub>4</sub> species  
6 are differentially sampled within tropical regions, and we can think of no reason why this should be  
7 the case. On the other hand, the phylogenetic tree is likely biased as taxa judged interesting for a  
8 variety of reasons would be preferentially sequenced. However, because the sampling of species  
9 from the phylogeny is filtered by the availability of GBIF, the final dataset is representative of  
10 GBIF without any bias from phylogeny (Table S1 and Figure S1).

11 To visualize the historical transitions between climatic types of C<sub>3</sub> and C<sub>4</sub> species, ancestral  
12 values were computed for climatic types using the *ace* function in the *ape* package in R (Pagel  
13 1994; Paradis *et al.* 2004) and the most likelihood climate of ancestors was mapped for each node  
14 on the phylogenetic tree using the *ggtree* package in R (Yu *et al.* 2017).

15

### 16 **Phylogenetic comparisons of temperature niches**

17 Phylogenetic generalized least squares (PGLS) were used to confirm that the photosynthetic type  
18 influences thermal maxima and minima as well as the breadth of the temperature niche, as  
19 suggested previously with smaller datasets (Lundgren *et al.* 2015; Aagesen *et al.* 2016).

20 Temperature was the independent variable, with photosynthetic type as the categorical predictor.

21 Phylogeny was incorporated to control for phylogenetic dependence and to estimate the  
22 phylogenetic signal using Pagel's  $\lambda$  (Pagel 1999; Freckleton *et al.* 2002).

23

24

# 1 RESULTS

## 2 Climatic distributions of C<sub>3</sub> and C<sub>4</sub> species

3 The percentage of C<sub>4</sub> species decreases from tropical to cold climates (Table S2), with more than  
4 ten per cent of C<sub>4</sub> species available in GBIF occurring in freezing temperate or cold climates (Table  
5 S1), the latter in our dataset of 2,133 species being colonized by C<sub>4</sub> species from three different  
6 subfamilies (Table S2), including a number of perennial species. The predominance of C<sub>3</sub> species in  
7 cold climates mainly reflects the success of members of the Pooideae subfamily, although other  
8 groups are also represented (Table S2). Yet only members of Pooideae can inhabit areas where the  
9 mean temperature of the coldest month is lower than -10 °C (Fig. 2).

10

## 11 Rates of transition among photosynthetic types and climates

12 Models were used to test whether climate influences transitions among photosynthetic types. They  
13 supported the hypothesis that C<sub>4</sub> origins are more frequent in tropical than temperate climates (rates  
14 of 1→5 > 2→6;  $p < 0.001$ ; Fig. 1; Table 1). The ancestral state reconstructions confirmed that C<sub>4</sub>  
15 photosynthesis evolved from C<sub>3</sub> PACMAD ancestors in tropical climates, while the ancestor of  
16 Danthonioideae moved to temperate climates, with descendants that remained C<sub>3</sub> (Fig. 3). The rate  
17 of C<sub>4</sub> origins is not significantly different from zero in freezing temperate and cold climates (rates  
18 of 3→7, 4→8 = 0;  $p > 0.05$ ; Fig. 1; Table 1), but it is greater than zero in non-freezing temperate  
19 climates (rate of 2→6 ≠ 0;  $p < 0.05$ ; Fig. 1; Table 1). Based on our models, the rate of transition  
20 from C<sub>4</sub> to C<sub>3</sub> types is not significantly different from zero under any climatic conditions (rates of  
21 5→1, 6→2, 7→3, 8→4 = 0;  $p > 0.05$ ; Fig. 1; Table 1), which is consistent with previous  
22 conclusions that the rate of reversal from C<sub>4</sub> to C<sub>3</sub> is extremely low or null in grasses (Christin *et al.*  
23 2010; Grass Phylogeny Working Group II 2012).

24 The same models were used to test whether the photosynthetic type influences transitions  
25 between climatic zones. The rates of transitions between tropical and non-freezing temperate

1 climates across the whole family and in the PACMAD clade are significantly higher in both  
2 directions in C<sub>4</sub> than C<sub>3</sub> taxa (rates of 5→6 > 1→2 and 6→5 > 2→1;  $p < 0.001$ ; Fig. 1, S2; Table 1,  
3 S3). The ancestral state reconstructions indicate that these transitions occurred many times since the  
4 split of Chloridoideae, Panicoideae and Aristidoideae (Fig. 3). Moreover, C<sub>4</sub> photosynthesis  
5 increases the rate of transition from temperate climates with freezing to those without freezing  
6 (rates of 7→6 > 3→2;  $p < 0.001$ ; Fig. 1; Table 1). The shift to occupy temperate climates without  
7 freezing during the winter therefore occurred more frequently in C<sub>4</sub> than C<sub>3</sub> taxa (Fig. 3). The rate  
8 of transition from temperate climates without freezing to climates with freezing was also higher in  
9 C<sub>4</sub> than C<sub>3</sub> types, but only in the analysis of the PACMAD clade (rates of 6→7 > 2→3;  $p < 0.05$ ;  
10 Fig. S2, Table S3). The transition from non-freezing to freezing conditions was found commonly in  
11 C<sub>4</sub> PACMAD lineages, but the shift also occurred frequently in C<sub>3</sub> Pooideae (Fig. 3), leading to  
12 equal rates when considering the grass family as a whole (rates of 6→7 = 2→3;  $p > 0.05$ ; Fig. 1;  
13 Table 1). However, transitions from temperate to cold climates are more frequent within C<sub>3</sub> than C<sub>4</sub>  
14 lineages (rates of 7→8 < 3→4;  $p < 0.001$ ; Fig. 1, Table 1), while the rate of transitions in the  
15 opposite direction, from cold to temperate climates, is independent of the photosynthetic type, with  
16 both C<sub>3</sub> and C<sub>4</sub> grasses moving at the same rate (rates of 4→3 = 8→7;  $p > 0.05$ ; Fig. 1; Table 1).  
17 Phylogenetic reconstructions suggest a few transitions to cold climates within C<sub>4</sub> groups, while the  
18 large Pooideae C<sub>3</sub> clade migrated early to cold climates and diversified there (Fig. 3).

