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LETTER

Climate, phylogeny and the ecological distribution of C4 grasses

Erika J. Edwards*,† **and**
Christopher J. Still
Department of Geography and the Institute for Computational Earth System Science University of California at Santa Barbara, Santa Barbara, CA 93106 USA
**Correspondence: E-mail: erika_edwards@brown.edu*
†Present address: Department of Ecology and Evolutionary Biology, Brown University, 80 Waterman St, Box G-W, Providence, RI 02906, USA

Abstract

'C4 photosynthesis' refers to a suite of traits that increase photosynthesis in high light and high temperature environments. Most C4 plants are grasses, which dominate tropical and subtropical grasslands and savannas but are conspicuously absent from cold growing season climates. Physiological attributes of C4 photosynthesis have been invoked to explain C4 grass biogeography; however, the pathway evolved exclusively in grass lineages of tropical origin, suggesting that the prevalence of C4 grasses in warm climates could be due to other traits inherited from their non-C4 ancestors. Here we investigate the relative influences of phylogeny and photosynthetic pathway in determining the ecological distributions of C4 grasses in Hawaii. We find that the restriction of C4 grasses to warmer areas is due largely to their evolutionary history as members of a warm-climate grass clade, but that the pathway does appear to confer a competitive advantage to grasses in more arid environments.

Keywords

Biogeography, C4 photosynthesis, ecological sorting, grassland, photorespiration, phylogenetic niche conservatism, water use efficiency.

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INTRODUCTION

The C4 photosynthetic pathway has evolved an estimated 45 times in terrestrial plants (Sage 2004), and is most prominent in grasses, which account for roughly 25% of global terrestrial primary production (Still *et al.* 2003) and include important crop and weed plants and potential biofuels such as maize, sugarcane, sorghum and switchgrass. The pathway differs from the more common 'C3' form of photosynthesis by concentrating CO_2 around the carbon-fixing enzyme Rubisco. This is accomplished via a series of anatomical and biochemical modifications to the C3 pathway that result in an energetically more expensive system, but one that virtually eliminates photorespiration, which can impose serious limitations to plant growth in high temperature and low CO_2 environments. The C4 pathway also confers a strong competitive advantage to plants in drought-prone areas, as the increased internal CO_2 concentrations allow a lower stomatal conductance for a given rate of photosynthesis (Sage 2004).

Molecular sequence data and phytolith assemblages suggest the C4 pathway first arose in grasses at least 20–30 MYA (Kellogg 1999; Stromberg 2005), which coincides with a general warming trend and falling atmospheric CO_2 levels throughout the Eocene and Oligocene epochs (Keeley & Rundel 2003; Osborne & Beerling 2006). However, C4 grasses did not become abundant until the late Miocene, approximately 5–8 MYA (Cerling *et al.* 1997). Their abrupt rise to ecological dominance has historically been attributed to further declines in atmospheric CO_2 concentrations, although geological evidence now suggests that atmospheric CO_2 levels were, if anything, increasing during this time (Pagani *et al.* 1999; Demicco *et al.* 2003). More recently, the development of expansive late Miocene C4 grasslands around the globe has been attributed to a more complex assortment of factors, including a shift to monsoonal climates, increased aridity and fire, and widespread forest fragmentation (Keeley & Rundel 2005; Osborne & Beerling 2006).

The present-day global distribution of C4 grasses is largely restricted to warmer climates, and strong positive relationships between C4 grass abundance and growing season temperature have been documented at continental scales (Teeri & Stowe 1976; Vogel *et al.* 1978; Hattersley 1983; Vogel *et al.* 1986) and along elevational gradients on tropical mountains across the globe (Chazdon 1978; Tieszen *et al.* 1979; Rundel 1980). Precipitation gradients seem to have much less impact on C3 and C4 grass distributions.

Most investigations relating C4 grass abundance and precipitation have focused on temperate mixed C3/C4 grasslands, where the timing of C4 growth is largely restricted to the hot summers and is thus augmented by increasing summertime rainfall (Hattersley 1983; Vogel *et al.* 1986; Paruelo & Lauenroth 1996; Epstein *et al.* 2002). The advantages of C4 photosynthesis in water-limited environments are thus not considered critical for establishing the distribution patterns of C3 and C4 grasses, and are more commonly invoked to explain the dominance of C4 eudicots in deserts and hypersaline areas (Sage 2004).

Explanations for the strong C3/C4 sorting along temperature gradients have focused on experimental measurements of effective quantum yield, which is a measure of light-use efficiency of leaves under light-limited conditions. In general, the quantum yield of C4 plants is reduced relative to C3 plants due to the increased metabolic costs of the C4 pathway; however, C4 quantum yield is indifferent to rising temperatures whereas C3 quantum yield declines under high leaf temperatures due to increased photorespiration (Ehleringer & Bjorkman 1977; Ehleringer 1978). These temperature responses are also predicted by mechanistic models of C3 and C4 photosynthesis (Collatz *et al.* 1998; Still *et al.* 2003). Under a range of light conditions, from light-limited to light-saturated (e.g., a dense or open grass canopy), carbon assimilation should be greater for C4 grasses compared with C3 grasses at higher temperatures, and vice versa at lower temperatures (Still *et al.* 2003). Because carbon uptake often determines ecological success, the now widely accepted 'crossover temperature hypothesis' was proposed to predict the threshold temperatures at various atmospheric CO_2 concentrations that will determine whether a grassland is dominated by C3 or C4 grasses (Ehleringer 1978; Ehleringer *et al.* 1997). Thus, variation in photosynthetic pathway is largely considered to be *the* driving factor in determining ecological distributions of grass species along temperature gradients. This assumption has important implications for inferring past climates and ecological conditions based on C3 and C4 carbon isotope signatures preserved in paleosols and fossil remains (Cerling *et al.* 1997; Fox & Koch 2004), as well as our understanding of the global carbon cycle (Still *et al.* 2003) and estimating future plant biogeographical and physiological responses to climate change (Collatz *et al.* 1998; Epstein *et al.* 2002; Keeley & Rundel 2003).

