#### SPECIAL TOPIC



# Some like it hot: the physiological ecology of C<sub>4</sub> plant evolution

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### Abstract

The evolution of  $C_4$  photosynthesis requires an intermediate phase where photorespiratory glycine produced in the mesophyll cells must flow to the vascular sheath cells for metabolism by glycine decarboxylase. This glycine flux concentrates photore-spired CO<sub>2</sub> within the sheath cells, allowing it to be efficiently refixed by sheath Rubisco. A modest  $C_4$  biochemical cycle is then upregulated, possibly to support the refixation of photorespired ammonia in sheath cells, with subsequent increases in  $C_4$  metabolism providing incremental benefits until an optimized  $C_4$  pathway is established. 'Why'  $C_4$  photosynthesis evolved is largely explained by ancestral  $C_3$  species exploiting photorespiratory CO<sub>2</sub> to improve carbon gain and thus enhance fitness. While photorespiration depresses  $C_3$  performance, it produces a resource (photorespired CO<sub>2</sub>) that can be exploited to build an evolutionary bridge to  $C_4$  photosynthesis. 'Where'  $C_4$  evolved is indicated by the habitat of species branching near  $C_3$ -to- $C_4$  transitions on phylogenetic trees. Consistent with the photorespiratory bridge hypothesis, transitional species show that the large majority of > 60  $C_4$  lineages arose in hot, dry, and/or saline regions where photorespiratory potential is high. 'When'  $C_4$  evolved has been clarified by molecular clock analyses using phylogenetic data, coupled with isotopic signatures from fossils. Nearly all  $C_4$  lineages arose after 25 Ma when atmospheric CO<sub>2</sub> levels had fallen to near current values. This reduction in CO<sub>2</sub>, coupled with persistent high temperature at low-to-mid-latitudes, met a precondition where photorespiration was elevated, thus facilitating the evolutionary selection pressure that led to  $C_4$  photosynthesis.

Keywords  $C_4$  photosynthesis  $\cdot C_3 - C_4$  intermediate  $\cdot$  Flaveria  $\cdot$  Photorespiration  $\cdot$  Photosynthetic evolution

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### Introduction

Since its discovery a half century ago, C<sub>4</sub> photosynthesis has been a major subject in the life sciences for reasons beyond its contribution to crop productivity. C4 plants produce a quarter of earth's terrestrial primary productivity and, in doing so, contribute to the success of grass and arid shrub biomes at low-to-mid-latitudes (Sage et al. 1999; Still et al. 2003; Edwards et al. 2010). The evolutionary radiation of  $C_4$  life forms is one of the most prolific examples of complex trait evolution with over 60 distinct evolutionary origins (Sage et al. 2011a; Sage 2016). The rise of  $C_4$  plants contributed to the diversification of numerous animal clades; for example, the expansion of  $C_4$  grasslands in Africa facilitated the origin of large grazing guilds (Cerling et al. 1997; Bobe and Behrensmeyer 2004). Expansion of  $C_4$  vegetation also affects climate, leading to drier regional climates, and possibly contributing to global cooling during the late Miocene (Beerling and Osborne 2006). Currently, C<sub>4</sub> photosynthesis supports global societies through efficient crop production that underpins much of our meat and sugar supply (Brown 1999; Sage and Zhu 2011). In the future, scientists are hoping to further exploit  $C_4$  photosynthesis by improving the efficiency and stress tolerance of the existing  $C_4$  crops, creating novel  $C_4$  crops by domesticating wild species, particularly for bioenergy and fiber production, and engineering the pathway into major  $C_3$  crops such as rice and wheat (Jones 2011; von Caemmerer et al. 2012; von Caemmerer and Furbank 2016).

Recognition of the broad significance of C<sub>4</sub> photosynthesis has promoted a longstanding desire to understand the details of C<sub>4</sub> evolution, namely, when, where, why, and how C<sub>4</sub> plants evolved (for example, Downton 1971; Monson and Moore 1989; Rawsthorne 1992). Monson et al. (1984) first proposed a mechanism for C<sub>4</sub> evolution involving the refixation of photorespired  $CO_2$  by Rubisco in  $C_3$ - $C_4$  intermediate leaves. Ehleringer and co-workers followed with a proposal that low atmospheric  $CO_2$  in the late Miocene triggered  $C_4$ evolution (Ehleringer et al. 1991), and discussed potential links between low  $CO_2$  and the mechanisms of  $C_4$  evolution (Ehleringer and Monson 1993; Ehleringer et al. 1997; see also Cerling et al. 1997). Since 1997, efforts to develop a more comprehensive view of C<sub>4</sub> evolution were supported by an increasing database from physiological, structural, and paleo-studies, and notably, phylogenetic analyses. An important synthesis was the contribution of Kellogg (1999), who highlighted the power of phylogenetic analyses to test hypotheses of C<sub>4</sub> evolution, yet noted the limited phylogenetic coverage of the C4 flora at that time. Her discussion of molecular clock approaches to C<sub>4</sub> evolution first indicated an early Miocene origin of C4 Andropogonae grasses at least 17 million years ago. Sage (2001, 2004) drew from this work and then-recent paleoclimate studies to hypothesize a late Oligocene origin for the first C<sub>4</sub> plants 25-30 million years ago, at a time when atmospheric  $CO_2$  levels were dropping from above 1000 ppm to near current values, and the global climate was becoming cooler and drier (Zachos et al. 2001). This hypothesis gained valuable support from molecular clock and paleontology studies that showed synchrony between CO<sub>2</sub> decline, climate deterioration, and the first C<sub>4</sub> origins in grasses (Tipple and Pagani 2007; Christin et al. 2008; Vicentini et al. 2008; Bouchenak-Khelladi et al. 2009) and eudicots (Christin et al. 2011b).

Since 2008, major advances have clarified our understanding of  $C_4$  evolution, with new colleagues and collaborations providing robust assessments of when and why  $C_4$  species arose. One collaboration of note was the NESCent  $C_4$ Grasses Consortium formed in 2009, and the Grass phylogeny Working Group (GPWG) which synthesized a wealth of phylogenetic, ecological, and paleo-research to provide new syntheses of  $C_4$  evolution in the grasses, including clarification of the phylogenetic distribution of the  $C_4$  clades within the difficult grass family (Edwards et al. 2010; Strömberg 2011; GPWG 2012; Osborne et al. 2014). Parallel efforts

with eudicot and sedge phylogenies clarified the diversity of C<sub>4</sub> lineages throughout the angiosperms (McKown et al. 2005; Akhani et al. 2007; Sage et al. 2007; Besnard et al. 2009; Feodorova et al. 2010; Roalson et al. 2010; Christin et al. 2011a), and led to a molecular clock analysis that estimated most C<sub>4</sub> origins occurred over the past 30 Ma (Christin et al. 2011b). Paleoecological work produced a wide range of isotope, microfossil, and proxy data which placed C<sub>4</sub> species on landscapes of the past 20 million years, thereby complimenting the molecular clock estimates (Fox and Koch 2003, 2004; McInerney et al. 2011; Strömberg 2011). Meanwhile, physiological studies identified many new  $C_3$ - $C_4$  intermediate species, which in tandem with phylogenetic work facilitated comparative approaches to test hypotheses of C<sub>4</sub> evolution (Marshall et al. 2007; Voznesenskaya et al. 2007, 2010, 2013; Muhaidat et al. 2011; Ocampo et al. 2010, 2013; Khoshravesh et al. 2012; Sage et al. 2011b, 2013; Lundgren et al. 2016). These advances informed a new generation of models that combined biochemical and evolutionary-landscape theory to predict evolutionary pathways from  $C_3$  to  $C_4$  photosynthesis (Williams et al. 2013; Heckmann et al. 2013; Heckmann 2016). Phylogenetic approaches have also been used to evaluate ecological drivers for C<sub>4</sub> evolution by reconstructing environmental conditions associated with the transition from  $C_3$  ancestors to C<sub>4</sub> lineages, and by assessing relative roles of gene mutation, duplication, and horizontal transfer in assembling the  $C_4$  genome from  $C_3$  predecessors (Monson 2003; Osborne and Freckleton 2009; Edwards and Smith 2010; Gowik and Westhoff 2011; Christin et al. 2012a; Schulze et al. 2013; Lundgren et al. 2015; Olofsson et al. 2016; Dunning et al. 2017; Lundgren and Christin 2017). Altogether, the extensive research on C<sub>4</sub> photosynthesis has produced a deep body of work that can now be used to evaluate various hypotheses concerning  $C_4$  origins.

With this progress in mind, the invitation by the editors of this special issue of Oecologia to honor the contributions of Jim Ehleringer led us to return to the topic of  $C_4$  evolution. C<sub>4</sub> ecophysiology and evolution have been one of Ehleringer's favorite topics, with his seminal work on quantum yields producing classical ecophysiological insights into the environmental conditions favoring the C4 versus C3 pathways, and the varying efficiencies of the multiple C<sub>4</sub> subpathways (Ehleringer and Björkman 1977; Ehleringer 1978; Ehleringer and Pearcy 1983; Ehleringer et al. 1997). In this contribution, our approach is to merge ecophysiological approaches with the latest developments in phylogenetics, paleobiology, and evolutionary studies to further argue that heat, in the context of low atmosphere CO<sub>2</sub>, was the primary driver of C<sub>4</sub> evolution, with drought and salinity commonly, but not always, acting as contributing factors. Similar perspectives of the evolutionary ecology of C<sub>4</sub> photosynthesis have recently been published (Kadereit et al. 2012; Osborne

and Sack 2012; Christin and Osborne 2014; Lundgren and Christin 2017). We add to these efforts by presenting a lineage-by-lineage consideration of the environments of the individual transitions from  $C_3$  to  $C_4$  photosynthesis. A key part of this effort is identification of transitional species that branch near the phylogenetic nodes separating  $C_3$  and  $C_4$  clades within as many phylogenies as possible.

### The value of transitional species

Comparative analyses of species branching near transitional nodes of phylogenetic trees provide a better assessment of selection environments favoring C<sub>4</sub> origins than binary comparisons of fully developed C<sub>3</sub> and C<sub>4</sub> species, which can be skewed by environmental specialization after acquisition of the  $C_4$  pathway (Lundgren et al. 2015). In studying  $C_4$  evolution, it is important to recognize that C<sub>4</sub> photosynthesis is the end point of hundreds to thousands of selection events over many millennia (Gowik et al. 2011). These selection events acted upon physiologies and structural traits that preceded C<sub>4</sub> photosynthesis, beginning with fully functional, ancestral C<sub>3</sub> species. As such, it is the selection upon these pre-C<sub>4</sub> forms that has critical relevance to understanding C<sub>4</sub> evolution, rather than selection upon fully formed C<sub>4</sub> plants in their respective environments. To characterize the critical selection events that modify  $C_3$ , and then  $C_3-C_4$  intermediate phenotypes, into C4 forms, it is necessary to use modern species that are either C3 species branching at nodes immediately sister to  $C_4$  lineages, are themselves  $C_3$ - $C_4$  intermediates branching between C3 and C4 clades, or are the basalbranching C<sub>4</sub> species within a clade. These "transitional" species, and the phylogenetic reconstructions of common ancestors they facilitate, provide the most valuable insights into the ecophysiology of  $C_4$  evolution (Fig. 1).

## Mechanisms of C<sub>4</sub> evolution photorespiratory CO<sub>2</sub> trapping (Fig. 2)

To identify the relevant selection factors in  $C_4$  evolution, it is useful to first understand the physiological and structural traits upon which selection acts. Natural selection is not simply a matter of removing deleterious  $C_3$  traits in novel environments, but must also innovate within the constraints of ancestral  $C_3$  genotypes to assemble novel physiologies that overcome environmental limitations. By understanding the fitness advantage of the traits assembled during the evolutionary process, it is possible to narrow down the ecological setting in which natural selection occurs.

