

Oligocene CO₂ Decline Promoted C₄ Photosynthesis in Grasses

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Summary

C₄ photosynthesis is an adaptation derived from the more common C₃ photosynthetic pathway that confers a higher productivity under warm temperature and low atmospheric CO₂ concentration [1, 2]. C₄ evolution has been seen as a consequence of past atmospheric CO₂ decline, such as the abrupt CO₂ fall 32–25 million years ago (Mya) [3–6]. This relationship has never been tested rigorously, mainly because of a lack of accurate estimates of divergence times for the different C₄ lineages [3]. In this study, we inferred a large phylogenetic tree for the grass family and estimated, through Bayesian molecular dating, the ages of the 17 to 18 independent grass C₄ lineages. The first transition from C₃ to C₄ photosynthesis occurred in the Chloridoideae subfamily, 32.0–25.0 Mya. The link between CO₂ decrease and transition to C₄ photosynthesis was tested by a novel maximum likelihood approach. We showed that the model incorporating the atmospheric CO₂ levels was significantly better than the null model, supporting the importance of CO₂ decline on C₄ photosynthesis evolvability. This finding is relevant for understanding the origin of C₄ photosynthesis in grasses, which is one of the most successful ecological and evolutionary innovations in plant history.

Results and Discussion

Number of Grass C₄ Lineages

The evolutionary transition from C₃ to C₄ photosynthesis occurred several times independently in the grass family [2, 7, 8], but the exact number of independent grass C₄ lineages remains unknown [7, 9]. This issue is addressed in this study by inferring a densely sampled phylogenetic tree of grasses and related monocots. The topology obtained was largely congruent with other published phylogenetic trees [7, 9–13] (Figures 1 and 2; see Supplemental Data available online). Based on a parsimony criterion, between 15 and 18 independent transitions from C₃ to C₄ photosynthesis occurred in the grass family, all in the well-recognized PACMAD clade (containing the subfamilies Panicoideae, Arundinoideae, Chloridoideae, Micrairoideae, Aristidoideae, and Danthonioideae [13]).

In three clades (Aristidoideae, Chloridoideae, and the *Paspalum-Ophiochloa* clade; Figure 2), it is equally parsimonious to hypothesize either a single C₄ appearance followed by C₄ to C₃ reversion or two independent appearances. Important clues on the origins of the C₄ trait in these three clades are given by the evolutionary history of genes encoding a C₄ enzyme (phosphoenolpyruvate carboxylase; PEPC [14]). In the core chloridoideae (Chloridoideae without *Centropodia* and *Merxmullera rangei*; Figure 2), the C₄-specific isoform of PEPC evolved through a gene duplication of a non-C₄ PEPC gene followed by neofunctionalization [14]. This duplication occurred after the divergence of *Centropodia*, which evolved a C₄-specific PEPC independently. Because this enzyme is a key component of the C₄ pathway [2], its independent evolutions indicate that the two lineages completed the evolution of the C₄ pathway independently. Similarly, in Aristidoideae, the PEPC from *Aristida* possessed several C₄-specific features absent from the *Stipagrostis* enzyme [14]. These two genera also have a different C₄ anatomy [15], which already suggests that the shared origin of their C₄ photosynthesis pathway could be questionable. Together with C₄ PEPC data, the evidence suggests two origins of the C₄ pathway in Aristidoideae. Concerning the *Paspalum-Ophiochloa* clade, it is not currently possible to determine the number of independent C₄ lineages because of a lack of information concerning C₄ gene evolution in these species.

With Chloridoideae and Aristidoideae both containing two C₄ lineages, there is a minimum of 17 C₄ lineages present in the grass family (Table 1; Figure 2). These clades at least completed the evolution of the C₄ pathway independently, but an earlier appearance of some C₄ characteristics cannot be excluded [2]. Morphological adaptations to drought, such as the reduction of mesophyll cell number and the expansion of bundle sheath size, could have facilitated the later specialization of bundle sheath and mesophyll regions [5]. A high number of gene duplicates could also have helped the recruitment

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Figure 1. Calibrated Phylogenetic Tree of Commelid Species

Branch lengths are proportional to time and scale is given in millions of years. The PACMAD clade is compressed. The uncompressed PACMAD tree is shown in Figure 2. Support values are available in Supplemental Data.

of enzymes for the C₄ function and their rapid adaptation under relaxed selective regimes [16]. If these morphological and genetic preadaptations to C₄ photosynthesis

appeared during the early PACMAD group diversification, this would explain the recurrent C₄ origins in the clade and the absence of C₄ species in other grass subfamilies.