However, it is important to consider where and in which lineages C4 photosynthesis evolved. Much progress has been made in resolving phylogenetic relationships among the major grass lineages, and most species belong to either the 'BEP' or 'PACCMAD' clades (Barker *et al.* 2001; Sanchez-Ken *et al.* 2007). The BEP clade includes the bamboos, rice and its relatives, and the Pooideae, a large group of *c.* 3300 species that has radiated extensively in open habitats of cold and temperate regions. The PACCMAD clade consists of *c.*5500 species that are ecologically diverse but largely restricted to open habitats in warmer regions. The C4 pathway is estimated to have evolved at least 11 times in grasses, and always in PACCMAD lineages (Sage 2004). Therefore, an alternative explanation for the strong positive correlation between temperature and C4 grass abundance is that C4 grasses live in warm places largely because they were pre-adapted to these environments, and are absent from the earth's cooler regions due to other traits (e.g. freezing intolerance) rather than limitations imposed by the C4 photosynthetic pathway *per se*. The first major study describing large-scale C4 distribution patterns precisely explained the problem, stating 'due to the complexity of temperature effects on physiology, it must be determined if the low temperature response is a result of the presence of the C4 pathway, or if it is due to other factors related to the apparent tropical origin of these taxa (Teeri & Stowe 1976)'. Others have since alluded to the conundrum (Hattersley 1983; Long 1999) but with the exception of two studies (Taub 2000; Stock *et al.* 2004) there has been no explicit investigation of the role that evolutionary history may play in the current distribution of C4 plants. Invariably, previous studies that have documented C3/C4 species turnover along temperature gradients have also inadvertently been documenting Pooideae/PACCMAD species turnover, due to the overwhelming dominance of Pooideae grasses in cooler climates. To understand the particular influence of photosynthetic pathway variation on plant ecological distribution, it is important to compare closely related C3 and C4 taxa that are likely to be more similar in other functional traits. In grasses, this means comparing the climate preferences of C3 and C4 members of the PACCMAD clade.

To investigate how photosynthetic pathway variation drives climate niche differentiation in grasses, we analysed environmental data from geo-referenced collection localities of 152 species of the Hawaiian grass flora within an explicit phylogenetic framework. Hawaii is well suited to this study: the grass flora is of moderate size but well sampled across the grass phylogeny, with multiple origins of C4 photosynthesis represented; the Hawaiian Islands are isolated and therefore present an easily defined species pool; they are of limited geographical size, but there is considerable climate variation both in temperature and precipitation; and a significant correlation of C4 grass distribution with warm temperatures in Hawaii has been previously documented (Rundel 1980). Furthermore, the Hawaiian grass flora is overwhelmingly non-native: over 75% of the *c.* 200 grass species were recently introduced by humans over the past 100–150 years (Wagner *et al.* 1999). Many of the native species are now rare or extinct, and the most commonly encountered grasses are invasive and found across all the major islands, indicating little to no dispersal limitations upon arrival in the archipelago. Considering that the current grass community in Hawaii has largely assembled very recently (*c.* 100 years ago to the present), we interpret current day grass distributions in Hawaii as mainly the product of ecological sorting processes, where the realized climate space of a given species is the result of intrinsic ecological preferences and relative competitive ability. If photosynthetic pathway is indeed the primary driver of ecological sorting along temperature gradients in Hawaiian grasses, then one would expect to see significant differences in the temperature niche of closely related C3 and C4 PACCMAD lineages.

MATERIALS AND METHODS

The environmental niche data

We utilized the databases of herbarium collections for Poaceae from the Smithsonian Flora of the Hawaiian Islands Website (Wagner *et al.* 2005) and from The Bishop Museum Herbarium (Oahu), which when combined, totalled 10,753 specimens. Of these, 9662 were located on one of the main islands (Hawaii, Maui, Kahoolawe, Lanai, Molokai, Oahu and Kauai) for which we had climate data. Of these, 1028 specimens already had latitude and longitude coordinates associated with them. Of the remainder, 4807 specimen descriptions included altitude data. We found that altitude information was essential in helping to estimate collection locations, and did not attempt to geo-reference any collections that did not include altitude. We initially tried to utilize the automated geo-referencing program Biogeomancer (http://www.biogeomancer.org) but ended up manually assigning coordinates to collection locations (using the Hawaii Atlas and Gazetteer, 1st edition, DeLorme 1999, Yarmouth, ME) because many descriptors used by collectors were present on our maps but not interpretable by Biogeomancer. The manual approach allowed for higher scrutiny of location estimations, which we felt was especially necessary in Hawaii due to extremely steep slopes that result in dramatic microclimate changes within short distances. Of the 4807 specimens with descriptions that included altitude data, we confidently geo-referenced 4040 of them, for a total of 5068 individual collections. Of these, 4087 were identified to the species level and also deemed to be non-duplicate specimens based on description locations, collector numbers and date of collection. For these 4087 points we used ARCGIS 9.1 to extract environmental data (monthly and annual precipitation and mean, maximum and minimum temperatures) from climate grids at a 250 m resolution (Giambelluca *et al.* 1986; T. Giambelluca, unpublished data). Climate data coverage was not complete in that some climate layers contained grid cells with missing data for some collection sites; most of these were due to specimens collected from small islets off the shores of Oahu and Maui. We discarded all points that did not have a complete sampling of climate data, as well as all points for species that were not represented by at least 4 independent collections; this resulted in a final data set of 3595 individual records, spread across 155 species (Table S1, Fig. 1). We used species' mean values for all climate variables in further analyses.