Monson et al. (1984) postulated that the critical development in  $C_4$  evolution is the formation of a mechanism to trap and refix photorespired  $CO_2$ , specifically within BS



**Fig. 1** Schematic of a phylogenetic tree showing a hypothetical branching patterns of the distinct phases of  $C_4$  evolution according to the *Flaveria* model of  $C_4$  origins (Fig. 2). In this treatment,  $C_3$ - $C_4$  intermediacy refers to species with intermediate traits between fully functional  $C_3$  and  $C_4$  plants, specifically, the proto-Kranz (PK),  $C_2$  (Type 1),  $C_2$ +(Type II), and  $C_4$ -like phases of  $C_4$  evolution. Transitional species include the  $C_3$ - $C_4$  intermediates,  $C_3$ +(=enabled  $C_3$ ) species, and the closely related sister  $C_4$  species of the intermediate types, termed "basal" (branching)  $C_4$  clades

cells (Fig. 2b). In this mechanism, the photorespiratory pathway becomes partitioned into two cellular compartments, the mesophyll tissue (M) and bundle sheath (BS), with the photorespiratory release of CO<sub>2</sub> occurring predominantly in the innermost (bundle sheath) compartment of the leaf. This hypothesis was supported in a seminal study by Hylton et al. (1988) and Rawsthorne et al. (1988) in which immunogold labeling with monospecific antibodies showed that the photorespiratory enzyme glycine decarboxylase (GDC), which was responsible for CO<sub>2</sub> release during photorespiration, was expressed exclusively in the BS of Moricandia arvensis, a  $C_3-C_4$  intermediate. In a  $C_3$  congener, *M. moricandioides*, GDC is present in both the M and BS cells. In C<sub>3</sub>-C<sub>4</sub> intermediate species with this type of physiology, glycine formed during photorespiration must diffuse from M to BS cells for metabolism, while the resulting serine returns to the M cells to complete the photorespiratory cycle (Fig. 2b; Monson et al. 1984; Rawsthorne 1992). Under conditions promoting high rates of photorespiration, the CO<sub>2</sub> released in the BS by GDC accumulates to levels two-to-three times that of the mesophyll, thus enhancing the efficiency of Rubisco in nearby chloroplasts (Keerberg et al. 2014). Ammonia is also released by GDC in the BS, creating a need for its rapid re-assimilation to prevent nitrogen (N) loss and ammonia toxicity. This need is proposed to favor the upregulation of a limited C<sub>4</sub> metabolic cycle to provide carbon skeletons to the BS for N re-assimilation, either in tandem with the rise of the glycine shuttle, or shortly after its establishment (Mallmann et al. 2014). Acquisition of a limited  $C_4$  cycle would establish a genetic and biochemical platform that could subsequently facilitate the establishment of a full  $C_4$ 



**Fig. 2** Evolutionary pathway from  $C_3$  to  $C_4$  photosynthesis, showing the main phases of the *Flaveria*-based model of  $C_4$  evolution. Select species corresponding to each phase are listed, beginning with the *Flaveria* clade, followed by eudicot and grass clades. Diagrams outlining the structure and physiology of the a) proto-Kranz, b) type I  $C_2$  with no  $C_4$  cycle, and c)  $C_2$  + (type II  $C_2$ , with a modest  $C_4$  cycle) are

cycle (Heckmann 2016). It is thus not simply the refixation of photorespired  $CO_2$  that enables  $C_4$  evolution, but also a need to efficiently recover photorespired nitrogen.

Because the mechanism for photorespiratory glycine shuttling and CO<sub>2</sub> trapping acts as a CO<sub>2</sub> concentrating mechanism that confers fitness, it has been termed C<sub>2</sub> photosynthesis, after the two carbons in the glycine molecules that shuttle CO<sub>2</sub> into the BS (Sage et al. 2012). In prior years, "C<sub>3</sub>–C<sub>4</sub> intermediate" was used to describe only plants using C<sub>2</sub> photosynthesis, but this use is now discouraged as C<sub>2</sub> metabolism is one of a number of traits in C<sub>3</sub>–C<sub>4</sub> intermediacy. In addition, many species with no close affiliation to C<sub>4</sub> photosynthesis are C<sub>2</sub> plants, demonstrating that C<sub>2</sub> is an adaptive, stable condition. "C<sub>3</sub>–C<sub>4</sub> intermediate" now

shown. Panel d) shows the evolutionary pathway as outlined by the *Flaveria* model of  $C_4$  evolution, with representative species listed that correspond to each phase. See text for explanation of the diagrams. *glc* glycolate, *gly* glycine, *HP* hydroxypyruvate, *ser* serine Modified from Monson and Rawsthorne (2000), Sage et al. (2012), Mallmann et al. (2014)

refers to species with intermediate traits that branch in sister positions between  $C_3$  and  $C_4$  clades in a phylogenetic tree, regardless of whether they exhibit the  $C_2$  trait or not (Fig. 1).

The evidence for a central role of C<sub>2</sub> photosynthesis in C<sub>4</sub> evolution is substantial. Phylogenetically, C<sub>2</sub> metabolism occurs in C<sub>3</sub>–C<sub>4</sub> intermediate taxa that branch in sister positions to both C<sub>3</sub> and C<sub>4</sub> species in over a dozen lineages of angiosperms (Table 1), and all known intermediates exhibit some form of glycine shuttling and photorespiratory CO<sub>2</sub> trapping (Monson and Rawsthorne 2000; Sage et al. 2014; Lundgren et al. 2016). Physiologically, C<sub>2</sub> photosynthesis improves carbon gain over C<sub>3</sub> photosynthesis in warm temperatures (> 25 °C) and reduced atmospheric CO<sub>2</sub> levels (< 400 ppm), demonstrating that it provides fitness

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Family	Number of $C_4$ lineages in clade (if > 1) and lineage name	C <sub>4</sub> genera/ C <sub>4</sub> species number	C <sub>3</sub> -C <sub>4</sub> Intermediate species in genus	Age of lineage, million years (± range)	Center of origin	Climate and soils in center of origin	References and notes
19 Angiosperm families	63						
EUDICOTS (16 families)	35						
Acanthaceae	(1) Blepharis	1/15	> 20 C <sub>2</sub> , 2 C <sub>4</sub> -like, some proto-Kranz	<b>6.8±0.9</b>	Southern Africa	Subtropical, semi-arid-to- arid savannah, desert or shrubland, often disturbed	Vollesen (2000), Fisher et al. (2015)
Aizoaceae	(2) Sesuvioideae	4/30	None known	21±1.0	Southern Africa	Subtropical, semi-arid-to- arid sand, gravel or rocky soils, often disturbed	Bohley et al. (2015)
Amaranthaceae ss	5	10/257					Sage et al. (2007)
	(3) Aerva	1/4	None known	8.5±6.0	North-east Africa/ Arabia	Very hot, arid, rocky slopes and flats	Thiv et al. (2006)
	(4) Alternanthera	1/17	$2-3 C_2$	6.7±0.8	South-central South America, and Car- ibbean basin	Weedy herbs, disturbed ground, often sand	Sanchez-Del Pino et al. (2012)
	(5) Amaranthus	1/90	None known	8.8±6.8	New World, prob- ably Mexico where diversity is high	Weedy species, disturbed ground	
	(6) Gomphrenoids	6/138	None known	7.8±0.8	South-central South America	Semi-arid savannah and disturbed ground	C <sub>3</sub> sisters in Brazilian cerrado; Bena et al. (2017)
	(7) Tidestromia	1/8	None known	8.1±4.9	Chihuahuan desert, Mexico	Arid, sandy, calcareous rocky soil, or gypsum	Sanchez del Pino and Motley (2010)
Asteraceae	4	8/138					
	(8) Flaveria clade A (9) Flaveria clade B	1/1	2 Proto-Kranz, 2–3 C <sub>4</sub> like 9 C <sub>2</sub>	(8) 2.0 (9) < 2.0	Clade A: semi-arid central Mexico; clade B: Texas Gulf coast	Warm, dry climate but often on moist saline soils, usu- ally as a pioneer species	McKown et al. (2005), Lyu et al. (2015)
	(10) Coreopsideae	6/41	None known	NA	Uncertain	Unclear where the C <sub>4</sub> tran- sition occurs	Kellogg (1999)
	(11) Pectis	1/90	None known	$10.2 \pm 1.0$	NW Mexico	Subtropical deserts on sand, gravel or rocky soil	Hansen (2012)
Boraginaceae	<ul><li>(12) Euploca</li><li>(=Heliotropium section</li><li>orthostachys)</li></ul>	1/130	2 Proto-Kranz, 5 C <sub>2</sub>	NA	Semiarid landscapes, SW North America	Drying mudflats, sand- dunes, desert flats. Summer active in warm climates	Hilger and Diane (2003), Sage et al. (2011a), Sage unpub- lished

Table 1 (continued)							
Family	Number of C <sub>4</sub> lineages in clade (if>1) and lineage name	C <sub>4</sub> genera/ C <sub>4</sub> species number	C <sub>3</sub> -C <sub>4</sub> Intermediate species in genus	Age of lineage, million years (± range)	Center of origin	Climate and soils in center of origin	References and notes
Cleomaceae	3 (13) Cleome gynandra (14) C. angustifolia (15) C. oxalidea	1/3 1/1 1/1	1 C <sub>2</sub>	$3.8 \pm 3.2$ $0.4 \pm 0.2$ $6.5^{a}$	<ul><li>(13) Southern Africa</li><li>(14) Afro-Arabia</li><li>(15) W. Australia</li></ul>	Subtropical 13) disturbed 14) arid, rocky and sandy 15) arid flats, rocky or sandy	Feodorova et al. (2010)
Caryophyllaceae Chenopodiaceae	(16) Polycarpaea 9	1/20 40/558	None known	8±2	Probably tropical or NE Africa	Disturbed soils, often sandy	Kool (2012)
4	(17) Arriplex	1/180	None known, but hybrids exist between C <sub>3</sub> and C <sub>4</sub> species	$15.3 \pm 0.4$	SW Asia to Central Asia	Disturbed, often salinized soils	Kadereit et al. (2010)
	(18) Bienertia	1/3	None known	11.7 <sup>a</sup>	SW Asia to Central Asia	Saline flats and saltmarshes	Schiitze et al. (2003), Kapralov et al. (2006)
	(19) Camphorosmeae	2/24	None known	14.9±1.8	SW Asia to Central Asia	Saline soils in semi-arid-to- arid landscapes	Kadereit et al. (2014), Kadereit and Freitag (2011)
	(20) Tecticornia	1/2	None known	6.5 <sup>a</sup>	Australia	Saline flats of semi-arid/ arid Australia	Shepard et al. (2005), Voznesenskaya et al. (2008), Kadereit et al. (2006, 2012)
	(21) Caroxylonae	13/157	None known	$20.9 \pm 2.5$	SW Asia to Central Asia	Halophyte and xerophytic habitat	Akhani et al. (2007), Kadereit et al. (2012)
	(22) Salsoleae s.s.	21/153	Proto-Kranz and C <sub>2</sub>	23.4±1.3	SW Asia to Central Asia	Saline soils in semiarid-to- arid basins of Asia	Akhani et al. (2007), Wen et al. (2010), Kadereit et al. (2012)
	Suaeda (23) sect. Borszczowia (24) sect. Salsina (25) sect. Schoberia	1/40 1 30 9	None known	$7.7^{a}$ 8.4±1.5 5.1±0.6	SW Asia to Central Asia	Saline soils and saltmarshes in arid to semiarid land- scapes	Schütze et al. (2003), Kapralov et al. (2006)
Euphorbiaceae	(26) Euphorbia	1/350	$2 C_2$	$19.3 \pm 4.0$	Texas, NW Mexico	Semi-arid limestone out- crops, caliche	Yang and Berry (2011)
Gisekiaceae	(27) Gisekia	1/1	None known	14.9 <sup>b</sup>	Southern Africa	Disturbed, sandy or rocky soils of hot regions, sum- mer active	Bissinger et al. (2014)
Molluginaceae	(28) Mollugo	1/2	1-2 C <sub>2</sub>	3.8±3.3	SW Africa	Disturbed soil, sandy, often very hot	Christin et al. (2011a)