19

## 20 **Evolution of temperature niche breadth**

21 In our study, temperature extremes and ranges were used to confirm that differences exist between  
22 C<sub>3</sub> and C<sub>4</sub> plants in the breadth of the temperature niche. The PGLS analyses indicate that C<sub>4</sub>  
23 evolution led to an expansion of the species-level temperature niche (Table 2). C<sub>4</sub> photosynthesis is  
24 specifically associated with increases in the upper bound of the species range toward higher  
25 temperatures during the warmest month (Table 2), mirroring previous conclusions with a smaller

1 species sampling (Aagesen *et al.* 2016). By contrast, the lower bounds of the temperature ranges  
2 within species did not differ significantly between C<sub>4</sub> and C<sub>3</sub> groups, which indicates that C<sub>4</sub>  
3 evolution does not affect the lower range of the temperatures that are occupied (Table 2). The  
4 conclusions remained the same whether the comparison was made across all grasses or just the  
5 PACMAD clade, which includes all C<sub>4</sub> lineages (Table 2).

6

## 7 **DISCUSSION**

### 8 **C<sub>4</sub> plants evolved in tropical climates, expanded to warmer and shifted to cooler environments**

9 Our analyses of evolutionary transitions across the whole grass phylogeny provide general  
10 statistical support for the hypothesis that C<sub>4</sub> photosynthesis in grasses evolved in tropical climates  
11 (Figs. 1 and 3), confirming previous work (Sage 2004; Edwards & Still 2008; Edwards & Smith  
12 2010). Hot climates, under the low-CO<sub>2</sub> atmosphere that prevailed for the last 30 million years  
13 (Pagani *et al.* 2005) exacerbated photorespiration, providing a selective pressure for novel  
14 photosynthetic physiologies that decrease the net cost of this process (Ehleringer & Björkman 1977;  
15 Osborne & Beerling 2006; Christin *et al.* 2008). Current models indicate that C<sub>4</sub> photosynthesis  
16 evolved via a series of intermediate stages, including photorespiratory bypasses and weak C<sub>4</sub> cycles,  
17 which progressively decreased the adverse effects of photorespiration (Sage 2004; Heckmann *et al.*  
18 2013; Mallmann *et al.* 2014). While it has been questioned whether extant taxa with an intermediate  
19 physiology are similar to those that enabled C<sub>4</sub> evolution, with some arguing that they might instead  
20 result from hybridization events (Kadereit *et al.* 2017), these intermediates are concentrated in  
21 hotter climates (Lundgren & Christin 2017). If states that preceded C<sub>4</sub> evolution were similarly  
22 restricted to hotter climates, C<sub>4</sub> origins would consequently be concentrated in warm climates, as  
23 observed here (Fig. 1). However, because the physiological effects of C<sub>4</sub> photosynthesis are broader  
24 than those of these intermediates (Vogan & Sage 2011; Christin & Osborne 2014), the ecological

1 consequences of C<sub>4</sub> evolution might not be limited to warm climates (Christin & Osborne 2014).  
2 Our analyses support this hypothesis.

3         Our modelling analysis shows for the first time that C<sub>4</sub> photosynthesis accelerated the  
4 migration of grass taxa between tropical and temperate climates in comparison with C<sub>3</sub> lineages  
5 (Fig. 1). Therefore, C<sub>4</sub> photosynthesis presents no inherent physiological barrier to the colonization  
6 of temperate environments. It has been hypothesised that cold acclimation in C<sub>4</sub> plants may be  
7 impeded by their leaf anatomy, which provides insufficient cellular volume to accumulate Rubisco  
8 protein (Sage & Kubien 2007), although this is debated (Long & Spence 2013). It has also been  
9 proposed that C<sub>4</sub> species should be excluded from low temperature regions by competition with C<sub>3</sub>  
10 plants, which have a higher photosynthetic efficiency than the C<sub>4</sub> type in cool environments,  
11 especially in low light conditions within dense leaf canopies or under cloudy skies (Ehleringer &  
12 Björkman 1977; Ehleringer 1978). However, modelling suggests that, under cloudless, high light  
13 conditions, the cost resulting from the extra C<sub>4</sub> reactions may be more than compensated at the  
14 canopy scale by light-saturated photosynthetic rates in sunlit leaves (Long 1999; Long & Spence  
15 2013). In addition, the C<sub>4</sub> syndrome provides advantages besides carbon-fixation efficiency. These  
16 include greater nitrogen- and water-use efficiencies than the C<sub>3</sub> type (Long 1999), and increased net  
17 assimilation rates enable investment into different growth strategies (Atkinson *et al.* 2016).  
18 Together, these properties might contribute to the success of C<sub>4</sub> species across a range of  
19 temperature conditions.

20         However, our analysis also shows that C<sub>4</sub> species are overall less likely than C<sub>3</sub> ones to  
21 migrate into continental, polar or alpine climates (grouped as “cold climates” in our analysis). A  
22 colonization of cold climates has previously been inferred early during the history of C<sub>3</sub> Pooideae  
23 (Edwards & Smith 2010), the group that dominate cold and temperate climates (Fig. 3; Table S2).  
24 This suggests that adaptation in this group to survive under prolonged cold conditions has been  
25 enabled by traits that evolved early during their history and may not characterise other grass

1 lineages (Sandve & Fjellheim 2010; Vigeland *et al.* 2013; Spriggs *et al.* 2014; McKeown *et al.*,  
2 2016). The early migration and adaptation to cold climates allowed the subsequent diversification of  
3 Pooideae in cold and temperate climates (Table S2, Fig. 3).