Tempo of C₃/C₄ Photosynthesis Transitions

A Bayesian molecular dating procedure was used to estimate all grass divergence times (Figures 1 and 2). The dates obtained for the major clades are very similar to other published estimates within the Poales [10, 12]. For all C₄ lineages, C₄ photosynthesis could have evolved at any time between the first node attributed to the C₄ type (crown group node) and its ancestral C₃ node (stem group node). These dates are given in Table 1. The only previous estimation of the date of C₄ origin in grasses [17, 18] compared only three C₄ grasses from Panicoideae (*Zea mays*, *Sorghum bicolor*, and *Pennisetum glaucum*), representing two independent C₄ lineages [2, 7] (lineages 7 and 12; Figure 2). Estimates of the ages of all known major grass C₄ lineages are reported for the first time in our study. The inclusion of poorly studied C₄ lineages, such as Aristidoideae, Chloridoideae, and Micrairoideae, allows a better understanding of C₄ photosynthesis evolution in the whole of the grass family. The oldest C₄ origin is found in Chloridoideae, dated between 32.0 (±4.5) Mya and 25.0 (±4.0) Mya (Table 1), which confirms the Oligocene origin of C₄ grasses [18]. The Chloridoideae divergence time indicates the first C₄ photosynthesis origin but most grass C₄ lineages evolved later, from 30 Mya to a recent past (Table 1). These ages overlap with the estimates of C₄ origins in Amaranthaceae and Chenopodiaceae (21.6–14.5 Mya [19]). The concurrent evolution of C₄ photosynthesis in distantly related plant orders (Chenopodiales and Poales) supports the hypothesis of a world-wide environmental change that shifted the physiological balance in favor of C₄ photosynthesis in certain environments.

CO₂ Levels and C₄ Photosynthesis Evolution

It has been hypothesized that C₄ photosynthesis evolved as a response to declining atmospheric CO₂ concentrations [1–6, 20]. During the Eocene, atmospheric CO₂ concentrations are estimated between 1000 and 1500 parts per million by volume (ppmv). These concentrations decreased in several steps during the Oligocene and reached modern levels by the late Oligocene [4]. An abrupt fall of CO₂ levels from more than 1000 ppmv to below 500 ppmv could have favored the initial evolution of C₄ photosynthesis. According to this hypothesis, C₄ plants should have appeared after the CO₂ fall about 32–25 Mya [3, 4]. Our molecular dating gives C₄ lineage ages that are equal or younger to this time interval (Table 1), supporting congruence between CO₂ decline and C₄ photosynthesis evolution timing. However, the relationship remains purely qualitative and linking CO₂ decrease and C₄ evolution requires deeper statistical considerations.

To address this shortcoming, we developed a novel likelihood-based approach. In this approach, the null model assumes that the forward and backward rates of change from C₃ to C₄ photosynthesis are the same along the whole grass evolutionary tree. The alternative model (TDR model) takes the CO₂ effect into account by incorporating different rates of change before and after a given time threshold. The optimum of the TDR model stands at 27.6 Mya (Figure 3), with a log-likelihood of –65.29, which was significantly better than the null model (log-likelihood of –74.83; chi-square = 19.08,