Table 1 (continued)							
Family	Number of C <sub>4</sub> lineages in clade (if > 1) and lineage name	C <sub>4</sub> genera/ C <sub>4</sub> species number	C <sub>3</sub> -C <sub>4</sub> Intermediate species in genus	Age of lineage, million years (± range)	Center of origin	Climate and soils in center of origin	References and notes
Nyctaginaceae	2 (29) Allionia (30) Boerhavia	3/44 1/2 2/42	None known None known	6.1ª 3.1±0.9	SW North America for both	Arid-to-semi-arid soils, often weedy on sandy, rocky or disturbed soil	Douglas and Manos (2007), Sage et al. (2011a)
Polygonaceae	(31) Calligonum	1/80	None known	$10.6 \pm 9.3$	Central or SW Asia or NE Africa/ Arabia	Arid sand or stony ground	Sage et al. (2011a)
Portulaceae	(32) Portulaca	1/100	1 C <sub>2</sub>	23.0°	New World, likely south-central S. America	Disturbed soils, often in semi-arid settings	Ocampo et al. (2010, 2013), Christin et al. (2014a)
Scrophulariaceae	(33) Anticharis	1/4	1–2 C <sub>2</sub>	NA	Southern Africa	Arid, rocky soil	Khoshravesh et al. (2012)
Zygophyllaceae	(34) Tribulus/Tribulopsis	3/37	None known	NA	Semi-arid Australia or Africa	Semi-arid soils, typically hot, can be sandy, often disturbed	Lauterbach, Kadereit, Ludwig, Sage unpub- lished data
	(35) Tetraena simplex	1/1	None known	8.6 <sup>a</sup>	Southern Africa	Disturbed soils in arid zones	Lauterbach et al. (2016)
MONOCOTS Cyperaceae	28 6	339/6368 16/1322					
	(36) Bulbostylis	2/211	None known	$14.9 \pm 4.7$	Uncertain	Uncertain	Besnard et al. (2009)
	(37) Cyperus	9/757	None known	9.7±1.3	Uncertain	Uncertain	Besnard et al. (2009), Larridon et al. (2013)
	(38) Eleocharis ser. Tenuis- simae	1/10	None known	6 <sup>d</sup>	New World Tropics and subtropics	Wet open areas	Roalson et al. (2010)
	(39) Eleocharis vivipara	1/1	None known; expresses C <sub>3</sub> in submerged leaves	4.4 <sup>a</sup>	Florida	Freshwater marsh in warm- temperate zone, summer active	Roalson et al. (2010)
	(40) Fimbrystylis	3/303	None known	$9.1 \pm 3.2$	Uncertain	Uncertain	Besnard et al. (2009)
	(41) Rhynchospora clade capitatae	1/40	None known	5.8±1.6	New World tropics	Savannah, rock outcrops	Bruhl and Wilson (2007), Thomas et al. (2009)
Poaceae	22	321/5044					GPWG II (2012), Osborne et al. (2014), Soreng et al. 2015
	(42) Aristida	1/288	None known	$16 \pm 3^{e}$	Tropical South America	Open savannah, often on sandy or disturbed areas	GPWG II (2012), Bes- nard et al. (2014)

	Number of $C_4$ lineages in slade (if > 1) and lineage name	C <sub>4</sub> genera/ C <sub>4</sub> species number	C <sub>3</sub> -C <sub>4</sub> Intermediate species in genus	Age of lineage, million years (± range)	Center of origin	Climate and soils in center of origin	References and notes
-	(43) Stipagrostis	1/56	None known	15±3°	Southern Africa	Semi-arid steppe, dry savannah, sandy soils.	Cerros-Tlatilpa et al. (2011), Besnard et al. (2014)
-	(44) Core Chloridoideae	145/1596	None known	$28.5 \pm 3.5$	Probably southern Africa	Dry sandy or rocky soils	Peterson et al. (2011)
-	(45) <i>Centropodia</i>	1/4	None known	$16.1 \pm 5.9$	Southern Africa	Dry sandy soils, arid to semiarid	Peterson et al. (2011)
-	(46) Eriachne	2/50	None known	9.0±2.5	Australia	Dry open landscapes, often sandy	GPWG II (2012)
-	(47) Tristachyideae	8/87	None known	NA	Central Africa or Central South America	Savannas	GPWG II (2012)
-	(48) Andropogonae	85/1228	None known	$19.5 \pm 2.5$	Uncertain	Uncertain	GPWG II (2012)
-	(49) Reynaudia	1/1	None known	NA	Caribbean Islands	Wet savannas	GPWG II (2012)
-	(50) Axonopus	3/90	None known	7.7±5.8	Central South America, Brazil	Sandy soils, rocky savannah	López and Morrone (2012)
-	(51) Paspalum	9/379	None known	$11.3 \pm 0.6$	South America	Subtropical-to-tropical savanna	GPWG II (2012)
-	(52) Anthaenantia	2/4	None known	7.2	South America	Dry savanna, sandy soils, often within open wood- lands	GPWG II (2012)
-	(53) Arthropoginae/ <i>Mesosetum</i> clade	6/35	One proto-Kranz, 3 C <sub>2</sub>	$11.8 \pm 0.5$	Tropical South America	Savannah	GPWG II (2012)
-	(54) Arthropoginae/ Onchorachis clade	1/2	None known	7.8	Semi-arid Brazil	Savannah, rocky soils	GPWG II (2012)
-	(55) Arthropoginae/ <i>Colaeteania</i> clade	1/7	None known	<b>8.4±2.0</b>	SE Brazil	Fire-prone savannah	GPWG II (2012)
-	(56) Anthephorinae	8/286	None known	14.7±6.5	Central to southern Africa, Tanzania	Sandy or laterite soils, grassland, savannah, or open woodland	GPWG II (2012)
-	(57) Echinochloa	1/35	None known	$9.1 \pm 4.8$	Uncertain	Uncertain	GPWG II (2012)
	<ul><li>(58) Neurachne munroi</li><li>(59) Neurachne muelleri</li></ul>	58) 1/1 59) 1/1	Two C <sub>2</sub> and one proto-Kranz	58)<4 59)<4	Subtropical arid Australia	Sandy or rocky soils, often shallow	Christin et al. (2012b)
-	(60) MPC (Melinidinae/ Panicinae/Cenchrinae)	43/889	None known	$17.5 \pm 1.1$	Uncertain	Uncertain	GPWG II (2012), Washburn et al. (2015)

 Table 1 (continued)

 Family

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Table 1 (continued)							
Family	Number of C <sub>4</sub> lineages in clade (if>1) and lineage name	C <sub>4</sub> genera/ C <sub>4</sub> species number	C <sub>3</sub> -C <sub>4</sub> Intermediate species in genus	Age of lineage, million years (± range)	Center of origin	Climate and soils in center of origin	References and notes
	<ul> <li>(61) Alloteropsis cimicina clade</li> <li>(62) A, semialata</li> <li>(63) A. angusta</li> </ul>	1/3 1 1	C <sub>2</sub> and C <sub>4</sub> -like populations in <i>Allotero-</i> pisis semialata	61) 12.0±2.5 62) < 3.0 63) < 7	Southern tropical Africa, Zambia and Tanzania	Seasonally dry savannah and woodlands, frequent fire	GPWG II (2012), Lun- dgren et al. (2015, 2016), Olofsson et al. (2016)
<i>sect</i> section, <i>ser</i> . seric <sup>a</sup> Only stem node ages <sup>b</sup> Crown age used due	es, s.s. sensu stricto s shown as crown age not avail t to high clade diversification	able					

<sup>1</sup>Assumed to be 60% of 10.5 Ma stem age in Christin et al. (2011b)

(2010) and Besnard et al. (2014)

<sup>2</sup>After Roalson et al.

<sup>2</sup>Approximation given in text of Ocampo et al. (2013)



**Fig. 3** Representative responses of net CO<sub>2</sub> assimilation rate to intercellular CO<sub>2</sub> concentration for five *Flaveria* species representing the distinct phases of C<sub>4</sub> evolution outlined in Fig. 2. The species are C<sub>3</sub>—*F. cronquistii*, proto-Kranz (PK)—*F. robusta*; C<sub>2</sub>—*F. angustifolia*; C<sub>2</sub>+(Type II intermediate)—*F. floridana*; C<sub>4</sub>-like—*F. brownii*; C<sub>4</sub>—*F. campestris*. Arrows indicate the intercellular CO<sub>2</sub> concentration (C<sub>1</sub>) corresponding to an ambient CO<sub>2</sub> concentration of either 400 ppm (current value, grey arrows) or 300 ppm (black arrows). Curves shown are representative of four to six curves per species, measured with a Li–Cor 6400 gas exchange machine at 33 °C and 1800 µmol photons m<sup>-2</sup> s<sup>-1</sup> on greenhouse-grown plants. To indicate the variance in the data, mean ± SE are shown for the C<sub>3</sub> and C<sub>4</sub> species

advantages that can stabilize the trait, thereby allowing it to become a platform for further evolutionary modification (Fig. 3; Monson and Jaeger 1991; Monson and Rawsthorne 2000; Vogan and Sage 2012). Theoretically, biochemical models of  $C_3$  and  $C_2$  photosynthesis have been incorporated into evolutionary landscape models to show how the acquisition of C<sub>2</sub> photosynthesis enhances fitness by improving carbon gain, while allowing for the gradual acquisition of C<sub>4</sub> biochemistry (von Caemmerer 1989; Heckmann et al. 2013; Heckmann 2016). The formation of the  $C_2$  mechanism also establishes the anatomical structure in which the  $C_4$ pathway can be assembled (McKown and Dengler 2007; Muhaidat et al. 2011; Sage et al. 2014). Key structural traits that arise with C<sub>2</sub> photosynthesis include Kranz-like BS cells with higher vein density. Organelle volume is enhanced in BS cells of  $C_2$  species over that of  $C_3$  species, and higher capacity trafficking networks appear in C2 species to rapidly transport metabolites between BS and mesophyll cells (Brown et al. 1983; Holaday et al. 1984; Monson and Rawsthorne 2000; Sage et al. 2012; Voznesenskaya et al. 2007, 2010, 2013; Khoshravesh et al. 2016).