4

#### 5 **Biogeography affects the current distribution of C<sub>4</sub> plants**

6 Evolutionary history, coupled with biogeographical pattern, explains the higher frequency of C<sub>4</sub>  
7 species in tropical or temperate climates (Table S2). C<sub>4</sub> origins happened predominantly in tropical  
8 climate regions (Fig. 1), allowing grasses to tolerate higher temperatures and expanding the  
9 temperature niche (Table 2), as shown previously (Christin & Osborne 2014; Lundgren *et al.* 2015;  
10 Aagesen *et al.* 2016; Bena *et al.* 2017). However, our analysis is the first to show that C<sub>4</sub>  
11 photosynthesis also increased the rate of transitions among climate types, with frequent migration  
12 into temperate climates without freezing (Fig. 1). The rate of C<sub>4</sub> plant migration into freezing  
13 temperate climates was also considerable, and was higher than that of close C<sub>3</sub> relatives within the  
14 PACMAD clade. When considered across the grass family as a whole it was comparable to the rates  
15 in Pooideae, indicating that C<sub>4</sub> lineages are physiologically capable of colonizing cold  
16 environments.

17 Geographical barriers could have played important roles in limiting the expansion of some  
18 C<sub>4</sub> groups into cold climates. Most tropical climate regions are geographically distant from cold  
19 climates (Donoghue 2008), presenting little opportunity for tropical plants to migrate into cooler  
20 environments (Edwards & Donoghue 2013). C<sub>4</sub> species of cold climates are therefore found mostly  
21 in high altitude habitats located at low latitudes, and only rarely at high latitudes (Long 1999; Sage  
22 & Monson 1999; Sage *et al.* 2010), but we argue that this pattern does not stem from physiological  
23 limitations, instead being the direct consequence of the increased rate of C<sub>4</sub> origins in tropical  
24 regions.

25

## 1 **CONCLUSIONS**

2 Using a large phylogeny for the grass phylogeny, we show for the first time that C<sub>4</sub> photosynthesis  
3 evolved primarily in tropical climates, and subsequently enhanced the rates of evolutionary  
4 transitions between tropical and temperate climates. When compared to close relatives, C<sub>4</sub> plants  
5 were furthermore more likely to colonize freezing environments. Our conclusions therefore  
6 contradict previous work based solely on geographical distributions and physiological theory. The  
7 macroevolutionary processes revealed in our large comparative study underpin the high ecological  
8 diversity and global expansion of C<sub>4</sub> species. Although there appear to be no physiological barriers  
9 to prevent C<sub>4</sub> plants from colonizing cooler environments, C<sub>4</sub> grass clades have still migrated less  
10 frequently from temperate to cold climate regions than members of some C<sub>3</sub> lineages (especially  
11 Pooideae). This pattern arises from the recent origins of C<sub>4</sub> photosynthesis in warm climates, in  
12 lineages with warm adapted traits, which contrasts with the ancient origin of cold adaptation in  
13 Pooideae. C<sub>4</sub> plants must therefore have both the time and the opportunities to acquire further traits  
14 needed to successfully colonize cold climates.