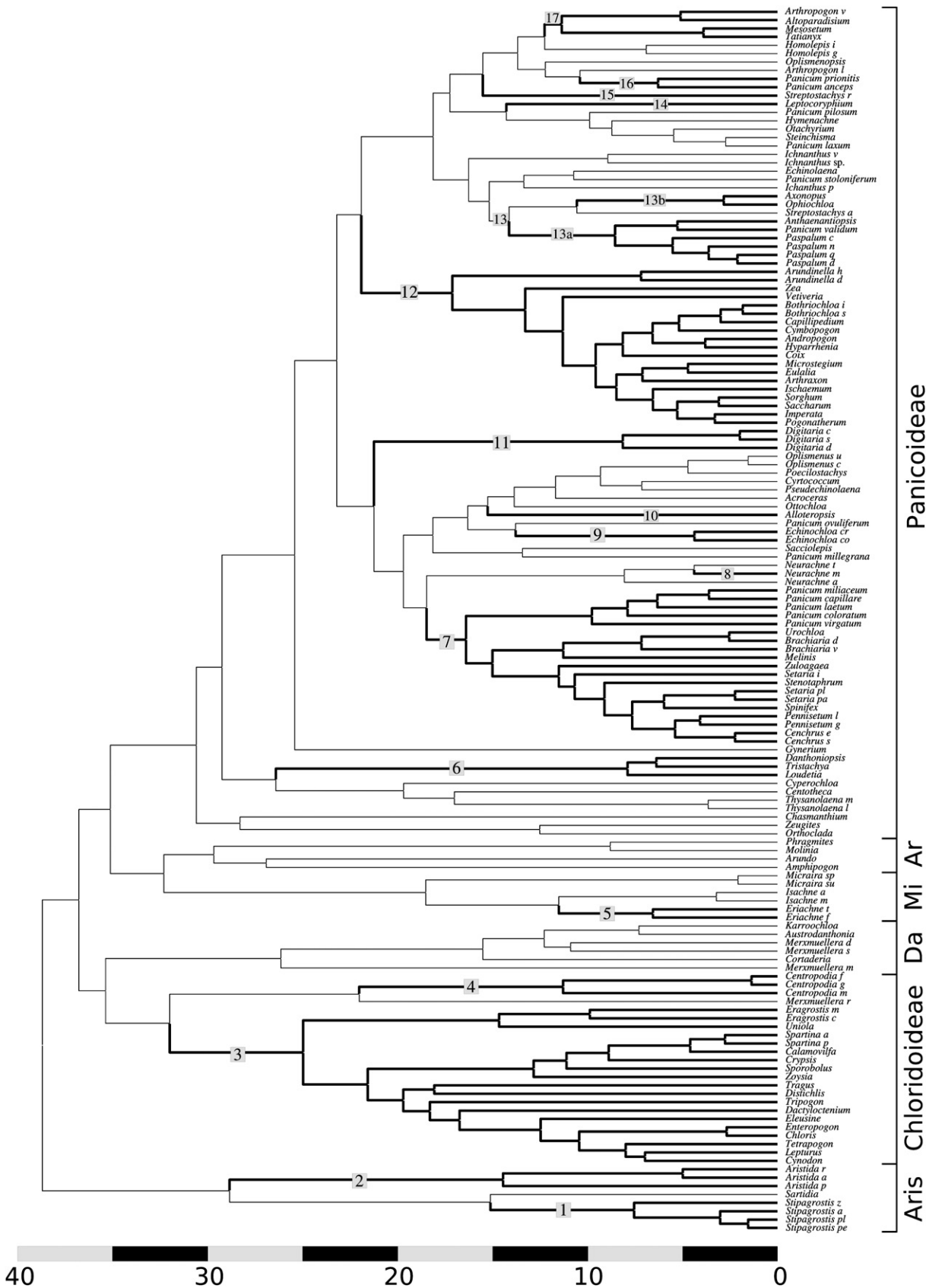
df = 2, p value < 0.0001). For the TDR model, the optimized rate of transition from C₃ to C₄ after 27.6 Mya was 6.58, whereas the back mutation rate was close to zero, indicating that the probability to evolve the C₄ trait from a C₃ state was largely superior to the reversion rate. Before the 27.6 Mya threshold, the modeled transition rate from C₃ to C₄ was null.