The realization that  $C_2$  photosynthesis plays a central role in  $C_4$  evolution allowed researchers to separately address the evolutionary transition from  $C_3$  to  $C_2$  photosynthesis, versus the acquisition of the  $C_4$  pathway from  $C_2$  ancestors (Edwards and Ku 1987). A simplified scheme that summarizes the key steps in a leading model is shown

in Fig. 2, with representative species for each major stage. This model has been referred to the Flaveria model, to highlight that it is heavily based on Flaveria research and to recognize the possibility that other  $C_3-C_4$  lineages may have followed different paths of evolutionary change (Schüssler et al. 2017). In the Flaveria model (Fig. 2), the starting point is a full  $C_3$  phenotype, where the photorespiratory cycle operates completely within single M cells, and metabolites cycle between adjacent chloroplasts, mitochondria, and peroxisomes along the M cell periphery facing intercellular air spaces. Bundle sheath cells in  $C_3$  plants are typically small in cross section, with a few organelles, and veins are usually over four M cells apart (Hattersley and Watson 1975; Dengler and Nelson 1999; Khoshravesh et al. 2016). Next, in the " $C_3$  enabled" stage (termed the  $C_3$  + stage for short), certain  $C_3$  taxa acquire features which predispose, or enable, the initiation of  $C_2$ evolution. These include duplicated genes, which allow for neofunctionalization of gene copies, more prominent BS cells with more organelles, and reduced distance between veins (Monson 2003; Muhaidat et al. 2011; Christin et al. 2013; Sage et al. 2011b, 2013, 2014; Williams et al. 2013; Voznesenskaya et al. 2007, 2010, 2013). In the BS cells of  $C_3$  + species, the organelles are usually positioned along the outer wall opposite the intercellular air spaces, in a pattern typical of C<sub>3</sub> M cells (Muhaidat et al. 2011; Sage et al. 2013, 2014). These BS cells are presumed to be more photosynthetically active than in a typical C<sub>3</sub> plant, and hence are "physiologically activated" (Schulze et al. 2013). Activated BS and close vein spacing are considered enabling traits, because they can facilitate a faster flux of metabolites to the BS, and have greater biochemical capacity to metabolize photorespiratory metabolites which may overflow from the M cells (Sage et al. 2012; Christin et al. 2013). The  $C_3$  + phenotype has been observed in the eudicot clades Flaveria, Euphorbia, and Heliotropium, and in the PACMAD grasses (Muhaidat et al. 2011; Sage et al. 2011b, 2013; Christin et al. 2013). Most of these enabled C<sub>3</sub> species are from hot, drought-prone environments, particularly in the eudicot clades. In such environments, greater vein density and enlarged, physiologically activated BS could be a response to high evaporative demand, as the higher vein density could improve water flux to individual M cells, while an activated BS could photosynthetically compensate for loss of M tissue that results from additional vascular tissue and its associated sheath (Sage 2004; Osborne and Sack 2012; Griffiths et al. 2013). In hot environments, enlarged BS may also act as a hydraulic buffer to provide water during transpirational surges, and to protect the vasculature from cavitation during high transpiration (Sage 2004; Osborne and Sack 2012). In this manner, the high evaporative demand associated with hot,

dry regions could establish the foundation that enables subsequent photorespiratory glycine flux.

The first distinct phase in C<sub>2</sub> evolution has been termed proto-Kranz (Fig. 2a). Proto-Kranz species are largely C<sub>3</sub> in function, with slightly enlarged, activated BS cells, and greater vein density (Sage et al. 2014). The key feature of the proto-Kranz phase is that mitochondria have been localized to the inner BS wall, against the vasculature (Muhaidat et al. 2011; Sage et al. 2013; Voznesenskaya et al. 2013; Khoshravesh et al. 2016; Schüssler et al. 2017). Why this occurs is uncertain; but, it has been observed in species that are close sisters to C<sub>2</sub> species in the eudicot clades Heliotropium, Flaveria, Salsola, and the monocot clades Homolepis and Steinchisma (Muhaidat et al. 2011; Sage et al. 2013; Khoshravesh et al. 2016; Mendonça 2017; Schüssler et al. 2017). Once in place, centripetally positioned mitochondria would force all photorespiratory glycine arising from centrifugal chloroplasts to migrate to the inner BS, since glycine decarboxylase (GDC) is mitochondria-specific (Fig. 2a). The resulting release of photorespired CO2 in the inner BS would create local zones of CO<sub>2</sub> enrichment that would improve Rubisco carboxylation efficiency in adjacent chloroplasts, which slightly enhances photosynthesis at low  $CO_2$  (Fig. 3). The proto-Kranz phase thus represents the establishment of a BS-specific, single-cell loop to trap and refix photorespired  $CO_2$ . It is also conceivable that, in hot conditions, excess photorespiratory glycine produced in the M cells could overflow into the BS, forming a weak, two-celled C<sub>2</sub> mechanism (Sage et al. 2012). The benefits of this additional  $CO_2$  could favor mutations in regulatory genes that further increase GDC investment in BS cells, which would then set the stage for GDC reduction in M cells by allowing BS cells to meet the GDC needs of both M and BS tissues.

To evolve from proto-Kranz to full C<sub>2</sub> photosynthesis requires substantial reduction of GDC expression in the M cells, which in Flaveria species resulted from the pseudofunctionalization of a GDC-P subunit gene expressed in M cells, while a second, BS-specific copy remains functional (Schulze et al. 2013). This loss of GDC in M cells appears gradual, as indicated by weaker C<sub>2</sub> species such as F. sonorensis and F. angustifolia which show some GDC in M cells and have a photosynthetic CO<sub>2</sub> compensation point between C<sub>3</sub> and typical C<sub>2</sub> species (Sage et al. 2013; Schulze et al. 2013). Coupled with the loss of GDC in M cells is an accumulation of mitochondria and chloroplasts in BS cells, which help to increase GDC capacity in the BS (Muhaidat et al. 2011; Sage et al. 2013). In full C<sub>2</sub> species, most organelles are centripetally placed, forming a rank of mitochondria at the inner cell periphery with chloroplasts either layered over them or intermixed within the mitochondrial rank (Holaday et al. 1984; Brown et al. 1983; Monson and Rawsthorne 2000; Voznesenskaya et al. 2007, 2010, 2013; Sage et al. 2014; Schüssler et al. 2017). This arrangement enables chloroplasts to rapidly reassimilate  $CO_2$  leaving the mitochondria (Hylton et al. 1988; Rawsthorne et al. 1988; Rawsthorne 1992). When photorespiration is high,  $CO_2$  levels within this inner BS are enhanced two-to-threefold over mesophyll levels, boosting photosynthesis below 400 ppm by 30% or more (Fig. 3; Ku et al. 1991; Vogan and Sage 2012; Keerberg et al. 2014).

To evolve from  $C_2$  to  $C_4$  photosynthesis, expression of the enzymes of the C<sub>4</sub> cycle must be upregulated in a coordinated manner to enable metabolic integration with the existing metabolism. Until recently, the selection benefit favoring the initial upregulation of the  $C_4$  cycle was unclear. To resolve this uncertainty, Mallmann et al. (2014) hypothesized that upregulation of the  $C_4$  cycle occurs to provide carbon skeletons to the BS to enable rapid re-assimilation of photorespired ammonium (Fig. 2c). The CO<sub>2</sub> released by the C<sub>4</sub> cycle could also be quickly refixed by BS chloroplasts. The fitness benefits arising from this incipient  $C_4$ metabolism could facilitate further  $C_4$  cycle enhancement, eventually leading to a condition where the C4 cycle dominates carbon flux to the BS (Heckmann et al. 2013; Mallmann et al. 2014). Species with a modest  $C_4$  cycle have been termed Type II C<sub>3</sub>-C<sub>4</sub> intermediates (type II C<sub>2</sub> species, or for simplicity,  $C_2$  + species) to distinguish them from Type I intermediates which lack a C<sub>4</sub> cycle (Edwards and Ku 1987). Competition between strong C<sub>3</sub> and C<sub>4</sub> cycles in M cells would be energetically expensive, so presumably a mutation that weakens C<sub>3</sub> expression in the M cells hastens the formation of a dominant C<sub>4</sub> cycle (Monson et al. 1986; Monson and Rawsthorne 2000). Modest  $C_4$  cycle enhancement is apparent in numerous  $C_2$  + species that are close sisters to C<sub>4</sub>-like species in the Flaveria phylogeny. Flaveria floridana (modest C<sub>4</sub> cycle), for example, is particularly close to *F. brownii* (C<sub>4</sub>-like) (Powell 1978; McKown et al. 2005). Photosynthesis responses to low  $CO_2$  in *Flaveria* show the relative impact of the transition from  $C_2$  to  $C_4$ -like species. Flaveria floridana has a greater rate of photosynthesis below 300 ppm than F. angustifolia ( $C_2$ ), but a lower rate than F. brownii (Fig. 3).

The final recognizable stage in  $C_4$  evolution before full  $C_4$  photosynthesis is the  $C_4$ -like stage, as observed in the  $C_4$ -like species *Flaveria brownii* and *F. palmeri* (Monson et al. 1987; Moore et al. 1989). These species are effectively  $C_4$  species as they run a strong  $C_4$  metabolic cycle and have enhanced water-use efficiency (WUE) and nitrogen-use efficiency (NUE), but they retain a weak  $C_3$  cycle in the M cells and do not  $CO_2$ -saturate photosynthesis below 500 ppm  $CO_2$  as does a full  $C_4$  plant (Monson et al. 1987; Moore et al. 1989; Kocacinar et al. 2008; Alonso-Cantabrana and von Caemmerer 2016). In the case of *F. brownii*, Rubisco, carbonic anhydrase, and PEP carboxylase still partially resemble the  $C_3$  isoforms, rather than the isoforms active in full  $C_4$  *Flaveria* species (Kubien et al. 2008; Gowik and Westhoff

2011; Ludwig 2011). The final phase of  $C_4$  evolution is an optimization phase where the compartmentalization of enzymes, their kinetics and regulation, and the cellular and organelle arrangements within their leaves are optimized to function in the  $C_4$  context (Sage et al. 2012).

The realization that  $C_2$  photosynthesis is a central step in C<sub>4</sub> evolution implicates photorespiration itself as the key physiology that allows the evolutionary process to proceed from  $C_3$  ancestors. The role of photorespiration, however, is not simply the depression of C<sub>3</sub> photosynthesis, but the production of a resource (photorespiratory  $CO_2$ ) that can be concentrated and exploited, and in so doing, promotes the assembly of the physiological and structural framework into which the  $C_2$  and  $C_4$  machinery can be introduced (Bauwe 2011). Suppression of  $C_3$  fitness by high photorespiration can only create niche space for alternative physiologies; it cannot produce the novel technologies. For this, the opportunistic scavenging of photorespiratory CO<sub>2</sub> and ammonia initiates the novelty that improves fitness, and ultimately suppresses photorespiration while enhancing Rubisco carboxylation. With this understanding, the major selection environments favoring  $C_4$  evolution must include conditions promoting high rates of photorespiration; for without these, it would be difficult to assemble the evolutionary bridge to C<sub>4</sub> photosynthesis (Bauwe 2011; Heckmann 2016).

# Ecological settings of transitional species in C<sub>4</sub> evolution

Environmental parameters promoting photorespiration are well understood and can be modelled using the following equation (Jordan and Ogren 1984):

Oxygenation/Carboxylation =  $v_0/v_c = O/(S_{rel} * C)$ . (1)

Photorespiration is initiated by the RuBP oxygenation reaction of Rubisco, which operates in competition with the carboxylation reaction. As shown by Eq. 1, the oxygenation rate  $(v_0)$  relative to the carboxylation rate  $(v_c)$  is a function of the  $O_2$  concentration in the chloroplast stroma (O) divided by the  $CO_2$  concentration in the stroma (C) and the specificity of Rubisco for  $CO_2$  relative to  $O_2(S_{rel})$ . Reduction of atmospheric  $CO_2$  concentration directly reduces C and hence the competition from the carboxylase reaction, allowing for greater oxygenase activity. Srel reflects the biochemical properties of Rubisco and can vary about 30% within the C<sub>3</sub> flora, but generally varies less within individual species (Galmés et al. 2005, 2014). The O value changes little in chloroplasts of C<sub>3</sub> plants, while C declines markedly with partial stomatal closure or following biogeochemical events that reduce atmospheric CO<sub>2</sub>. Other than direct change in CO<sub>2</sub>, the main environmental parameter affecting  $v_0/v_c$  is temperature. With a rise in leaf temperature, the specificity of Rubisco for CO<sub>2</sub> relative to O<sub>2</sub> declines, and *C/O* declines. For example, at 35 °C, the value of  $S_{rel}$  is 55% of that at 15 °C, while the solubility of CO<sub>2</sub> relative to O<sub>2</sub> is 18% less at 35 °C than 15 °C (Jordan and Ogren 1984; Galmés et al. 2005). Together, these effects nearly double  $v_o/v_c$  from 15 to 35 °C at ambient CO<sub>2</sub> levels of 340 ppm (Jordan and Ogren 1984; Sage 2013). Even greater enhancement of photorespiration occurs above 40 °C, particularly in depleted atmospheric CO<sub>2</sub> or conditions causing partial stomatal closure, such as drought and salinity (Ehleringer et al. 1991).