15

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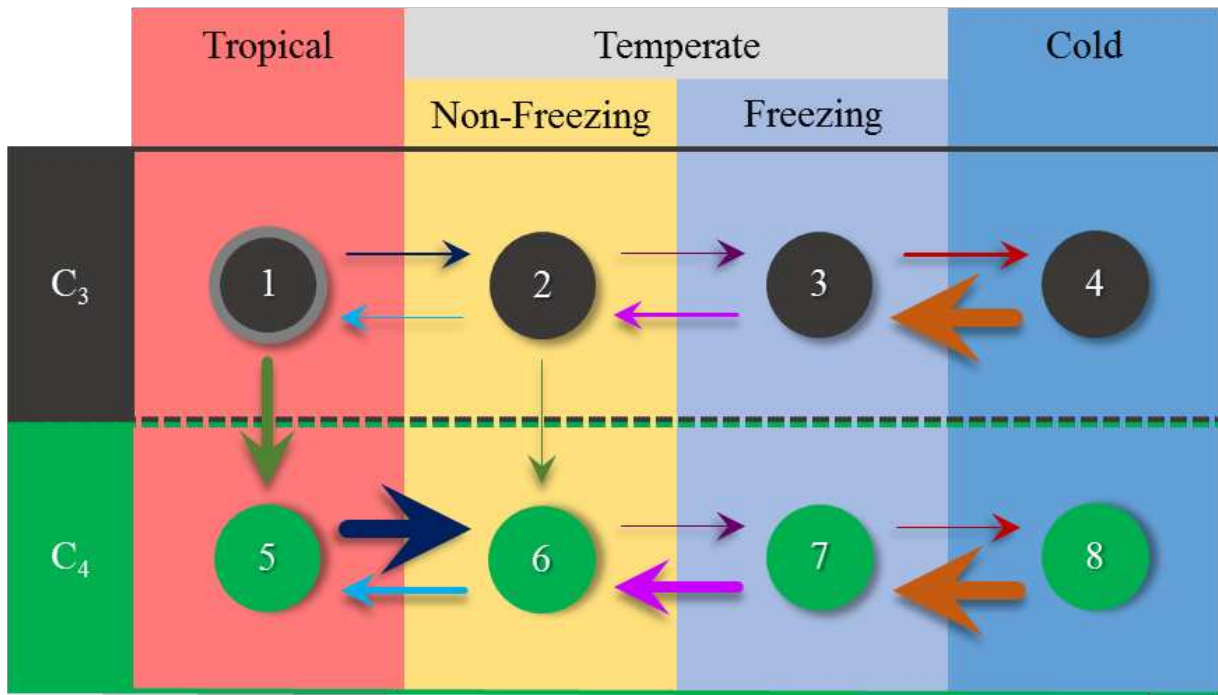
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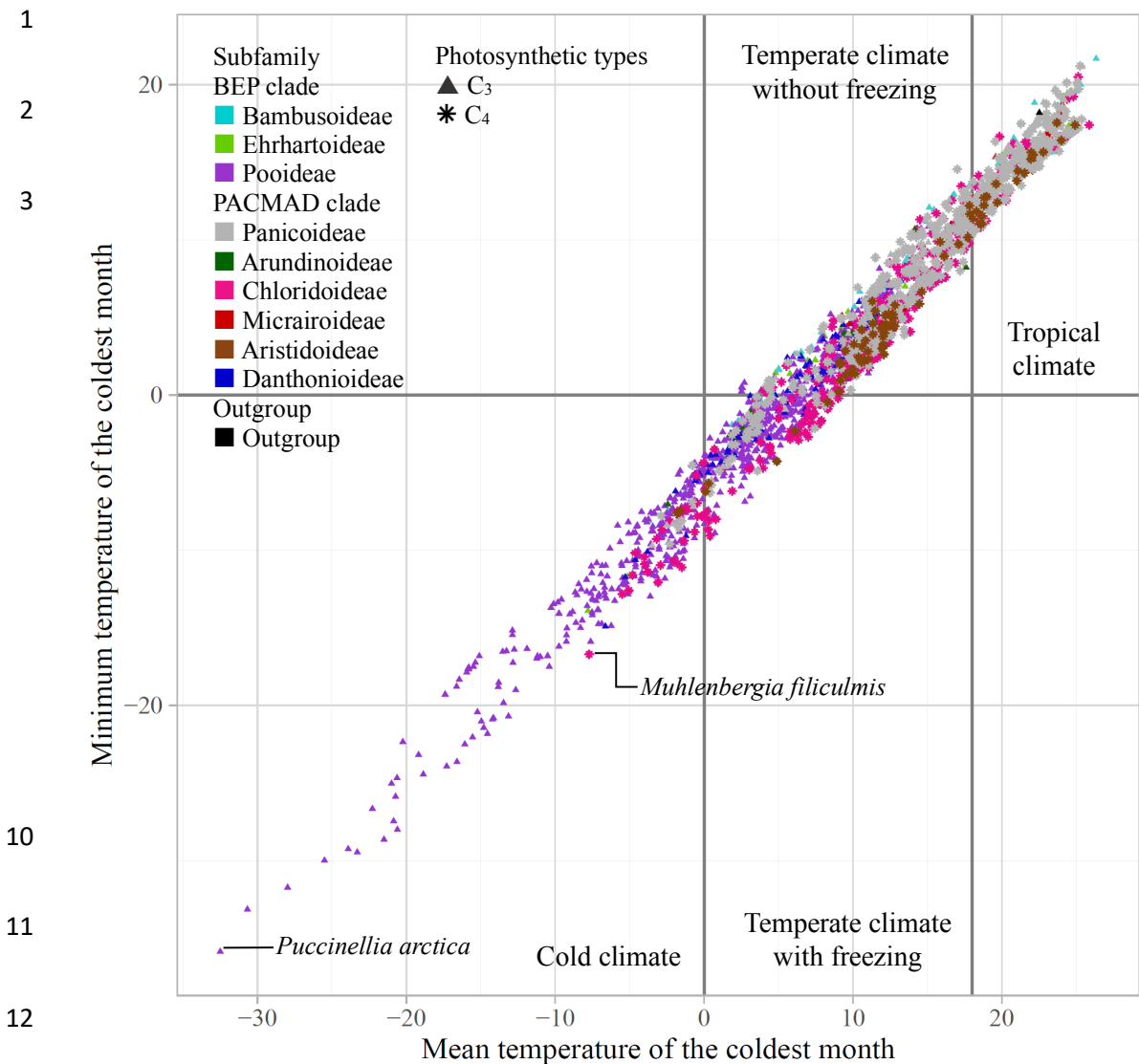
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5 **Figure 1** Model of coevolution of photosynthetic types and temperature niches. Sizes of arrows  
6 indicate transition rates among climate and photosynthetic types. The most likely ancestral  
7 condition is indicated by the grey outer circle.

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14 **Figure 2** Climatic distributions of C<sub>4</sub> and C<sub>3</sub> species from various subfamilies indicated by

15 temperature regimes. The thick grey lines indicate the boundaries between climatic types. The C<sub>3</sub>