These results point to an increased probability to evolve C₄ photosynthesis during the last 27 My, a period when CO₂ levels first reached less than 500 ppmv (Figure 3) [4], the concentration below which C₄ photosynthesis has been proposed to become advantageous in some terrestrial environments [1, 20]. Indeed, C₄ photosynthesis presents an advantage over the C₃ pathway mainly at high temperature, when photorespiration decreases C₃ photosynthesis efficiency. The temperature at which C₄ overcome C₃ plants, the crossover temperature, is correlated with atmospheric CO₂ levels [1, 3]. The Oligocene CO₂ fall decreased the crossover temperature from more than 40°C to 17°C–21°C [3], rendering C₄ photosynthesis advantageous in warm environments and favoring its appearance. The initial step of C₄ evolution is likely to be an enhancement of glycine decarboxylase in the bundle sheath tissue of C₃ plants [21]. This first step is followed by an increased expression of Rubisco in the same compartment and later by the enhancement of PEPC expression in the mesophyll [5]. Mutations modifying the expression patterns of these enzymes would be counterselected under high CO₂ concentrations. However, after the Oligocene fall of CO₂, the same mutations would confer a photosynthetic advantage in warm environments by reducing photorespiration cost. Natural selection might then favor such mutations, initiating the C₄ character evolution. With subsequent acquisitions of other C₄ features, some species would be driven toward the acquisition of an achieved C₄ pathway.

The independent C₄ origins do not all match the Oligocene CO₂ decline, but occurred between 30 Mya and the present (Table 1; Figure 3). The Oligocene CO₂ decline created an environment enabling C₄ evolution, which persisted at least until the Industrial Revolution, with atmospheric CO₂ concentrations constantly lower than 500 ppmv [4]. Under this low CO₂ environment, a plant population encountering ecological conditions favoring C₄ photosynthesis (see below), after either an environmental change or a migration event, might develop the C₄ adaptive suite. However, the appearance of favorable mutations is also necessary to initiate C₄ evolution. The occurrence of mutations, local environmental changes, and migration events, are primarily stochastic and did not occur simultaneously in the different grass species, which could explain the continuous emergence of novel C₄ lineages until recent times.

The Ecological Dominance of C₄ Grasses Occurred Long after They First Evolved

According to our molecular dating, the first C₄ grasses, in Chloridoideae, appeared 32.0–25.0 Mya. However, the first undisputed evidence of C₄ anatomy in the fossil record (in a grass also belonging to Chloridoideae) is much more recent and dates from 12.5 Mya [22]. The absence of older C₄ fossils suggests that C₄ grasses remained rare during a long period after their first



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Table 1. Age of the Independent Grass C₄ Lineages

Lineage	n	Stem Group Age	Crown Group Age
<i>Stipagrostis</i>	1	15.1 (±4.6)	7.5 (±3.1)
<i>Aristida</i>	2	28.8 (±5.2)	14.4 (±4.7)
Core Chloridoideae	3	32.0 (±4.4)	25.0 (±4.0)
<i>Centropodia</i>	4	22.0 (±4.6)	11.3 (±5.5)
<i>Eriachne</i>	5	11.5 (±3.6)	6.6 (±2.8)
Arundinelleae	6	26.4 (±4.4)	7.9 (±3.4)
Main C ₄ Paniceae	7	18.5 (±3.7)	16.4 (±3.6)
<i>Neurachne munroi</i>	8	4.4 (±3.3)	NA
<i>Echinochloa</i>	9	13.8 (±3.5)	4.4 (±2.8)
<i>Alloteropsis semialata</i>	10	15.3 (±3.5)	NA
<i>Digitaria</i>	11	21.2 (±3.9)	8.1 (±3.4)
Andropogoneae	12	21.9 (±3.9)	17.1 (±4.1)
<i>Paspalum</i> clade ^a	13a	14.1 (±3.4)	8.5 (±3.1)
<i>Ophiochloa</i> clade ^a	13b	10.6 (±3.3)	2.8 (±1.9)
<i>Paspalum-Ophiochloa</i> clade ^a	13	15.2 (±3.5)	14.1 (±3.4)
<i>Leptocoryphium lanatum</i>	14	14.3 (±3.5)	NA
<i>Streptostachys ramosa</i>	15	15.5 (±3.5)	NA
<i>Panicum prionitis</i> – <i>P. anceps</i>	16	10.4 (±2.9)	6.3 (±2.7)
<i>Mesosetum</i> clade	17	12.3 (±3.2)	11.3 (±3.0)

Lineage numbers (n) correspond to those on Figure 2. The age of each C₄ lineage is comprised between the stem and crown ages. Each date is given with the associated standard deviation in brackets. When one clade is formed of a single species, NA (not available) is indicated for the crown group age.