Within C<sub>3</sub> plants, atmospheric CO<sub>2</sub> levels above 1000 ppm and 30 °C are modelled to reduce  $v_q/v_c$  to a level where production of photorespired CO<sub>2</sub> is less than 10% of photosynthetically assimilated CO<sub>2</sub>; by contrast, at 230 ppm  $CO_2$  (the late-Pleistocene average), photorespired  $CO_2$  production is > 40% of photosynthetic CO<sub>2</sub> uptake (Ehleringer et al. 1991; Sage and Stata 2015). The associated photorespiratory metabolism above 1000 ppm would produce relatively small amounts of glycine for GDC, and the CO<sub>2</sub> produced by GDC would have less proportional impact, due to the high background  $CO_2$  concentration. As shown in Fig. 3, there is a little enhancement of photosynthesis in C<sub>2</sub> and proto-Kranz species relative to  $C_3$  species at elevated  $CO_2$ . Even if GDC levels in M cells were reduced, any remaining activity could handle the low glycine flux occurring at elevated CO<sub>2</sub>, such that little would overflow into the BS cells. There would thus be little opportunity for a C2-type mechanism to be favored by selection. By contrast, in warm environments at the low CO<sub>2</sub> levels of recent geological time (180–400 ppm in the past 4 million years; Bartoli et al. 2011; Zhang et al. 2013; Higgins et al. 2015), glycine production would be substantial enough to support glycine shuttling from M to BS, and the resulting improvements in carbon gain in the BS would be proportionally larger given the low ambient CO<sub>2</sub> (Ehleringer et al. 1991; Rawsthorne 1992; Sage and Stata 2015). Thus, the reduction in atmospheric  $CO_2$  levels from near 1000 ppm over 30 million years ago to as low as 180 ppm in the last 100,000 years (Fig. 4a) is viewed as meeting a precondition for the inception of glycine shuttling and the rise of C<sub>2</sub> and  $C_4$  photosynthesis (Sage 2001; Osborne and Beerling 2006; Tipple and Pagani 2007). As shown in Fig. 4a, this reduction in CO<sub>2</sub> is predicted to have increased the percentage of photorespiratory inhibition of photosynthesis at 30 °C from below 10% around 30 million years ago to over 25% in the past 20 million years.

Associated with this reduction in  $CO_2$  was an increase in aridity in low-to-mid-latitudes due to a generally drier atmosphere, which occurs in response to a weakened greenhouse effect (Zachos et al. 2001; Edwards et al. 2010). Drier atmospheres expand arid zones, and reduce humidity around vegetation, both of which would reduce stomatal conductance to the diffusion of  $CO_2$ . Increasing aridity is also associated with expansion of regions with high soil salinity and other mineral-rich soil types, such as gypsum, alkaline clays, and caliche. Salinity and other forms of mineral excess also induce stomatal closure, as plants reduce transpiration to lower salt accumulation in leaves (Sage and Pearcy 2000; Flowers and Colmer 2008).



**Fig. 4** Relationship between estimated lineage age and number of species in (**a**) 23 monocot C<sub>4</sub> lineages and (**b**) 30 eudicot C<sub>4</sub> lineages. In panel (**a**), triangles show grass clades, and diamonds show sedge clades. Also shown in panel a are the estimated CO<sub>2</sub> values over the past 30 million years (Zhang et al. 2013—grey line for proxy and modelled values, and Higgins et al. 2015—black circles for ice core data), and the % photorespiratory inhibition corresponding to estimated CO<sub>2</sub> levels at 30 °C (dashed line). The % photorespiratory inhibition was calculated as  $(0.5V_o/V_c)$  100% (Sage 2013). Lineage age data originally published in Christin et al. (2011b) and

Sage (2016) are taken as the midpoint between stem and crown node age estimates, except where noted otherwise in Table 1. Abbreviations: *Photoresp.* photorespiration, *And* Andropogonae, *Atr Atriplex, Car* Caryxylonae, *Chl* Chloridoideae, *Cyp Cyperus, Eup Euphorbia, MPC* Melinidinae, Panicinae, Cenchrinae, *Port Portulaca, Sal* Salsoloideae, *Ses* Sesuviodeae. Note, *y*-axis scales in panels (**a**) and (**b**) differ. Regressions: monocots:  $y=102.2-27.7x+2.9x^2$ ,  $R^2=0.65$ , p<0.001; eudicots: y=7.5x-21.4,  $R^2=0.37$ , p=0.003. Data for the C<sub>4</sub> lineages reprinted from Sage (2016) by permission

With reduced stomatal conductance,  $CO_2$  concentrations in chloroplasts decline, amplifying the impacts of low atmospheric  $CO_2$ . Warm conditions aggravate these effects, by increasing the vapor pressure difference between leaf and air (VPD), which also reduces stomatal conductance (Schulze and Hall, 1982). Interactions among high air temperature, drought and salinity will also enhance leaf temperatures. Stomatal closure reduces evaporative cooling, driving leaf temperatures higher, while exposed or rocky soils in hot, dry, and saline settings can reflect sunshine or reradiate thermal radiation into leaves. Altogether, these considerations lead to the hypothesis that regions where these factors combine to maximize photorespiratory potential will be the areas where  $C_2$  and  $C_4$  plants are most likely to evolve (Sage 2004). A lineage-by-lineage assessment of  $C_4$  centers of origin allows us to evaluate this hypothesis.

# A lineage-by-lineage assessment of C<sub>4</sub> origins

Table 1 lists 63 independent lineages of  $C_4$  photosynthesis, which represents nearly all the  $C_4$  lineages in monocots and eudicots that can be currently documented using phylogenetic inferences. The table also lists regions where  $C_4$  photosynthesis is predicted to have evolved, for 55 lineages, using habitat distributions of transitional species, or if this is not clear, habitats of  $C_4$  species within a lineage if they are not widely distributed (for example,



**Fig. 5** Estimated regions of origins for 48  $C_4$  lineages of monocots and eudicots, mapped onto a Köppen-Geiger climate diagram showing regional climates across the Earth (Peel et al. 2007). The six major regions of origin are highlighted by ovals and the corresponding  $C_4$  lineages are listed in boxes. Centers of origin from other lineages that can be hypothesized are also shown. The codes for the climate diagram are as follows: Af, tropical rainforest; Am, tropical monsoon forest; Aw, tropical savanna; Bwh, hot arid desert; Bwk, cold arid desert; BSh, hot semi-arid steppe; BsK, cold semi-arid steppe; Csa, hot summer Mediterranean climate, with dry summer; Csb, temperate with warm dry summer; Cwa, temperate with dry winter and hot summer; Cwb, temperate with dry winter and warm summer; Cwc, temperature with dry winter and cold summer; Cfa, temperate with no dry season and hot summer; Cfb, temperate with no dry season and warm summer; Cfc, temperate with no dry season and a cold summer; Dsa, cold wet winter with hot dry summer; Dsb, cold wet winter with warm dry summer; Dsc, cold wet winter with dry cold summer; Dsd, very cold wet winter with dry summer; Dwa, cold dry winter and hot summers; Dwb, cold dry winter and warm summers; Dwc, cold dry winter with cold summer; Dwd, very cold wet winter cold summer; Dfa, no dry season, cold winter, hot summer; Dfb, no dry season, cold winter and warm summer; Dfc, no dry season, cold winter and summer; Dfd, no dry season, very cold winter, cold summer; ET, polar tundra; EF, polar ice cap. Temperature corresponding to these climate classifications is presented in Peel et al. (2007) and on Wikipedia (https://en.wikipedia.org/wiki/ K%C3%B6ppen\_climate\_classification) in *Tidestromia*, a small  $C_4$  lineage in the Amaranthaceae that is restricted to SW North America; Sanchez del Pino and Motley 2010). These regions of origins are mapped in Fig. 5 along with Köppen-Geiger climate zones (Peel et al. 2007).

Six major geographic regions of  $C_4$  diversity are indicated in Fig. 5. These are (1) southwestern North America corresponding to the hot deserts and steppes of North Central Mexico and the adjacent USA (8 origins); (2) south-central South America in the dry inland regions of tropical Brazil to northern Argentina (8 origins); (3) the hot deserts of southwestern Africa to the dry steppes of southeastern Africa (10 origins); (4) the hot deserts of northeastern Africa and adjacent Arabia (2-3 origins); (5) the inland arid (and often salinized) basins of southwestern to central Asia (9 origins), and (6) the hot desert and steppe region of subtropical-towarm-temperate Australia (6 origins). These six regions account for 44 of the 63 postulated lineages  $C_4$  photosynthesis (Table 1), and largely correspond to Köppen-Geiger climate classifications for tropical savanna (Aw), dry climates with warm-to-hot summers (the B group of classifications), or temperate with hot summers (Cwa and Cfa). Of the lineages estimated to have arisen outside the six regions of C<sub>4</sub> origin, all are in warm environments of lower latitude. Some are associated with seasonally dry tropical forests with drought-deciduous canopies (Alloteropsis semialata, and probably other grass clades; Lundgren et al. 2015), while others are in coastal salt marshes of the warm-temperate zone (Flaveria brownii). Of note, sedges in the Eleocharis clades probably evolved  $C_4$  in freshwater wetlands where most of their  $C_4$  members now occur (Table 1; Roalson et al. 2010).

Numerous studies have characterized the regional climates corresponding to centers of origins, using annual or summer mean temperatures and precipitation data. An example of this approach is provided for the Blepharis lineage, where 15  $C_4$  species are documented within the *Blepharis* section Acanthodium, a large clade of 80 species (Fig. 6). Of approximately 65 species in Blepharis section Acanthodium that are not fully  $C_4$  most appear to be  $C_2$ , while a few are  $C_4$ -like or  $C_3$  + (Fisher et al. 2015; Stata and Sage unpublished). With the inclusion of a few basal-branching C<sub>4</sub> species, the section Acanthodium provides a high number of transitional species to map onto climate diagrams. Climactic parameters corresponding to the geographic coordinates of these species show that the large majority of points cluster between mean annual temperatures (MAT) of 15° to 23 °C, and below mean annual precipitation (MAP) of 700 mm (Fig. 6a). Using similar approaches for grasses and the eudicot Mollugo clade, Christin and Osborne (2014) show relatives of C4 clades cluster in warmer and drier areas between a MAT of 10° to 30 °C and below MAP of 3000 m. In a study that pooled distributions of transitional species of many eudicot and monocot lineages, Lundgren and Christin (2017) similarly observed the species clustered between MAT of 15° to 25 °C, and below MAP of 2000 mm. Each of these approaches shows that C4 origins occur toward the warmer and drier ends of climate gradients.