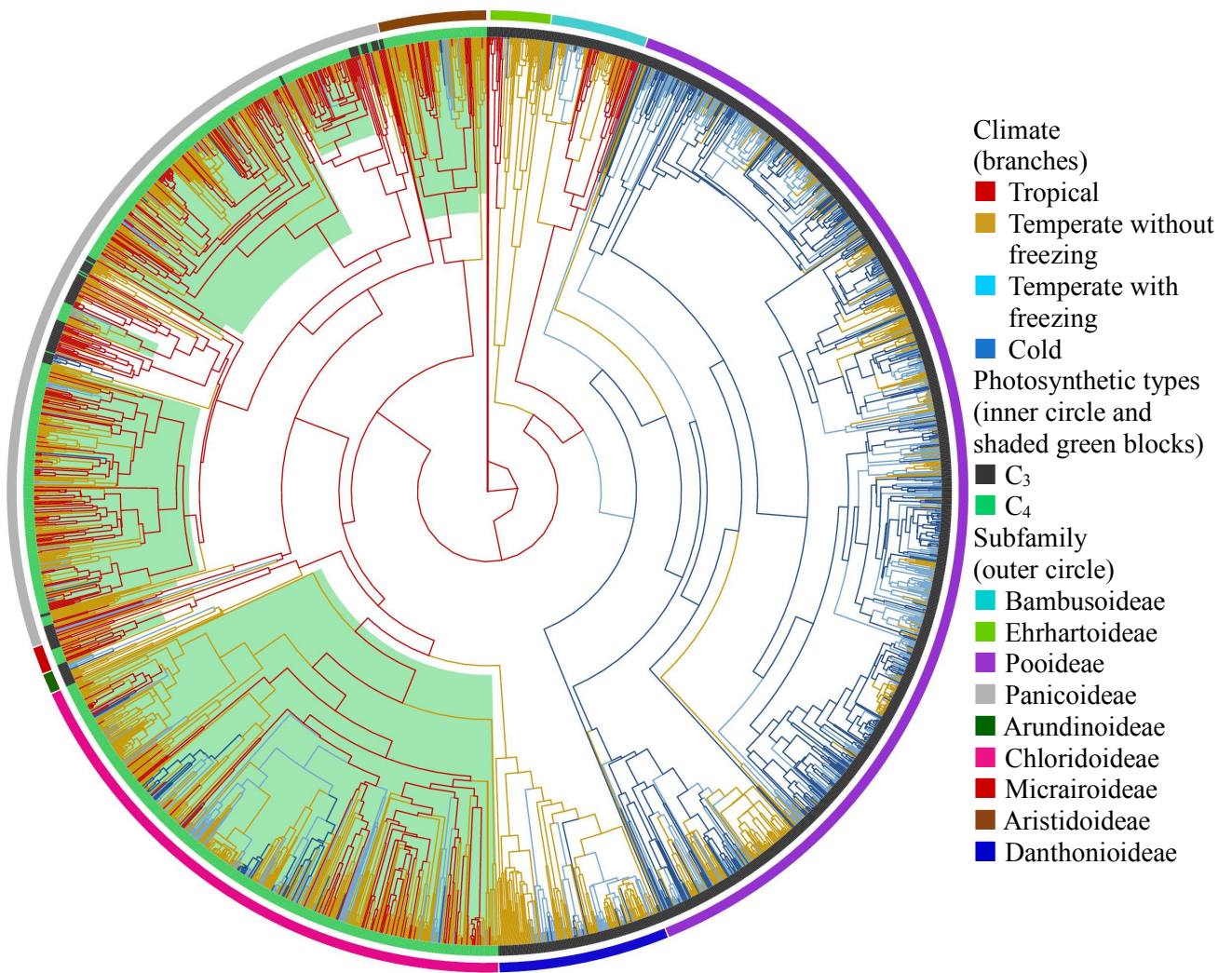
16 and C<sub>4</sub> species in the lowest temperature regimes are indicated.

1 **Table 1** Rates of transitions determined from point estimates of models.

State	Rate	Estimated rates
Tropical vs. Temperate climates and C <sub>3</sub> vs. C <sub>4</sub> photosynthesis		
Transition from tropical to temperate climate		
C <sub>3</sub>	Rate <sub>1→2</sub>	0.02200
C <sub>4</sub>	Rate <sub>5→6</sub>	0.11663
C <sub>4</sub> – C <sub>3</sub>	Rate <sub>5→6</sub> – Rate <sub>1→2</sub>	0.09464***
Transition from temperate to tropical climate		
C <sub>3</sub>	Rate <sub>2→1</sub>	0.00004
C <sub>4</sub>	Rate <sub>6→5</sub>	0.04316
C <sub>4</sub> – C <sub>3</sub>	Rate <sub>6→5</sub> – Rate <sub>2→1</sub>	0.04313***
Transition from C <sub>3</sub> to C <sub>4</sub> photosynthesis		
Tropical	Rate <sub>1→5</sub>	0.00749
Temperate	Rate <sub>2→6</sub>	0.00016
Tropical – Temperate	Rate <sub>1→5</sub> – Rate <sub>2→6</sub>	0.00734***
Temperate = 0	Rate <sub>2→6</sub> – 0	0.00016*
Transition from C <sub>4</sub> to C <sub>3</sub> photosynthesis		
Tropical	Rate <sub>5→1</sub>	0
Temperate	Rate <sub>6→2</sub>	0
Tropical = Temperate = 0	Rate <sub>5→1</sub> + Rate <sub>6→2</sub> – 0	0 <sup>ns</sup>
Temperate climates without freezing vs. with freezing and C <sub>3</sub> vs. C <sub>4</sub> photosynthesis		
Transition from temperate climate without freezing to with freezing		
C <sub>3</sub>	Rate <sub>2→3</sub>	0.01095
C <sub>4</sub>	Rate <sub>6→7</sub>	0.01356
C <sub>4</sub> – C <sub>3</sub>	Rate <sub>6→7</sub> – Rate <sub>2→3</sub>	0.00262 <sup>ns</sup>
Transition from temperate climates with freezing to without freezing		
C <sub>3</sub>	Rate <sub>3→2</sub>	0.03054
C <sub>4</sub>	Rate <sub>7→6</sub>	0.07954
C <sub>4</sub> – C <sub>3</sub>	Rate <sub>7→6</sub> – Rate <sub>3→2</sub>	0.04900***
Transition from C <sub>3</sub> to C <sub>4</sub> photosynthesis		
Non-freezing	Rate <sub>2→6</sub>	0.00355
Freezing	Rate <sub>3→7</sub>	0.00000
Non-freezing – Freezing	Rate <sub>2→6</sub> – Rate <sub>3→7</sub>	0.00355***
Freezing = 0	Rate <sub>3→7</sub> – 0	0 <sup>ns</sup>
Transition from C <sub>4</sub> to C <sub>3</sub> photosynthesis		
Non-freezing	Rate <sub>6→2</sub>	0
Freezing	Rate <sub>7→3</sub>	0
Non-freezing = Freezing = 0	Rate <sub>6→2</sub> + Rate <sub>7→3</sub> – 0	0 <sup>ns</sup>
Temperate vs. Cold climates and C <sub>3</sub> vs. C <sub>4</sub> photosynthesis		
Transition from temperate to cold climates		
C <sub>3</sub>	Rate <sub>3→4</sub>	0.03118
C <sub>4</sub>	Rate <sub>7→8</sub>	0.00421
C <sub>4</sub> – C <sub>3</sub>	Rate <sub>7→8</sub> – Rate <sub>3→4</sub>	-0.02697***
Transition from cold to temperate climates		
C <sub>3</sub>	Rate <sub>4→3</sub>	0.13183
C <sub>4</sub>	Rate <sub>8→7</sub>	0.13628
C <sub>4</sub> – C <sub>3</sub>	Rate <sub>8→7</sub> – Rate <sub>4→3</sub>	0.00445 <sup>ns</sup>
Transition from C <sub>3</sub> to C <sub>4</sub> photosynthesis		
Temperate	Rate <sub>3→7</sub>	0.00233
Cold	Rate <sub>4→8</sub>	0.00000
Temperate – Cold	Rate <sub>3→7</sub> – Rate <sub>4→8</sub>	0.00233*
Cold = 0	Rate <sub>4→8</sub> – 0	0 <sup>ns</sup>
Transition from C <sub>4</sub> to C <sub>3</sub> photosynthesis		
Temperate	Rate <sub>7→3</sub>	0
Cold	Rate <sub>8→4</sub>	0
Temperate = Cold = 0	Rate <sub>7→3</sub> + Rate <sub>8→4</sub> – 0	0 <sup>ns</sup>