^aIt was not possible to state whether C₄ photosynthesis evolved twice in the *Paspalum-Ophiochloa* clade or if a single appearance was followed by a reversal. Consequently, both scenarios are presented (i.e., lineage 13 corresponding to a single appearance whereas 13a and 13b represent the two putative independent C₄ lineages).

transition from C₃ or were found in habitats that were not conducive to fossilization. Carbon isotopes in palaeosols indicate the presence of C₄ plants in North America great plains since the early Miocene (~23 Mya) [23]. However, C₄ plants came to dominate certain terrestrial ecosystems only 8–5 Mya [3, 6, 23, 24], emphasizing the possible minor ecological importance, at a global scale, of C₄ grasses during the first 10–20 My of their evolutionary history [2, 18].

The low atmospheric CO₂ concentration alone is not sufficient for C₄ plants to expand their geographical and ecological range by outcompeting the C₃ plants previously established. A combination of ecological factors is needed to allow the development of a C₄ flora (e.g., high light and warm temperature [25]). C₄ plants are unlikely to colonize wooded habitats, where light at ground levels are generally extremely low and temperature is lower than in open habitats. The ecological rise of C₄ plants corresponds to the emergence of herbaceous plant-dominated biomes, such as savannah [3, 26, 27]. These ecosystems appeared and were maintained by external disturbances such as fire [26, 27], herbivore grazing, and climatic changes [28]. In the newly disturbed environment, C₃ trees were replaced by herbaceous species, and the high light and warm temperature conditions favored C₄ over C₃ taxa [27]. During their

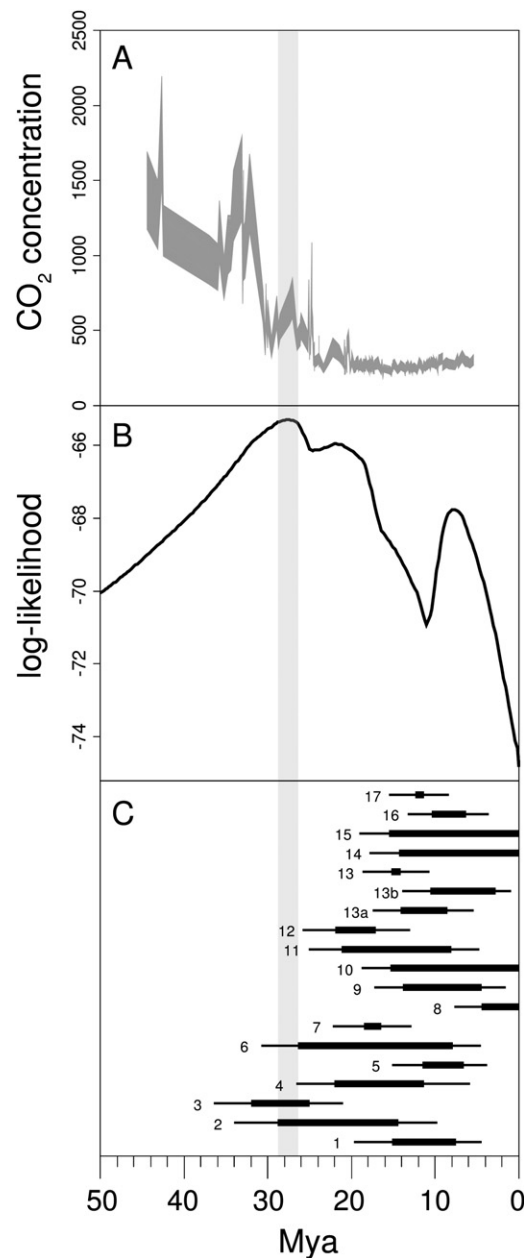


Figure 3. Paleogene CO₂ Levels and C₄ Photosynthesis Evolution (A) CO₂ concentrations (ppmv) during the Paleogene. Redrawn from [4, 32] with permission. The surface of the curve represents maximal and minimal estimates. (B) Likelihood of the TDR model with a threshold varying between 50 Mya and the present. (C) Ages of the different C₄ grass lineages. Thick bars represent the interval between stem and crown group nodes of each C₄ lineage and thin bars give the standard deviations. C₄ lineage numbers correspond to those on Figure 2. The vertical gray bar across the three panels stands at 27.6 Mya, the optimal threshold when the probability of evolving the C₄ pathway increased, according to our models.