Phylogenetic reconstructions of the habitats occupied by the last common ancestor of  $C_3$  and  $C_4$  clades may best





**Fig. 6** Climate parameters for the collection locales of 445 herbarium specimens of *Blepharis* species. **a** Mean annual precipitation and mean annual temperature associated with collections of 93 *Blepharis* species. Data are grouped into  $C_3$  *Blepharis* species not in section *Acanthodium* (non-Acan),  $C_4$  species in section *Acanthodium*, and a series of transitional species as follows:  $C_3$  species in section *Acanthodium* (C3 Acan), sister  $C_3$ – $C_4$  intermediate species in section *Acanthodium* (sister C3–C4),  $C_4$ -like species, and basal-branching  $C_4$  species in the section *Acanthodium* (basal C4). The oval

surrounds most collections for the transitional species, and thus infers the climate space where  $C_4$  photosynthesis evolved in *Blepharis*. **b** Mean February precipitation and daily maximum temperature for species of *Blepharis* section *Acanthodium* considered to be transitional species. Climate data from the WorldClim database (Hijmans et al. 2005; http://www.worldclim.org/) downloaded from the Global Biodiversity Information Facility (GBIF). Reprinted from Fisher et al. (2015) by permission

narrow the climate envelopes associated with  $C_4$  evolution, as is demonstrated with *Alloteropsis semialata* in which  $C_3$ ,  $C_2$ ,  $C_4$ -like, and  $C_4$  genotypes are present. Phylogenetic reconstructions indicate the center of  $C_4$  origin in *A. semialata* was among the dry forests and savannahs of central and eastern Africa (Lundgren et al. 2015). Inclusion of all *A. semialata* collections produces a climate map where MAP ranges from about 500–2800 mm, and MAT ranges between 13 and 28 °C. Using phylogenetic reconstructions to estimate the climate of the last common ancestor of the existing  $C_3$  and  $C_4$  clades, the climate of probable  $C_4$  origins is considerably narrowed, to an MAT near 20 °C and an MAP around 1000 mm (Lundgren et al. 2015). In modern grasslands,  $C_4$  grasses dominate above MAT near 20 °C, while  $C_3$ species are favored by cooler conditions (Sage et al. 1999).

Mean annual data are widely used in climate reconstructions because of widespread data availability and the simplicity of ignoring microclimate and phenology. MAT data are limiting, however, in that the night and dormant-season measurements included in the MAT calculation obscure the daytime temperatures during the growing season that directly influence photosynthesis and photorespiration. In turn, MAP does not account for seasonality and intensity of precipitation events, nor soil water availability. For example, dry climates such as those in the C<sub>4</sub> regions of origins are noted for large thermal oscillations, between cool nights and hot days, while semi-arid monsoon climates can have a dry dormant season that alternates with warm, episodically wet growing seasons (Oke 1987). For a clearer picture, it is useful to narrow the climate window by acquiring growth season values and ultimately, by measuring diurnal leaf temperatures, water status, and photosynthetic performance within the microclimate of transitional species in the field. In the case of Blepharis, the transitional species are mainly summer active (Fisher et al. 2015). The climate data corresponding to summer (February in southern Africa) show the transitional species of Blepharis cluster where mean maximum daily temperatures exceed 25-30 °C (Fig. 6b). Field measurements of the microsites where transitional species grow are uncommon, but where they do exist, they show that their physiological activity corresponds to conditions favoring high levels of photorespiration. Monson and Jaeger (1991) published the only known gas exchange study of a  $C_3-C_4$ intermediate in its natural habitat. In the C2 plant Flaveria floridana growing along the Gulf of Mexico coastline in central Florida, leaf temperatures in May were observed to reach the mid 30 °C range before noon, and were above 42 °C by early afternoon. Similarly, high daytime temperatures (>35 °C) were measured in the field habitat in C<sub>2</sub> Euphorbia acuta (Sage et al. 2011b) and Heliotropium clades in west Texas (Sage, unpublished). As shown for the  $C_2$  species H. convolvulaceum, a sand dune specialist, August leaf temperatures exceed 40 °C by mid-morning and approach 50 °C by early afternoon (Online-only supplemental Fig. S1). By comparison, MAT at the nearest climate station (Las Vegas) is 21 °C, while mean August maximum temperature is 39 °C (www.usclimatedata.com). Because of moisture stored in the dunes, *H. convolvulaceum* is able to maintain physiological activity during these hot summer days with very low humidity, such that photorespiration would be substantial (Sage, Vogan, and Coiner unpublished).

### The field ecology of transitional species

Over the years, numerous groups have examined field environments for transitional species from a dozen or so  $C_3-C_4$  lineages. These case studies build upon broad climate envelope surveys and phylogenetic analyses by providing details of local habitats. Below, we highlight some of the key observations for seven of these lineages, beginning with eudicot clades. (Climate data are from www.usclimatedata.com).

Blepharis (Acanthaceae, online-only supplemental Fig. S2 A, B; Vollesen 2000; Fisher et al. 2015; Stata and Sage unpublished): Fifteen C<sub>4</sub> species are present in Blepharis section Acanthodium, an old world section that occurs across arid-to-semi-arid landscapes of Africa and Southwestern Asia. Blepharis section Acanthodium also includes over a dozen C2 species, two C4-like species, a few putative proto-Kranz species, and a few C<sub>3</sub> species, which may be of the "enabled" type. The bulk of this diversity is southern African, with all intermediate and enabled C<sub>3</sub> forms occurring in arid-to-semi-arid environments, particularly in the deserts of Namibia and arid scrub to dry savannahs of northern South Africa (Fig. S2 A, B). These regions have dry winters, and in summer receive episodic moisture from summer monsoons that sweep across southern Africa from the Indian Ocean. Most species in section Acanthodium are xerophytic herbs or subshrubs, and are active in the summer to mid-autumn. The plants commonly occur on open desert surfaces with sand, gravel or rocky substrates, or rock outcrops and disturbed soils of dry savannahs and xerophytic scrub (Fig. S2 A, B). Blepharis is estimated to have acquired C<sub>4</sub> photosynthesis around 8 million years ago, when aridity was expanding in southern Africa due to intensification of the cold ocean currents along southwestern Africa (Feakins and deMenocal 2010; Fisher et al. 2015).

*Euphorbia* (Euphorbiaceae; Fig. S2 C; Webster et al. 1975; Mayfield 1991; Sage et al. 2011b; Yang and Berry 2011): C<sub>4</sub> photosynthesis evolved in subgenus *Chamaesyce* of the *Euphorbia* genus about 19 million years ago, which makes it one of the older eudicot lineages of C<sub>4</sub> plants. All species in subgenus *Chamaesyce* are C<sub>4</sub> except for three closely related species from the northern Chihuahuan desert region in Texas and adjacent Mexico, where two C<sub>2</sub> species and one C<sub>3</sub> species occur within the *Acuta* clade. This

distribution, along with a northern Mexico to Texas center of diversity for  $C_{4}$  Euphorbia species, indicate  $C_{4}$  photosynthesis in Euphorbia originated in the hot and dry landscapes of southwestern North America. The C<sub>2</sub> species *E. acuta* and *E.* johnstonii occur as sprawling herbs on open, often disturbed soils among desert scrub. The plants typically grow on dry calcareous clay, shallow limestone outcrops, or sandy loam. Both species are summer active in regions where summer highs average 35-36 °C, and are especially active following monsoon rain events. The leaves of each species occur close to the ground, and are substantially warmed by the soil boundary layer and reflected radiation on sunny days. In southwest Texas, Sage et al. (2011b) recorded field leaf temperatures of E. acuta approaching 40 °C on days when air temperature peaked at 35 °C in mid-July. Based on these observations, C<sub>4</sub> origins in Euphorbia occurred in environments that favor high rates of photorespiration.

Flaveria (Asteraceae; Fig. S2 D, E; Powell 1978; Monson and Jaeger 1991; Monson and Rawsthorne 2000; McKown et al. 2005; Sudderth et al. 2009; Sage et al. 2013): Flaveria is the leading model for C<sub>4</sub> evolution due to its having more confirmed intermediates (>10) than any other lineage. Twoto-three  $C_4$  origins are evident in *Flaveria*, one or two in a clade A that includes all of the fully C4 species, and another in a clade B where one C<sub>4</sub>-like species, F. brownii, evolved. Flaveria brownii exhibits a very strong C4 cycle, and has the high WUE and NUE of C<sub>4</sub> photosynthesis, so although it retains vestiges of  $C_3$  photosynthesis in the mesophyll, it operates a version of C<sub>4</sub> photosynthesis (Monson et al. 1987). Flaveria brownii probably arose in the past million years from C2 ancestors of the F. linearis complex (Christin et al. 2011b). The origin of F. brownii is relatively easy to place, as it is restricted to saline soils along the Texas Gulf coast, often along roadsides and saltmarshes. Its sistertaxa in the F. linearis complex includes two  $C_2$  + species (F. floridana, F. linearis) from disturbed, saline, and sandy or marshy habitats along the coastlines of Florida and the Caribbean (Fig. S2D). Daily summer highs in the Florida/ Texas habitats of the F. linearis complex average around 33 °C, while leaf temperatures exceed 42 °C (Monson and Jaeger 1991). Photosynthesis rates remain high in F. floridana at 40 °C, being depressed just 10% relative to values at the thermal optimum of 30 °C (Monson and Jaeger 1991). In addition, photosynthesis above 40 °C in F. floridana is approximately fourfold greater than observed in a nearby  $C_3$  species, *Eustoma exaltatum*. Two other species in the complex are F. pubescens and F. oppositifolia of northern Mexico, which occur inland on disturbed, saline or gypsum soils, often in moist microsites. The sister species to the F. linearis complex is F. chlorifolia, a summer active species in the northern Chihuahua desert region that also grows in moist saline soils. These occurrences of clade B species clearly indicate a C<sub>4</sub> origin in a hot climate where salinity

(or gypsum) and community disturbance are present, but not necessarily soil drought.

In clade A, the key transitional species F. ramosissima is a type II intermediate that is sister to five  $C_4$  species arising in two distinct branches that split from one another 1-2 million years ago. It occurs in disturbed, sandy, or gypseous soils of seasonally dry habitats of south-central Mexico, along with its sister species F. robusta and F. pringlei (both exhibiting the proto-Kranz trait), and F. vaginata, which has been postulated to be a C<sub>4</sub>-like species. This region is the likely place of origin for one line of C<sub>4</sub> photosynthesis in clade A. These species are typically found along roadsides, abandoned fields, and other disturbed and exposed sites in a thorn-scrub, semi-arid vegetation zone (Fig. S2D). Closely related to these  $C_4$ -like species are the widespread F. bidentis and F. trinervia, both of which are fully C<sub>4</sub> species growing as weeds on disturbed soils in tropical to warm-temperate Americas. A second branch in clade A includes F. palmeri, a C<sub>4</sub>-like species of disturbed gypsum soils in semi-arid northern Mexico, and F. campestris, a fully C4 species that is specialized for moist, alkaline soils in the American Southwest. All clade A species are active in the summer when hot days are common in their subtropical habitats. Drought and disturbance appear to be the more critical co-factors for C<sub>4</sub> evolution in clade A, except possibly for the ancestor of F. *campestris* which may have independently evolved C<sub>4</sub> traits from a  $C_2$  ancestor in moist gypsum or alkaline soils.

Heliotropium (Boraginaceae; Fig. S1A; Frohlich 1978; Förther 1998; Vogan et al. 2007; Muhaidat et al. 2011; Vogan and Sage 2012): in the genus Heliotropium section Orthostachys (a group now recognized as the genus Euploca), approximately 100 C<sub>4</sub> species exist, along with at least five C<sub>2</sub> species, three proto-Kranz species, and a halfdozen  $C_3$  + species. An enabled  $C_3$  species (*H. tenellum*), two proto-Kranz species (H. procumbens and H. karwinsky), two C<sub>2</sub> species (H. racemosum and H. convolvulaceum), and the basal C<sub>4</sub> species occur in a phylogenetic cluster distributed in arid-to-semi-arid regions of Mexico and adjacent regions in the southwestern USA, indicating that  $C_4$  arose in Heliotropium within this region. The locales of these species mirror those from Flaveria clade A species, clustering in seasonally dry areas of northern and south-central Mexico. Soils are often harsh in this area, being of caliche, limestone, or sand. Frohlich (1978) notes many of the species identified as transitional occur in ephemeral habitats such as roadsides, stream banks, and margins of ephemeral ponds. The C<sub>2</sub> species H. lagoense, for example, is found on drying streambeds or pond margins, where competition from established vegetation is limited. Heliotropium convolvulaceum and H. racemosum occur on deep sand in the American southwest, where daily leaf temperatures often exceed 40 °C (Fig. S1B). Water stress is minimal in these two species as they tap into deep reservoirs within the sand that are replenished by the

episodic monsoon showers. In laboratory grown *H. convol-vulaceum*, the net  $CO_2$  assimilation rate at 40–45 °C was within 80% of the value at the thermal optimum near 30 °C at both 380 and 180 ppm  $CO_2$ , indicating that this species can maintain robust photosynthesis rates under the hot conditions of its midday habitat.