2 Asterisk indicates the differences between rates of transitions, \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , <sup>ns</sup> indicates no  
3 differences between rates of transitions

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2 **Figure 3** Maximum likelihood reconstruction of the transitions between climatic regions: tropical,  
 3 temperate without freezing, temperate with freezing and cold climates. Photosynthetic types and  
 4 subfamilies are indicated.



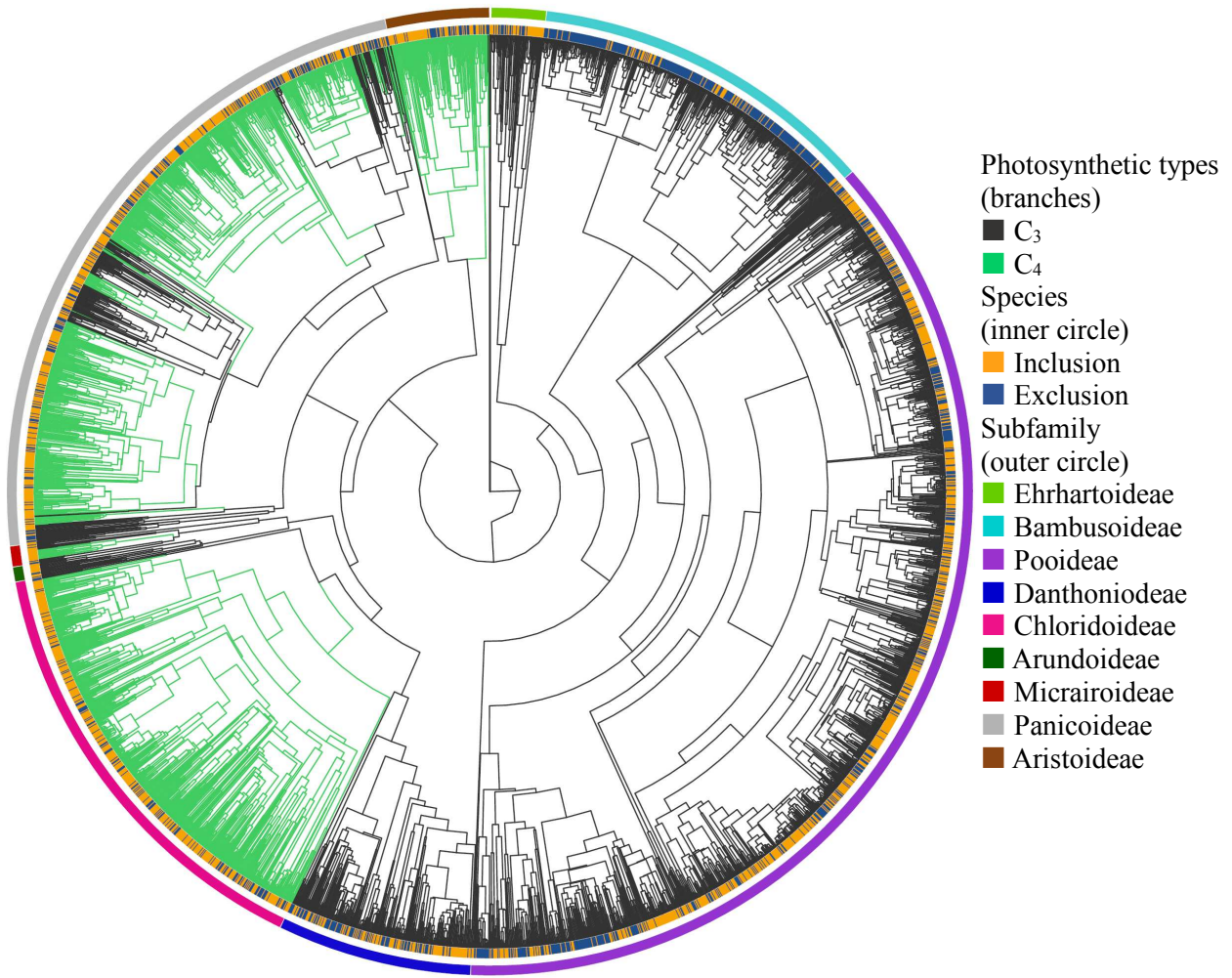
1 **Table 2** Results from phylogenetic generalized least square regression testing for an association  
 2 between photosynthetic pathway and climate, and making statistical comparisons between  
 3 photosynthetic types.