The phylogeny

Poaceae phylogeny has received considerable attention in recent years (Catalan *et al.* 1997; Barker *et al.* 2001; Giussani *et al.* 2001; Hilu & Alice 2001; Mathews *et al.* 2002; Aliscioni *et al.* 2003; Catalan *et al.* 2004; Quintanar *et al.* 2007; Sanchez-Ken *et al.* 2007), and we used the wealth of molecular sequence data stored in the NCBI public archives (http://www.ncbi.nlm.nih.gov/) to reconstruct evolutionary relationships among our study species. We first generated a Poaceae sequence database using the PhyLoTA Browser (http://loco.biosci.arizona.edu/phylota), which retrieves clusters of homologous gene sequences from NCBI for a specified taxon. We then searched this subset of gene clusters for representatives of our Hawaiian taxa at both species and genus levels, and found that the greatest taxon diversity in sequenced grasses was found in seven commonly used gene regions for plant phylogenetics: *rbc*L, *mat*K, *ndh*F, the *trn*L-F intergenic spacer and the *rpo*C2 intron from the chloroplast genome, and the ITS and GBSSI (waxy) regions from the nuclear genome. Among these markers, 85 of our sampled species were represented and an additional 57 species were represented by congeneric taxa. We built initial matrices for each of the seven genes using the software program MUSCLE (Edgar 2004) to align the sequences and the program Gblocks (available at http://molevol.ibmb.csic.es/software.html) to trim the alignment. These initial matrices included multiple sequences of the same region for the same taxon if there was more than one record in the GenBank database. Using the software program SCAFOS (Roure *et al.* 2007), we purged duplicate sequences by selecting only the longest sequence for inclusion in the final matrix. We also used SCaFoS to create chimeric taxa for some genera with multiple species that each had few to one sequence, and then concatenated all regions into one large matrix. This final matrix consisted of 7740 characters and 103 taxa, nine of which were chimeric and 15 of which were 'placeholder' taxa that were con-generic species of our sampled Hawaiian taxa (see Table S2 for text file of GenBank identification numbers for all sequences used in our data matrix).

We performed Bayesian analyses on this matrix using the program BEAST (Drummond & Rambaut 2007). We imple-