Mollugo (Molluginaceae, Fig. S2F; Kennedy et al. 1980; Christin et al. 2011b): There are two  $C_4$  species in the eudicot Mollugo genus—M. cerviana and M. fragilis. Mollugo cerviana forms two clades, one with just M. cerviana, and a second with an *M. cerviana*-like genotype that is sister to a second  $C_4$  species, *M. fragilis*. These clades are separated in the phylogeny by a putative C<sub>2</sub> species, Mollugo spergulacea, which is from hot, arid landscapes in southern Namibia (similar to that in Fig. S2A). This separation indicates two independent  $C_4$  origins. Mollugo cerviana from both the cerviana and fragilis groups is widespread across hot arid zones around the Earth, largely on sandy or rocky soils. Mollugo fragilis is restricted to the coastal strand of Angola, also on sand. Sister to these species is a clade of multiple xerophytic genera from southern Africa (the Adenogramma/ *Pharnaceum* clade) that together with *M. spergulacea* indicate the C<sub>4</sub> lineages arose in this region, probably in hot arid microsites, on rocky or sandy soils such as those where *M. spergulacea* and the  $C_4$  *Mollugo* species currently grow.

Two additional  $C_2$  species are present in Molluginaceae. The weedy herb M. nudicaulis occurs in a sister position to the complex of Adenogramma/M. spergulacea/M. cerviana, suggesting deep  $C_2$  ancestry at the base of this clade. It is a pantropical weed of open, disturbed ground, often on sandy soils and active in summer. A second C<sub>2</sub> species, the common carpet-weed M. verticillata arose at the opposite end of the Mollugo phylogeny and is not closely related to the C<sub>4</sub> clades. It is also widespread as a weed on disturbed, often sandy soils, and is, perhaps, the most commonly encountered  $C_2$  species in the world. It can grow into the cool temperate zone of Europe and southern Canada, leading to suggestions that C2 species can be tolerant of cool climates (Lundgren and Christin 2017). In the case of *M. verticillata*, its activity at the northern end of its range is restricted to the warmest months of the year. In southern Canada and Michigan, we have observed it only in July-September, as a sidewalk, driveway, and patio weed, growing on surfaces that become quite warm on sunny days (Fig. S2F). It was particularly notable for the senior author, who burned his bare feet while photographing a Michigan M. verticillata plant sprawling across an asphalt driveway (inset of Fig. 8F). Although the central Michigan climate is considered cool temperate (MAT =  $8 \,^{\circ}$ C) and July is warm (mean daily maximum =  $28 \degree C$ ), the leaves of *M. verticillata* in Fig. S2F were in the boundary layer of a dark surface that exceeded 50 °C. The prostrate nature of the plant leads to its common name "carpet-weed", which highlights how this

 $C_2$  species has evolved a growth form and phenology that allows it to exploit hot microsites, thereby persisting even in mild climates.

Neurachne (family Poaceae; Fig. S2G-J; Blake 1972; Hattersley et al. 1986; Prendergast and Hattersley 1985; Christin et al. 2012b): Neurachne, a grass genus with seven species from Australia, is one of only two monocot genera known to contain closely related C<sub>3</sub>, C<sub>2</sub>, and C<sub>4</sub> species. Two independent C<sub>4</sub> origins occur in *Neurachne*, probably within the past 5 million years. One C<sub>2</sub> species (N. minor) is known from this genus, branching in a sister position to one of the  $C_4$  clades. Most *Neurachne* species and the three C<sub>3</sub> species in its sister genus Thyridolepis are restricted to warm-temperate to subtropical Australia, in semi-arid-toarid grassland, rocky outcrops or scrublands, almost exclusively in open, high-light environments. The C<sub>2</sub> species N. minor occurs on rocky outcrops, sandy grasslands, and shallow loamy soils in dry glades (Fig. S2J). The shallow substrate often aggravates drought stress for this species in its dry habitat by restricting root depth. A close relative with C3-isotopic values, N. annularis, occurs in inland areas of Western Australia in open savannahs with sandy soils (Fig. S2H), while a second (N. lanigera) occurs in dry, sandy soils of west Australian grasslands, typically following fire. One C<sub>4</sub> species, N. munroi, is largely present on open, rocky soils and outcrops (Fig. S2G), while the other C<sub>4</sub>, N. muelleri, is present on open dunes and sandy soils along the northern length of inland Australia (Fig. S2J). These regions are affected by episodic summer monsoon precipitation, allowing growth of plants during the hot summers months, when intense solar radiation coupled with hot soil surfaces would further increase leaf temperature.

Alloteropsis semialata (Christin et al. 2012a; Lundgren et al. 2015, 2016; Olofsson et al. 2016; Dunning et al. 2017): Alloteropsis is a small genus with four fully  $C_4$  species and one species (A. semialata) that contains fully  $C_4$ , fully  $C_3$ , and a range of C<sub>3</sub>-C<sub>4</sub> intermediate phenotypes. Given the range of photosynthetic diversity, it has attracted much attention recently, leading to detailed phylogenomic and ecophysiological advances, such that now it is one of the best-studied C4 lineages. This work demonstrates the complex nature of the transition to  $C_4$ . Multiple  $C_4$  origins are apparent in Alloteropsis. In the A. cimicina clade that contains three fully C<sub>4</sub> species, the C<sub>4</sub> anatomy and biochemistry appears to have evolved once. In the A. semialata clade, which includes all A. semialata types and  $C_4$  A. angusta,  $C_4$  anatomy was likely acquired once near the base of the clade, while C<sub>4</sub> biochemistry arose three times, once in the branch leading to A. angusta and twice in the  $C_4$  genotypes of A. semialata (once in a Tanzanian clade F, and once in Zambezian-centered clade DE). Lateral transfer of C<sub>4</sub> cycle genes between ancestral A. semialata and fully developed C<sub>4</sub> lineages such as *Cenchrus* and *Setaria* are proposed to have facilitated the assembly of a functional  $C_4$  cycle. The center of origin of each lineage is probably central Africa, in seasonally dry-wooded savannahs. The split between the A. semialata/angusta clade and the A. cimicina clade is around 10-12 mya, indicating a late Miocene acquisition of  $C_4$  in the A. cimicina clade. The split between A. semialata and A. angusta centers around 7 mya, so C<sub>4</sub> arose in the A. angusta clade and A. semialata F clade after this time. The origin of  $C_4$  in the core A. semialata clade is better defined in space and time, probably due to a recent acquisition of fully expressed  $C_4$  that has allowed many intermediate genotypes to persist. Phylogenetic reconstructions place the separation of non-C<sub>4</sub> and C<sub>4</sub> clades after 2-3 Ma, in the seasonally dry, open woodlands of the Zambezi region of central Africa, at MAT of 20 °C and MAP centered around 1000 mm. This is the most precise estimate of time, place, and climate of origin currently in the literature, enabled in part by the presence of C<sub>4</sub> and non-C<sub>4</sub> states in a single species, and the combination of efforts in genomics, field ecology, and physiology by Alloteropsis researchers. Collections of C<sub>2</sub>-type A. semialata are from sunny as well as partially shaded woodland habitats, suggesting a less extreme selection environment than observed in Neurachne and the eudicots. Similarly, C<sub>2</sub> Homolepis aturensis occurs in habitats that can include shady locations within the seasonally dry savannahs of Central and South America (Khoshravesh et al. 2016). It is possible that episodic fire and severe drought, which are common to these environments, created open, stressful habitats that increased photorespiration rates, or alternatively, the very low atmospheric CO<sub>2</sub> concentrations of the past 2 million years (< 300 ppm) was sufficient to drive  $C_4$  evolution in less open and milder environments.

Eleocharis (Cyperaceae; Ueno et al. 1988; Besnard et al. 2009; Roalson et al. 2010): The evidence supports twoto-three  $C_4$  origins in the sedge genus *Eleocharis*, one in the monospecific  $C_4$  lineage *E. vivipara*, and one or two in the Eleocharis section tenuissimae. Eleocharis vivipara is notable for its ability to produce C<sub>3</sub> leaves under water and C<sub>4</sub> leaves above water, and is generally found in freshwater marshy habitats of Florida and adjacent US states, indicating that this area is where its C<sub>4</sub> pathway evolved. Species in *Eleocharis* section *tenuissimae* occur in tropical Africa and in the warm climates of the Americas, with the basal species in the C<sub>4</sub> clade(s) largely occurring in the wetlands of central America, northern South America, and the Caribbean islands. These locations indicate a wet, neotropical origin(s) for  $C_4$  in section *tenuissimae*.  $C_4$  is estimated to have evolved in E. vivipara and the E. tenuissimae clades in the past 4–6 million years, when atmospheric CO<sub>2</sub> concentrations were approaching the low levels of the past 2 million years (Table 1; Bartoli et al. 2011). The wetland origin of  $C_4$ in *Eleocharis* does not support the hypothesis that C<sub>4</sub> evolution requires a combination of heat, low CO<sub>2</sub>, and stress conditions that would drive high rates of photorespiration. Although periodic fire disturbance may help to maintain open landscapes, as noted for *E. vivipara* (Roalson et al. 2010), the *Eleocharis* case indicates that low CO<sub>2</sub> alone coupled with a warm climate was sufficient to support C<sub>4</sub> evolution. Given this anomaly, *Eleocharis* would be an excellent genus for further population-level studies of C<sub>4</sub> evolution, as are being conducted for *Alloteropsis*. Alternative evolutionary pathways to the *Flaveria* model may exist here, possibly aided by ancestral patterns of C<sub>4</sub> acid metabolism that can occur in submerged environments (Bowes 2011).

### Paleo-environments of C<sub>4</sub> evolution

As shown in Fig. 4,  $C_4$  photosynthesis is estimated to have repeatedly evolved over the past 30 Ma, when atmospheric  $CO_2$  levels were declining or had reached low levels similar to our recent era. While these origin dates have to be considered as rough estimates given the inherent uncertainty in molecular clock procedures (Vicentini et al. 2008; Christin et al. 2014b), they do provide a window for evaluating climatic and atmospheric conditions for the time when  $C_4$  lineages are estimated to have appeared. In particular, the dates allow us to evaluate whether the six major geographic regions of  $C_4$  origin, at the time of origin, had the  $C_4$ -selection environments indicated by modern transitional species.

The paleo-record indicates the time before the Oligocene epoch (> 35 Ma) was not favorable to  $C_4$  evolution as Earth's climate was warm and wet, and the atmosphere was enriched with CO<sub>2</sub>, perhaps, to over 1000 ppm (Prothero 1994; Zachos et al. 2001; Tripati et al. 2009; Zhang et al. 2013). During the Oligocene, the earth's climate cooled and CO<sub>2</sub> declined, such that, by the beginning of the Miocene epoch 24 Ma, CO<sub>2</sub> levels were similar to today (Fig. 2a). At low latitude, climates in the C<sub>4</sub> regions of origin became drier in the early Miocene, causing localized aridification (Zachos et al. 2001, 2008). In the  $C_4$  region of origin, the drier conditions led to localized patches of semi-arid habitat such as savannah and xerophytic scrub, which allowed many xerophytic clades of higher plants to diversify (Becerra 2005; Moore and Jansen 2006; Senut et al. 2009; Graham 2010; Strömberg 2011; Pound et al. 2012). The early Miocene climates were warmer than today (Zachos et al. 2008), and with  $CO_2$  levels around 350–450 ppm, the combination of warmth, reduced CO<sub>2</sub>, and increasing aridity (and salinity) likely favored sufficient photorespiration to promote the first series of  $C_4$  origins.