Clade	Variable <sup>1</sup>	C <sub>3</sub>	C <sub>4</sub>	<i>p</i> -value <sup>2</sup>	$\lambda$
All grasses	MTCM max	17.0	17.7	0.5154	0.78
	MTCM min	9.0	8.0	0.5191	0.80
	MTWM max	26.0	27.8	0.0142*	0.69
	MTWM min	20.1	20.8	0.3995	0.81
	Range	15.3	18.3	0.0126*	0.79
PACMAD	MTCM max	17.7	18.3	0.5577	0.68
	MTCM min	9.6	8.4	0.3873	0.72
	MTWM max	26.7	28.4	0.0031**	0.64
	MTWM min	20.3	21.0	0.3748	0.75
	Range	16.2	19.4	0.0057**	0.68

4 <sup>1</sup> MTCM max = 95th mean temperature of the coldest month; MTCM min = 5th mean temperature  
 5 of the coldest month; MTWM max = 95th mean temperature of the warmest month; MTWM min =  
 6 5th mean temperature of the warmest month; Range = difference between MTWM max and MTCM  
 7 min. <sup>2</sup> \* *p* <0.05, \*\* *p* <0.01.

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3 **Figure S1** Phylogenetic tree showing the distribution of species excluded due to lacking occurrence  
4 data.

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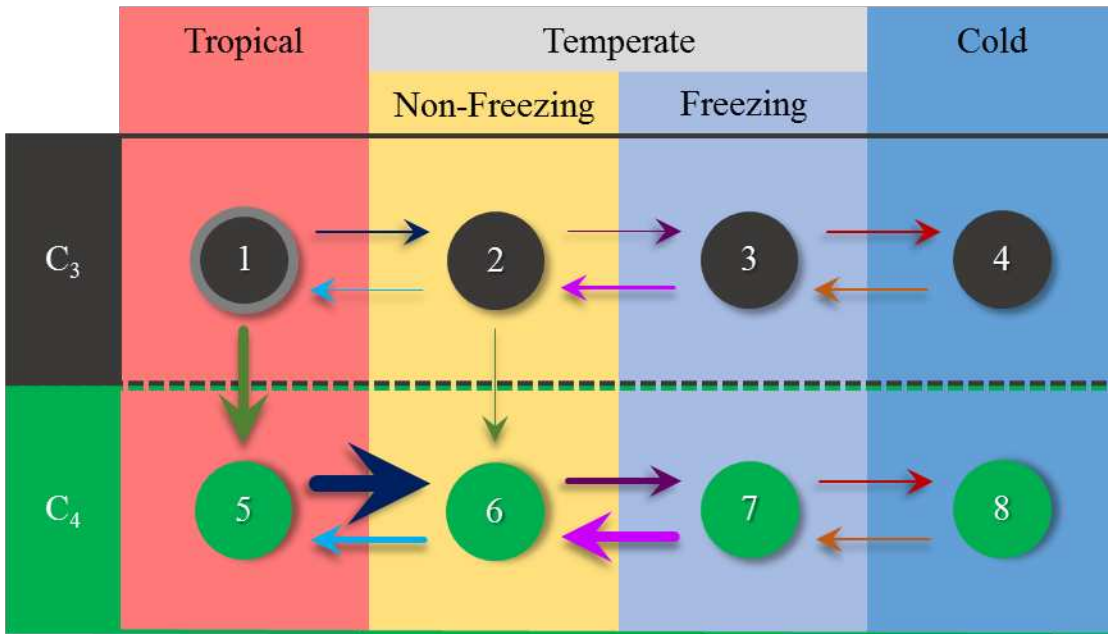
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**Figure S2** Model of coevolution of photosynthetic types and temperature niches in the PACMAD clade. Size of arrows indicate transition rates among climate and photosynthetic types. The most likely ancestral condition is indicated by the grey outer circle.

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**Table S1** Distribution of C<sub>3</sub> and C<sub>4</sub> photosynthesis among climatic types before and after fitting into the phylogenetic tree.

Group	Number of species				Total
	Tropical	Temperate		Cold	
		Non-freezing	Freezing		
Before fitting into phylogenetic tree					
C <sub>3</sub>	210 (5%)	859 (21%)	623 (15%)	475 (11%)	2,167 (52%)
C <sub>4</sub>	710 (17%)	1,097 (26%)	154 (4%)	60 (1%)	2,021 (48%)
Total	920	1,956	777	535	4,188
After fitting into phylogenetic tree					
C <sub>3</sub>	83 (4%)	439 (21%)	370 (17%)	293 (14%)	1,185 (56%)
C <sub>4</sub>	263 (12%)	542 (25%)	105 (5%)	38 (2%)	948 (44%)
Total	346	981	475	331	2,133

1 **Table S2** Distribution of grass subfamilies and C<sub>4</sub> photosynthesis among climatic types

Group	Number of species (Number of C <sub>4</sub> species)			
	Tropical	Temperate		Cold
		Non-freezing	Freezing	
Bambusoideae	18 (0)	37 (0)	12 (0)	3 (0)
Ehrhartoideae	10 (0)	28 (0)	3 (0)	3 (0)
Pooideae	0 (0)	234 (0)	314 (0)	272 (0)
Panicoideae	227 (179)	288 (243)	41 (36)	11 (7)
Arundinoideae	1 (0)	9 (0)	4 (0)	1 (0)
Chloridoideae	57 (57)	244 (244)	64 (64)	29 (29)
Micrairoideae	10 (7)	10 (5)	0 (0)	0 (0)
Aristidoideae	21 (20)	50 (50)	5 (5)	2 (2)
Danthonioideae	0 (0)	81 (0)	32 (0)	10 (0)
Outgroup	2 (0)	0 (0)	0 (0)	0 (0)
Total	346 (263)	981 (542)	475 (105)	331 (38)

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1 **Table S3** Rates of transitions in the PACMAD clade determined from point estimates of models.