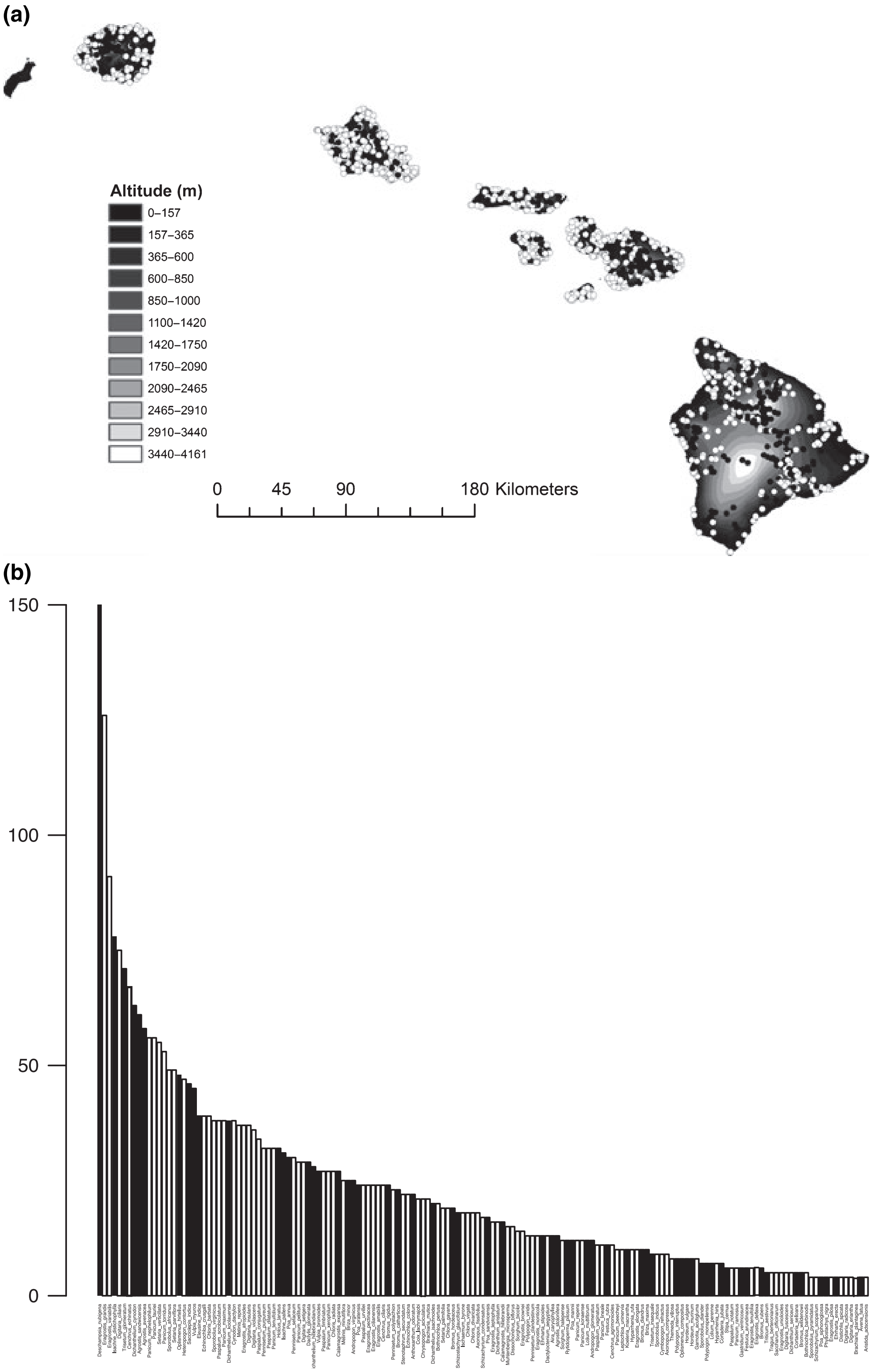

Figure 1 (a) Collection locations for the 3595 geo-referenced specimens used in analyses. Black dots represent C3 species; white dots represent C4 species. The specimens are overlaid on a digital elevation map of the main Hawaiian Islands. (b) Distribution of number of specimen collections per species. Black bars are C3 species, white bars are C4 species.

mented a GTR + G + I model of sequence evolution, with six estimated alpha categories, and enforced a molecular clock with a fixed substitution rate of 1.0. This resulted in a posterior distribution of ultrametric trees, which means that branch lengths are proportional to relative time (but not absolute time, as we did not include calibration points in our priors). We performed eight independent runs of 2 000 000 chains each, sampling every 1000 generations. We viewed statistics of each run using Tracer (Rambaut & Drummond 2007) and, after removing burn-in samples (generally between 200 and 350 000 generations), combined the eight runs into one log file. We generated a majority rule consensus tree of all combined trees (*c.* 16 000), and to this tree added our remaining taxa where they could be unambiguously placed. Genera were assumed to be monophyletic unless specifically demonstrated in other phylogenetic studies (e.g. *Panicum*, *Festuca*, *Vulpia*, *Pennisetum*, *Cenchrus*). Congeneric groups of Hawaiian endemic species (*Isachne*, *Panicum*, *Dicanthelium* and *Eragrostis*) were also each considered to be monophyletic; preliminary phylogenetic studies on *Panicum* and *Dicanthelium* (Aliscioni *et al.* 2003) and *Eragrostis* (E. Edwards, unpublished data) support this assumption. The two Hawaiian endemic species of *Calamagrostis* are purported to be due to two separate colonization events (Wagner *et al.* 1999); however they are the only *Calamagrostis* species in our analysis, so they are grouped here as monophyletic. In cases where taxa were being added to a lineage that contained at least two tips, the new species was added as a polytomy with a branch length identical to its sister taxa (i.e. the additional Hawaiian *Panicum* species). In cases where taxa were being added to a single tip, the new species was attached at the midpoint of the branch (i.e. *Dichanthium sericeum*). Three taxa were discarded for analyses: there was no molecular sequence data available to act as a placeholder at the genus level for *Dissochondrus biflorus* and *Garnotia acutigluma*, and *Panicum maximum* could not confidently be placed in the tree because *Panicum* is not monophyletic and there was no sequence information available for this taxon. Both the final, aligned matrix and the .xml file specifying all parameters of our Bayesian search are available as Appendix S1.

Climate niche analyses

Our full climate dataset consisted of monthly and annual means of precipitation and minimum, maximum and average air temperatures; however, we found high autocorrelation between all temperature (average $R = 0.993$) and precipitation (average $R = 0.941$) variables. We therefore selected mean annual temperature and mean annual precipitation as the parameters to represent the temperature and precipitation axes of climate space.

Divergence width

To calculate divergences in temperature and precipitation between taxa across the tree, we used the AOT module in PHYLOCOM v. 3.1 (Moles *et al.* 2005; Webb *et al.* 2005). This software is unique in that it uses the standard deviation of the values of descendant nodes rather than the absolute difference between descendant nodes [the more commonly used independent contrast (Felsenstein 1985)], which allows the calculation of divergence width at nodes with more than two direct descendants (i.e. at polytomies). Specifically, trait divergence at a given node (i) was calculated as:

$$D_i = \sqrt{\frac{\sum (A_{i,j} - A_i)^2}{V_i}}$$

where A_i is the estimated value of node i calculated as:

$$A_i = \frac{\sum_{j=1}^{V_i} \frac{A_{i,j}}{b_{i,j}}}{\sum_{j=1}^{V_i} \frac{1}{b_{i,j}}}$$

and A_{ij} is the value of descendant j of node i, b_{ij} is the branch length separating nodes i and j, and V_i is the number of descendants of node i.