By the late Miocene (5–12 MA), a second phase of global cooling set in, possibly accompanied by further slippage in atmospheric CO<sub>2</sub> concentration (Zachos et al. 2001; Kürschner et al. 2008; Tripati et al. 2009; Zhang et al. 2013).

This led to widespread expansion of arid, semi-arid, and seasonally dry environments at low-to-mid-latitudes, which although cooler than before remained warmer than today. The six  $C_4$  regions of origin were markedly affected by this climate deterioration, as indicated by expansion of semi-arid grasslands and xerophytic scrub, and many of the modern deserts such as the Sahara came into existence (van Devender 2000; Schuster et al. 2006; Senut et al. 2009; Strömberg 2011; Pound et al. 2012; Amidon et al. 2017). In addition to the appearance of a large number of new  $C_4$ lineages (> 20; Fig. 4; Sage 2016), the late Miocene was characterized by the widespread expansion of  $C_4$ -dominated grasslands at low-to-mid-latitudes (Cerling et al. 1997; Edwards et al. 2010; Strömberg 2011 l; Bouchnek-Khelladi et al. 2014). This simultaneous ecological expansion and evolutionary proliferation of C<sub>4</sub> lineages indicates a global driver favored C4 success in the late Miocene (Ehleringer et al. 1991; 1997). This driver was probably the combined expansion of arid-to-semi-arid landscapes, coupled with the still warm climates at low latitude, and further reductions in CO<sub>2</sub> (Edwards et al. 2010; Arakaki et al. 2011; Herbert et al. 2016). Increased seasonality and fire probably contributed by expanding landscapes that favored C<sub>4</sub> plants (Keeley and Rundel 2005; Beerling and Osborne 2006; Hoetzel et al. 2013). The expanding C<sub>4</sub> grasslands may also have contributed to the regional aridification by promoting climate change and fire (Beerling and Osborne 2006).

In the past 4 Ma, the earth entered another phase of climate cooling and drying which ended with the advent of the Pleistocene Ice Ages, and minimal atmospheric  $CO_2$  levels that oscillated between 180 and 320 ppm (Zachos et al. 2001; Higgins et al. 2015). Modern landscape came into existence at low latitude, with expansive grasslands and deserts across the subtropics (Martin 2006; Salzmann et al. 2011; Feakins et al. 2013; Liddy et al. 2016). Despite the climate cooling, hot days prevailed in the low latitude summers, and with the minimal  $CO_2$  levels of recent geological times, would have promoted the origins of the youngest  $C_4$  lineages, such as *Flaveria, Neurachne*, and *Alloteropsis* which are now important systems for studying how  $C_4$  evolved.

# Quantum yield and resource use efficiency as drivers of C<sub>4</sub> evolution?

As a final note, we return to a frequently discussed issue, notably whether differences in resource use efficiency and photosynthetic quantum yield were important drivers of adaptations associated with  $C_4$  evolution. The  $CO_2$ -concentrating mechanism of  $C_4$  plants generally provides the potential for higher water-use efficiency (WUE), nitrogen-use efficiency (NUE), and light-use efficiency (as measured by the maximum quantum yield of photosynthesis), as compared to  $C_3$  plants (Pearcy and Ehleringer 1984). Because of this, the  $C_4$  pathway has often been suggested as an adaptation to water- or nitrogen-limited habitats, or habitats where warm temperatures increase the potential for photorespiration and, in doing so, reduce the quantum yield of the C<sub>3</sub> pathway (Ehleringer and Monson 1993). However, as discussed above, phylogenetically controlled analyses of C<sub>3</sub> and C<sub>4</sub> species divergence show that ecological niches are not altered as species transition through intermediate phases of  $C_4$  evolution (Lundgren et al. 2015). Rather, it is only after emergence of a fully expressed  $C_{4}$ pathway that niche expansion occurs (Lundgren et al. 2015). Furthermore, physiological analyses of traits in C<sub>3</sub>-C<sub>4</sub> intermediates indicate that adaptations to habitats with limited water or nitrogen availability, such as improved WUE or NUE, are not often observed in C3-C4 intermediates relative to C<sub>3</sub> species (Monson 1989; Vogan et al. 2007; Kocacinar et al. 2008; Vogan and Sage 2011-although improvements have been observed in some species, Brown 1978; Brown and Simmons 1979). It is, therefore, difficult to conclude that adaptive features of the fully expressed C4 pathway, such as improved WUE and NUE, drove evolution and niche expansion during the  $C_2$  stages of  $C_4$  evolution. Instead, higher NUE and WUE that arose in the later stages of C<sub>4</sub> evolution likely contributed to niche expansion that occurred after fully formed  $C_4$  species appeared.

Quantum yield differences between  $C_3$  and  $C_4$  species are effective in modelling the distribution of  $C_4$  plants along climate gradients (Ehleringer 1978), and predicting CO<sub>2</sub> and thermal conditions that would favor C<sub>4</sub> evolution (Ehleringer et al. 1997). Monson et al. (1986) observed that, in some of the intermediate phases of C<sub>3</sub>-C<sub>4</sub> photosynthesis, the quantum yield is reduced relative to either fully expressed  $C_3$  or C<sub>4</sub> species due to futile cycling of metabolic intermediates. Instead, the quantum yield advantages of  $C_4$  photosynthesis in warm environments appear only in the final phases of C<sub>4</sub> evolution. Given that the quantum yield differences can predict when  $C_4$  evolution is favored (Ehleringer et al. 1997), or where  $C_4$  vegetation is favored (Ehleringer 1978), it is useful to consider why this parameter is an effective indicator of C<sub>4</sub> success. Sage and Kubien (2003) demonstrated that quantum yield is inversely related to photorespiratory potential as follows:

Maximum quantum yield = 
$$\frac{dA_g}{dI_g} = \frac{0.125 - 0.0625v_o/v_c}{1 + v_o/v_c}$$
, (2)

where  $dA_g$  is a change in gross photosynthesis relative to  $dI_g$ , the change in the absorbed light intensity at low light,  $v_0$  is the oxygenation velocity, and  $v_c$  is the carboxylation velocity.  $v_0/v_c$  reflects the photorespiratory potential. The relationship between quantum yield and  $v_0/v_c$  is slightly curvilinear, declining from a quantum yield above 0.12 when  $v_0/v_c$  is zero to 0.031 when  $v_0/v_c$  is 1.0 (Online-only supplemental Fig. S3). This relationship demonstrates that the reduction in maximum quantum yield is an effective predictor of conditions favoring photorespiration relative to photosynthesis, and hence environments where photorespiration rates would be high enough to favor evolutionary assembly of the C<sub>2</sub>, and subsequently C<sub>4</sub>, pathways. Once C<sub>4</sub> species evolved, quantum yield could then predict where C<sub>3</sub> photosynthesis is inhibited by increasing photorespiration, and hence could identify conditions where the C<sub>4</sub> pathway provides performance advantages that influence competitive outcomes.

### Summary and conclusion

The lineage-by-lineage comparison demonstrates C<sub>4</sub> photosynthesis consistently evolved in taxa of warm-to-hot environments at low latitude, or warm seasons and hot microsites at temperate latitudes. Leaf temperatures > 30 °C are routine, and often exceed 40 °C during the peak growing season. Arid and semi-arid conditions are common, although for numerous lineages, particularly in the Chenopodiaceae and Flaveria clade B, salinity may be the more important co-factor as indicated by transitional species associated with moist, but saline soils. Disturbance is a common theme as are harsh substrates comprised of sand, gravel, and rock, limestone derived hard-pans, or mineral-rich soils derived from alkali, gypsum, and sodic evaporites. In the case of certain sedges,  $C_4$  evolution appears to occur in warm environments with abundant freshwater, demonstrating that it is incorrect to conclude that aridity and/or salinity stress are absolute requirements for C<sub>4</sub> evolution. Sunny, open environments may also not be an absolute, as some C2 grass clades may have appeared in the partial shade of dry forest interiors in the warm tropics (Lundgren et al. 2015). Summer monsoons are common where most  $C_4$  clades originate, although they are not strong enough to prevent frequent drought, salinity stress or high evaporative demand. Seasonal rain showers from monsoons are important co-factors in C<sub>4</sub> evolution, because they provide moisture in an otherwise hot and dry climate, thereby allowing for high rates of photorespiration and photosynthesis, simultaneously, on hot days. Without this moisture, vegetation would be summer dormant with negligible photosynthesis, as observed in Mediterranean climate zones, where no C<sub>4</sub> origins are identified, and modern C<sub>4</sub> plants fail to dominant except where water is locally available in summer (Sage et al. 1999).

When placed into the context of the paleo-environments in which the  $C_4$  lineages actually evolved, the settings are consistent with that indicated by modern transitional species. All  $C_4$  origins save, perhaps, the Chloridoideae grasses probably arose in depleted CO<sub>2</sub> atmospheres, following climate deterioration that left the  $C_4$  regions of origin drier, and with patches of elevated salinity. While low CO<sub>2</sub> is widely implicated in promoting C4 origins, it did not reach its lowest levels until the past 2 Ma, after most C<sub>4</sub> lineages evolved. This implies most C4 origins occurred in more moderate CO<sub>2</sub> atmospheres of 350–550 ppm, in which case, the greater warmth of the Miocene climate, coupled with reduced stomatal aperture caused by increased aridity and salinity, could have been critical in facilitating C<sub>4</sub> origins via the photorespiratory bridge mechanism. The possible origin of C<sub>4</sub> photosynthesis in the core Chloridoids around 28 Ma raises the possibility that C<sub>4</sub> could evolve in a higher CO<sub>2</sub> setting, perhaps, 800 ppm as indicated by Zhang et al. (2013) for this time. (Other elevated  $CO_2$  origins are possible if older molecular clock estimates, or early putative  $C_4$  fossil dates, are valid; Vicentini et al. 2008; Urban et al. 2010). At elevated  $CO_2$ , the possibility of the photorespiratory bridge is reduced, but if the ancestral species operated at very low stomatal conductances, scenarios for  $C_4$  origin based on high photorespiration remain possible given the warm conditions of the time. In the case of the Chloridoids, the closest C<sub>3</sub> relatives grow in semi-arid-to-arid landscapes of South Africa (Peterson et al. 2011). It would be interesting to characterize the physiological ecology of these species to see if they maintain unusually low stomatal conductance and intercellular CO<sub>2</sub> levels.

In closing, Ehleringer's co-authors wish to observe that for those fortunate enough to work with Jim, he has been a source of inspiration and energy and a wealth of new insights. During the preparation of this manuscript, Jim noted that he greatly benefited from interactions with many colleagues, particularly those who brought perspectives from other disciplines. Ehleringer stated that "one's career always benefits from interactions and forming bridges", which is among the best advice that anyone could give to younger colleagues. Jim's comment is particularly appropriate for the C<sub>4</sub> plant community, which benefited from interactions between colleagues across a broad disciplinary spectrum. The advances reviewed in this work became possible through the combined efforts of paleontologists, biochemists, molecular biologists, evolutionary ecologists, phylogeneticists, theoretical modellers, and plant physiological ecologists. It is hoped that, in the future, C<sub>4</sub> colleagues will maintain Ehleringer's ethic of collaboration, and in doing so, enlarge the understanding of C4 evolution well beyond the synthesis presented here.

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measured all new data for Fig. 3 and provided editorial feedback on the final draft.

#### **Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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