Figure 2. Calibrated Phylogenetic Tree of PACMAD Species

C₄ lineages as inferred by parsimony and with our knowledge of C₄ PEPC gene evolution (see Discussion) are in bold. Branch lengths are proportional to time and scale is given in millions of years. Support values are available in Supplemental Data. Aris, Aristidoideae; Da, Danthioideae; Mi, Micraioideae; Ar, Arundinoideae.

early history, C₄ plants could have remained isolated in some extreme and open habitats where they had a competitive advantage over C₃ plants [5] until further environmental changes gave them the opportunity to rise to ecological dominance in many tropical and subtropical biomes.

Concluding Remarks

The calibrated phylogenetic tree presented in this study includes species from all known major grass C₄ lineages and represents the most complete molecular phylogenetic tree currently available for C₄ grasses and their C₃ relatives. The dense sampling and the accurate date estimates give a strong basis for further macro-evolutionary hypotheses testing. Here, we used this phylogenetic information deduced from two plastid markers to address the evolutionary history of the C₄ adaptive suite and to demonstrate the effect of past climatic changes on the evolution of a morphological and biochemical character. This was reached by integrating palaeontological, physiological, and ecological knowledge in a common phylogenetic context.

Our findings supporting the effect of atmospheric CO₂ levels on the evolution of C₄ photosynthesis are important given the current global climatic changes. Beside its influence on climatic variables, increased CO₂ concentration could trigger important ecological changes in major terrestrial ecosystems by affecting the distribution of C₄-dominated biomes and the affiliated flora and fauna. It is thus essential to investigate the ecological factors that are at work in shaping species distributions. In that sense, a comparative approach via phylogenetics will enable comparison between species and will highlight patterns and processes involved in these ecological changes. However, in order to do that, we also need good data on species distribution and we need to further develop the link between GIS modeling and phylogenetics [29]. Integrating phylogenetic trees into nontaxonomic fields of biology is of prime importance nowadays and will continue to be an intense area of research in the future [30].

Experimental Procedures

The sampling strategy, phylogenetic analyses, and molecular dating are described in [Supplemental Data](#).

CO₂ Modeling

Evolution of C₃ and C₄ photosynthetic types was modeled under a maximum-likelihood criterion (MLtree software, <http://www2.unil.ch/phylo/software.html>), with the topology inferred from the two plastid sequence regions. Branch lengths proportional to the mutational rates, rather than to time, were used. The former are more representative of the generation time, as well as the evolutionary rates, which are susceptible to affect the probability to evolve new characters, such as C₄ photosynthesis. The first model used has two parameters and is similar to the Assym2p model implemented in Mesquite [31], with state frequencies estimated from the transition probabilities. The two estimated parameters are the rates of forward and backward transitions between C₃ and C₄. The second model, TDR model (time-dependent rates), was specifically developed for this study and has four parameters. It incorporates the information on past atmospheric CO₂ levels [4] by using two independent Assym2p rate matrices, one before and one after a particular CO₂ threshold is reached. For branches crossing this threshold, two different rate matrices were attributed to the portion of the branch before and after the threshold, respectively. The two models are nested and can be

compared through a likelihood ratio test (LRT). To determine the optimal age of this threshold, given the data, we calculated the likelihood under this model by varying the threshold from 50 Mya to the present.

Supplemental Data

Two figures, two tables, and Experimental Procedures are available at <http://www.current-biology.com/cgi/content/full/18/1/DC1/>.

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Accession Numbers

The accession numbers assigned to the sequences we submitted to GenBank are AM849121–AM849216, AM849330–AM849422, and AM887870–AM887888. See full list in Table S1.