Branch lengths are an integral component of the calculation of D_i, but there is still great uncertainty about what branch lengths are meaningful for analyses of character change across phylogenies (O'meara *et al.* 2006). Ideally, branches should represent the distance between taxa in terms of time since divergence, which is why we constrained our Bayesian searches to enforce a strict molecular clock. However, this is an estimate that is likely to contain error (Arbogast *et al.* 2002). To test the robustness of our results to uncertainty both in branch lengths and topology, we built a composite phylogeny of the 155 study species using current best opinion of Poaceae phylogeny (Catalan *et al.* 1997; Barker *et al.* 2001; Giussani *et al.* 2001; Hilu & Alice 2001; Mathews *et al.* 2002; Aliscioni *et al.* 2003; Catalan *et al.* 2004; Quintanar *et al.* 2007; Sanchez-Ken *et al.* 2007). Uncertain nodes or nodes that received weak statistical support in previous analyses were left as polytomies. From this starting tree, we generated three individual sets of 333 trees with branch lengths randomly altered with a variance factor ranging from 0.1 to 0.5, using the software package Mesquite (Maddison & Maddison 2006). We then retransformed these 999 trees back into ultrametric trees using non-parametric rate smoothing in the software program TREEEDIT (Rambaut & Charleston 2002). This resulted in a distribution of trees with branch lengths proportional to relative time, but varying widely in the relative ages of the internal nodes. We ran divergence width analyses across all trees and compared the values of D_i with that of our Bayesian consensus tree.

We inferred the C3/C4 character state of internal nodes using maximum likelihood, implementing a two rate Markov model (allowing a different rate between shifts in the C3–C4 direction, and the C4–C3 direction) in Mesquite. With the exception of the node subtending the PACCMAD lineage, reconstruction was unambiguous, with all nodes assigned to one state or the other with a proportional likelihood of at least 90%. After reconstructing character states, we then assigned each node as a particular 'divergence type': (i) a 'C3–C3' node, where all descendant nodes are reconstructed as C3; (ii) a 'C3–C4' node, where there is at least one C3 descendant and one C4 descendant; and (iii) a 'C4–C4' node, where all descendant nodes are constructed as C4. Of the 101 nodes across the tree, 41 were designated as C3–C3 splits, 55 as C4–C4 splits, and five as C3–C4 splits.

To test for significant differences in divergence width between the three categories, we used Tukey's Honest Significant Difference test for unplanned comparisons of means with unequal sample sizes (Sokal & Rohlf 1995), implemented in the statistical package R.

Tests for rate heterogeneity

We used maximum likelihood rate estimators and likelihood ratio tests [implemented in the software program BROWNIE V.2.0 B7 (O'meara *et al.* 2006)] to evaluate the relative lability of temperature and precipitation niche in different parts of the phylogeny. The likelihood ratio test compares likelihood scores of two models of character evolution: a one-rate model, where the rate of character change is constant across the tree, and a two-rate model, where the character is allowed to evolve at a different rate in one part of the tree relative to another. We evaluated the significance of the likelihood scores in two ways. We compared Akaike Information Criterion scores of the two models, using a minimum difference of ten as indication of strong support for a two-rate model. We also evaluated the test statistic $2[(-log\ L_{one\ rate}) - (-log\ L_{two\ rates})]$ using a chi-squared distribution, with a significant ($P < 0.01$) score rejecting the null (one-rate) model.

RESULTS

When analysed across species, clear differences in average temperature ranges between C3 and C4 species emerged,

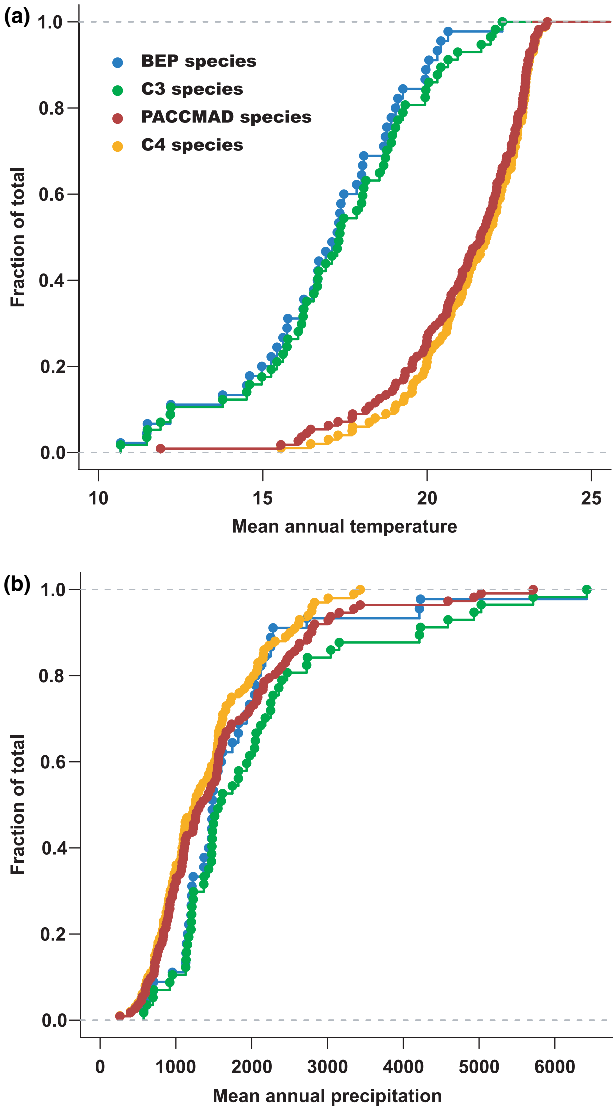

Figure 2 Cumulative distribution curves for temperature and precipitation parameters for geo-referenced specimens of the Hawaiian grass flora represented by at least four collections. Each point represents the mean value of one species. Species are sorted by photosynthetic type (C3 vs. C4) as well as by major grass lineage (BEP vs. PACCMAD). (a) Mean annual temperature, showing that C3–C4 sorting and BEP-PACCMAD sorting mirror one another. (b) Mean annual precipitation, showing little apparent sorting by species or photosynthetic pathway.