State	Rate	Estimated rates
Tropical vs. Temperate climates and C <sub>3</sub> vs. C <sub>4</sub> photosynthesis		
Transition from tropical to temperate climate		
C <sub>3</sub>	Rate <sub>1→2</sub>	0.02355
C <sub>4</sub>	Rate <sub>5→6</sub>	0.11088
C <sub>4</sub> – C <sub>3</sub>	Rate <sub>5→6</sub> – Rate <sub>1→2</sub>	0.08733***
Transition from temperate to tropical climate		
C <sub>3</sub>	Rate <sub>2→1</sub>	0.00104
C <sub>4</sub>	Rate <sub>6→5</sub>	0.03975
C <sub>4</sub> – C <sub>3</sub>	Rate <sub>6→5</sub> – Rate <sub>2→1</sub>	0.03872***
Transition from C <sub>3</sub> to C <sub>4</sub> photosynthesis		
Tropical	Rate <sub>1→5</sub>	0.01161
Temperate	Rate <sub>2→6</sub>	0.00100
Tropical – Temperate	Rate <sub>1→5</sub> – Rate <sub>2→6</sub>	0.01061***
Temperate = 0	Rate <sub>2→6</sub> – 0	0.00100**
Transition from C <sub>4</sub> to C <sub>3</sub> photosynthesis		
Tropical	Rate <sub>5→1</sub>	0
Temperate	Rate <sub>6→2</sub>	0
Tropical = Temperate = 0	Rate <sub>5→1</sub> + Rate <sub>6→2</sub> – 0	0 <sup>ns</sup>
Temperate climates without freezing vs. with freezing and C <sub>3</sub> vs. C <sub>4</sub> photosynthesis		
Transition from temperate climate without freezing to with freezing		
C <sub>3</sub>	Rate <sub>2→3</sub>	0.00510
C <sub>4</sub>	Rate <sub>6→7</sub>	0.01390
C <sub>4</sub> – C <sub>3</sub>	Rate <sub>6→7</sub> – Rate <sub>2→3</sub>	0.00881**
Transition from temperate climates with freezing to without freezing		
C <sub>3</sub>	Rate <sub>3→2</sub>	0.04660
C <sub>4</sub>	Rate <sub>7→6</sub>	0.08201
C <sub>4</sub> – C <sub>3</sub>	Rate <sub>7→6</sub> – Rate <sub>3→2</sub>	0.03541*
Transition from C <sub>3</sub> to C <sub>4</sub> photosynthesis		
Non-freezing	Rate <sub>2→6</sub>	0.00695
Freezing	Rate <sub>3→7</sub>	0.00000
Non-freezing – Freezing	Rate <sub>2→6</sub> – Rate <sub>3→7</sub>	0.00695*
Freezing = 0	Rate <sub>3→7</sub> – 0	0 <sup>ns</sup>
Transition from C <sub>4</sub> to C <sub>3</sub> photosynthesis		
Non-freezing	Rate <sub>6→2</sub>	0
Freezing	Rate <sub>7→3</sub>	0
Non-freezing = Freezing = 0	Rate <sub>6→2</sub> + Rate <sub>7→3</sub> – 0	0 <sup>ns</sup>
Temperate vs. Cold climates and C <sub>3</sub> vs. C <sub>4</sub> photosynthesis		
Transition from temperate to cold climates		
C <sub>3</sub>	Rate <sub>3→4</sub>	0.01196
C <sub>4</sub>	Rate <sub>7→8</sub>	0.00403
C <sub>4</sub> – C <sub>3</sub>	Rate <sub>7→8</sub> – Rate <sub>3→4</sub>	-0.00793 <sup>ns</sup>
Transition from cold to temperate climates		
C <sub>3</sub>	Rate <sub>4→3</sub>	0.24525
C <sub>4</sub>	Rate <sub>8→7</sub>	0.12936
C <sub>4</sub> – C <sub>3</sub>	Rate <sub>8→7</sub> – Rate <sub>4→3</sub>	-0.11590 <sup>ns</sup>
Transition from C <sub>3</sub> to C <sub>4</sub> photosynthesis		
Temperate	Rate <sub>3→7</sub>	0.00596
Cold	Rate <sub>4→8</sub>	0.00000
Temperate – Cold	Rate <sub>3→7</sub> – Rate <sub>4→8</sub>	0.00596 <sup>ns</sup>
Cold = 0	Rate <sub>4→8</sub> – 0	0 <sup>ns</sup>
Transition from C <sub>4</sub> to C <sub>3</sub> photosynthesis		
Temperate	Rate <sub>7→3</sub>	0
Cold	Rate <sub>8→4</sub>	0
Temperate = Cold = 0	Rate <sub>7→3</sub> + Rate <sub>8→4</sub> – 0	0 <sup>ns</sup>

2 Asterisk indicates the differences between rates of transitions, \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , <sup>ns</sup> indicates no  
3 differences between rates of transitions

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