Table 1 Summary of climate data by photosynthetic type and major lineage

	No. total species	No. native species	Mean annual temperature (± 1SE)	Mean annual precipitation (± 1SE)
C3 BEP	46	10	17.0 (± 0.40)	1711 (± 142)
C3 PACCMAD	12	6	18.4 (± 0.83)	3361 (± 444)
C4 PACCMAD	97	23	21.3 (± 0.18)	1404 (± 1)

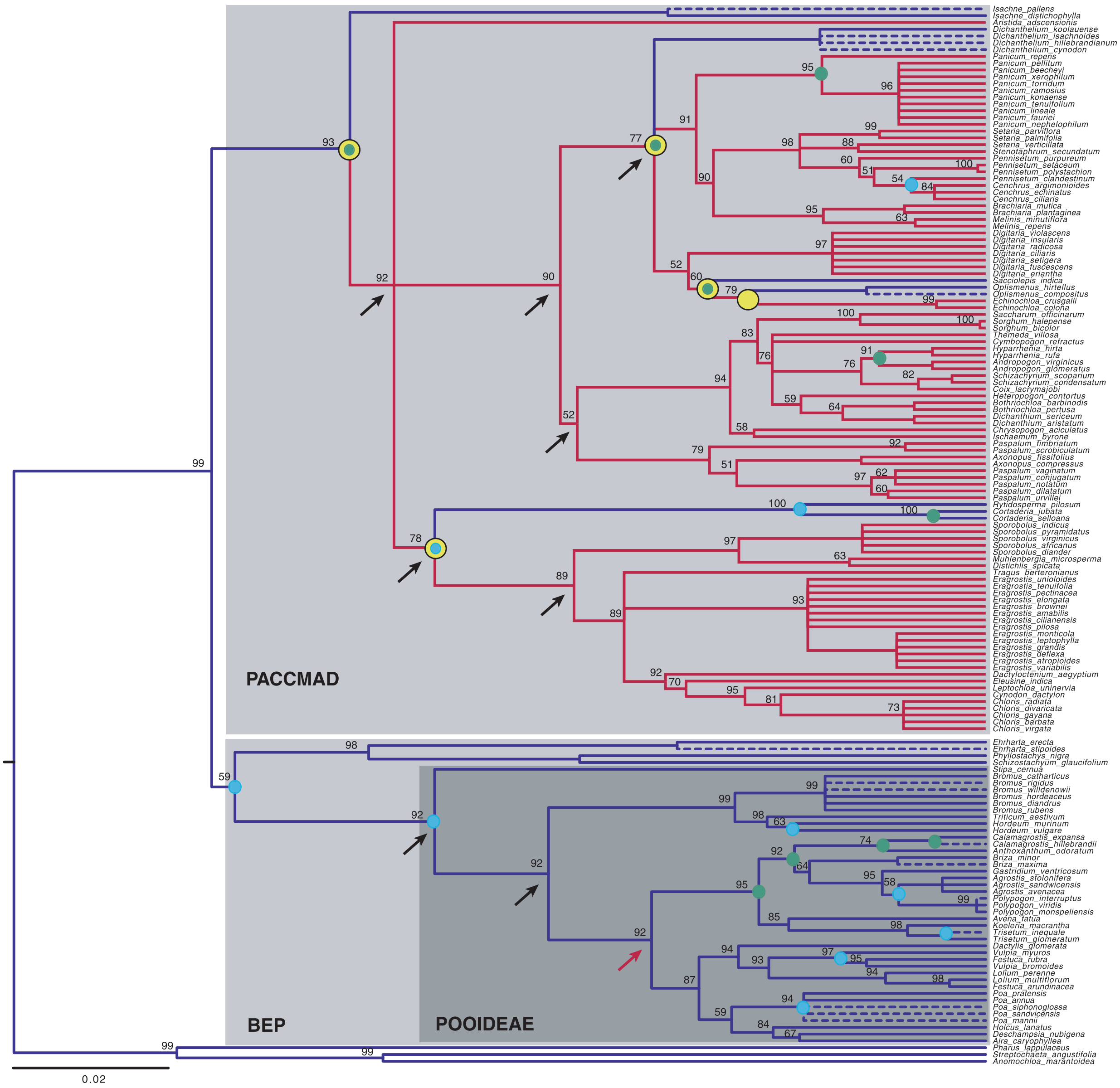

Figure 3 Majority rule consensus of a Bayesian posterior tree distribution, inferred using seven nuclear and chloroplast gene regions and enforcing a strict molecular clock. Numbers above branches are clade posterior probabilities; branch lengths are the mean branch lengths of the posterior distribution, with the scale bar indicating mean substitutions per site. Dashed branches indicate taxa that were added to the tree after phylogenetic analysis. Yellow dots indicate nodes that were characterized as C3/C4 divergences. Green dots represent the 10 largest divergences across the phylogeny in precipitation; blue dots represent the 10 largest divergences in temperature. Arrows indicate regions on the tree that were tested for changes in rate of character change for the two climate variables; the only significant rate change detected was an increase in lability of temperature niche in the representatives of Aveneae/Pooeae, indicated by the red arrow.

though the pattern was indistinguishable from a similar sorting among BEP and PACCMAD species (Table 1, Fig. 2a). From this strong signal we infer that our dataset of geo-referenced herbarium collections captures the same distinct ecological pattern demonstrated in previous elevation transect studies (Chazdon 1978; Tieszen *et al.* 1979; Rundel 1980), and any potential biases inherent in the method of data collection (e.g. plant collectors are more likely to sample near roads or urban centers) do not differentially affect BEP, PACCMAD, C3 or C4 grass distribution estimations.

Analysing climate divergence within a phylogenetic context, however, produced very different results. Our Bayesian analyses produced a relatively well-supported tree

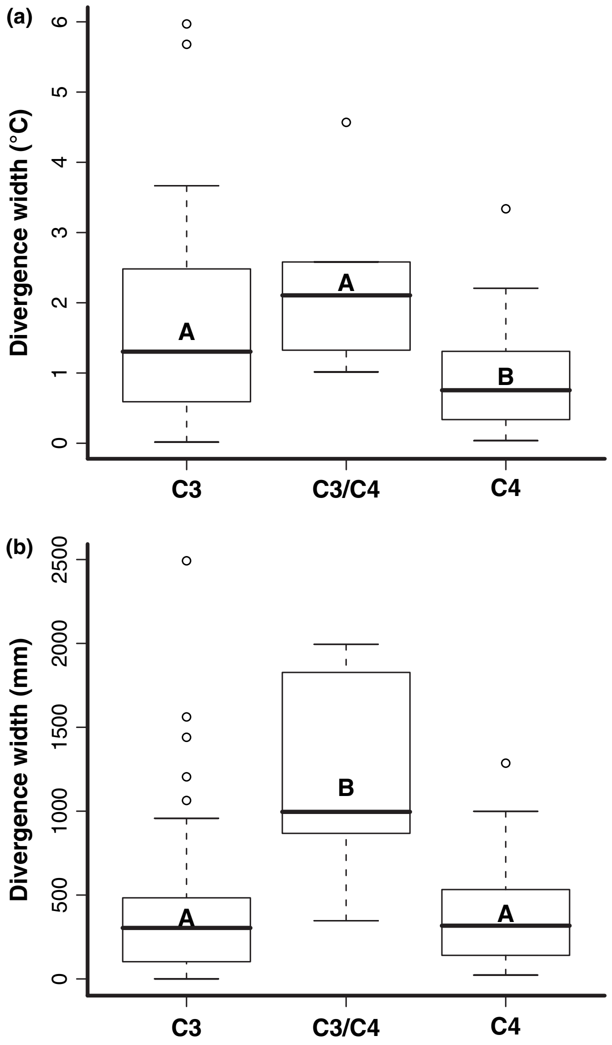

Figure 4 Box plots of divergence width of climate variables, categorized by divergence type. Lines represent median values, 95% confidence intervals are represented by the dashed lines, and dots are outliers. (a) Mean annual temperature. (b) Mean annual precipitation. Values with different letters are significantly different from one another at $P < 0.05$.

topology that is highly congruent with current best opinion of Poaceae phylogeny (Catalan *et al.* 1997; Barker *et al.* 2001; Giussani *et al.* 2001; Hilu & Alice 2001; Mathews *et al.* 2002; Aliscioni *et al.* 2003; Catalan *et al.* 2004; Quintanar *et al.* 2007; Sanchez-Ken *et al.* 2007; Fig. 3). Figure 4a depicts mean divergence width in mean annual temperature for 101 focal nodes across the phylogeny. In general, C4 taxa occupied the narrowest range of temperatures, and divergences between sister C4 lineages were significantly smaller than between C3–C4 ($P = 0.02$) and C3–C3 ($P = 0.002$) splits. However, divergences between C3 and C4 lineages were not significantly greater than those between C3 and C3 ($P = 0.43$). These results were robust to branch length estimation, as average D_i values across our population of trees with randomized branch lengths were similar (C3 vs. C3–C4 $P = 0.59$, C4 vs. C3–C4 $P = 0.04$, C3 vs. C4 $P = 0.003$). From these analyses, we infer that photosynthetic pathway variation is not the major factor driving temperature niche differentiation among the Hawaiian grass taxa. In fact, most of the largest divergences in temperature are captured within the strictly C3 BEP lineage (Fig. 3).

This suggests that in Hawaii BEP grasses may have a less restricted niche space, with regard to temperature, than their PACCMAD counterparts. Using a relative rates test designed to pinpoint locations on a tree where the rate of change of a character increases or decreases relative to another clade (O'meara *et al.* 2006), we examined the relative lability of temperature niche for several key lineages across the phylogeny. We found only one instance of rate change that was strongly supported by both AIC and chi-squared tests: a significant increase in the lineage representing Aveneae and Pooeae clades, within Pooideae (Fig. 4).

The sorting of Hawaiian grass taxa along precipitation gradients provides a strong contrast to the temperature data. When analysed across species, Hawaiian grasses did not appear to sort along a precipitation gradient by either photosynthetic type or major lineage (Table 1, Fig. 2b), echoing many earlier studies that found no impact of precipitation gradients on C3 and C4 grass distributions. However, patterns in mean annual precipitation divergence width were strikingly different, with C3–C4 divergences being significantly larger than C3–C3 or C4–C4 splits (C3 vs. C3–C4, $P = 0.0006$, C4 vs. C3–C4, $P = 0.0001$, Fig. 2b). Similar to the temperature analyses, these results were also robust to branch length variation as estimated by our randomized branch length analysis (C3 vs. C3–C4, $P = 0.0002$, C4 vs. C3–C4, $P = 0.0002$). In all cases, the C4 lineage lived in areas with lower mean annual precipitation than its C3 counterpart. Also in contrast to the temperature data, there was no obvious difference in lability of precipitation niche between photosynthetic types, as there was no significant difference in mean divergence value between C3–C3 and C4–C4 taxa ($P = 0.71$, randomized branch lengths $P = 0.99$) and our relative rates tests did not uncover any substantial differences among lineages. On the whole, C3 PACCMAD species live in much wetter places than either C3 BEP or C4 PACCMAD species (Table 1, Fig. 3).

DISCUSSION

While our data clearly demonstrate that C4 grasses are restricted to warmer regions in Hawaii, this pattern appears to be due in part to the near exclusivity of the BEP lineage (particularly the Pooideae) to cooler climates and the PACCMAD lineage to warmer climates. Within the PACCMAD clade, the striking differences in precipitation niche between closely related C3 and C4 taxa suggest that the higher water-use efficiency conferred by the C4 pathway (Pearcy & Ehleringer 1984) may be playing a large role in the sorting of C3 and C4 grasses along a precipitation gradient. We doubt that this result is unique to Hawaii, as the tendency for C3 PACCMAD grasses to grow in hot, wet environments has been noted in other regions (Ellis *et al.* 1980; Hattersley 1983). Our analyses uncover a remarkably strong trend of C3–C4 grass sorting along a precipitation gradient that mirrors trends in C4 eudicots and that would not have been discovered without an explicit phylogenetic analysis (i.e. as illustrated in Fig. 2b).

We are not arguing that C4 photosynthesis is not adaptive in high temperature environments; at the very least, the fact that C4 photosynthesis evolved only in warm-adapted clades implies that selection for reduced photorespiration is stronger in hot climates. It is also undeniable that C4 PACCMAD species have been more 'successful' than their C3 PACCMAD counterparts, both in terms of lineage diversification and ecological abundance. Furthermore, the significantly lower mean temperature divergence width between C4 taxa compared with the other categories (Fig. 4) suggest that the C4 pathway may be fixing the ecological role of these species as warm climate specialists. This supports the hypothesis that C4 photosynthesis, as a more complicated and energetically expensive photosynthetic system, may limit the range of environments available to a species (Sage & Mckown 2006). These results are also relevant to recent empirical work demonstrating Rubisco-limited growth of C4 species at low temperatures (Kubien *et al.* 2003; Kubien & Sage 2004).

However, our data do suggest that invoking a causal link between C4 photosynthesis and temperature to explain the current biogeographical patterns of C4 grasslands may not be warranted: a shared preference for warmer climates between C3 and C4 PACCMAD taxa means that we would likely be documenting similar patterns of species turnover along temperature gradients even if those taxa had never evolved the C4 pathway. In other words, the global patterns of C3/C4 abundance are really patterns of Pooideae/PACCMAD abundance, and it is not clear to what extent variation in photosynthetic pathway contributes to that trend; in Hawaii, it appears to contribute very little. Our data set only allowed for five independent contrasts between pathway types in PACCMAD grasses, but still presented striking evidence for strong ecological sorting of photosynthetic pathway along a precipitation, but not a temperature, gradient. If this pattern holds in other regions of the globe and with the inclusion of additional taxa, it would substantiate recent criticisms of the 'crossover-temperature hypothesis' as an overly simplistic explanation of C4 grass distributions (Keeley & Rundel 2005; Osborne & Beerling 2006; Osborne 2008). Our data support the view that the widespread expansion of C4 grasses in the late Miocene and into their present day distributions was driven primarily by their higher water use efficiency, which allowed them to exploit high-light, fire-prone areas in a cooler, but increasingly seasonal and arid world (Fox & Koch 2004; Huang *et al.* 2007).

In conclusion, there may be a number of traits currently attributed to the C4 pathway that are, as documented in this study, instead characteristic of PACCMAD grasses in general (Edwards *et al.* 2007). C4 photosynthesis is a fundamental innovation in land plant physiology that has evolved numerous times, and clearly carries with it significant ecological consequence. In grasses, the most common and ecologically important C4 species, it is critical to compare C3 and C4 sister taxa in the PACCMAD clade to determine the physiological and ecological advantages and limitations of C4 photosynthesis. In addition, shifting the comparative focus from traditional plant functional groups to phylogenetic lineages may reveal new relationships between functional traits and climate. Rephrasing the question from 'Why are there no C4 grasses at high latitudes?' to 'Why do the Pooideae, but not PACCMAD grasses, dominate cold-climate regions?' may uncover key differences between the major grass lineages that will better predict both their historical and current distributions, as well as their future responses to climate change.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article:

Table S1 Specimen location and climate data used in analyses.

Table S2 GI numbers for all sequences used in phylogenetic analyses.

Appendix S1 Additional supplemental files – edwards_still.nex, edwards_still.xml.

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