SYSTEMATICS AND PHYLOGENY

# Photosynthesis metabolism in the Compositae: Current knowledge and future directions

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**Abstract** Compositae accounts for ca. 10% of all flowering plants, being found in nearly all types of habitats worldwide. The family is particularly diverse in markedly dry and seasonal habitats, raising questions about what processes led to diversification in these challenging environments. The presence of $C_4$ and CAM metabolism in some Compositae taxa has been known since the 1970s, and although some of these taxa have been extensively studied, such as *Flaveria*, there has been no systematic effort in gathering and reviewing data about photosynthesis metabolism in the family in the last few decades. In the present paper, we gathered data from more than 50 articles, spanning several different methods, raising information on photosynthesis metabolism for more than 400 Compositae species. We also present newly acquired carbon isotope data for 66 species, mostly from previously unsampled tribes. We analyzed photosynthesis metabolism in the tribe Tageteae using a phylogeny and ancestral character reconstruction. $C_4$ photosynthesis in Compositae is restricted to two tribes: Tageteae, where it had two independent origins, and Coreopsideae, where it seems to have arisen only once. CAM metabolism is found in succulent members of tribe Senecioneae, and in Astereae and Eupatorieae, with an unknown number of evolutionary origins. We discuss the evolutionary implications of the results and propose future directions for studying photosynthesis metabolism in the family.

**Keywords** Asteraceae; $C_3$; $C_4$; CAM; character reconstruction

**Supporting Information** may be found online in the Supporting Information section at the end of the article.

## ■ INTRODUCTION

Compositae is one of the most diverse families of flowering plants, accounting for ca. 10% of all extant angiosperm species. Representatives are found on all continents except Antarctica and in nearly all types of habitats, with much of this diversity appearing to be the result of numerous relatively recent radiations within the family (Mandel & al., 2019). The adaptive processes or circumstances that may have allowed such widespread and repeated diversification remain mostly speculative, ranging from the generation of large quantities of seed and the presence of a pappus to facilitate dispersal to the generalist nature of pollination syndromes, rapid generation times, and the rampant occurrence of polyploidy (Barker & al., 2016; Panero & Crozier, 2016). However, it has also been observed that Compositae are particularly ubiquitous in dry and markedly seasonal environments such as deserts, prairies, montane regions and savannas, suggesting a high level of specialization to environmental conditions unsuitable to many other groups of plants.

Diversification into challenging seasonal habitats can be found across lineages and geographic regions. Examples include Barnadesieae on the dry Andean slopes, Vernonieae in the tropical savanna of central Brazil, several tribes of the Heliantheae Alliance in North American deserts, Cardueae in the Mediterranean and mountainous Asian regions, Arctotideae in southern Africa, and Gnaphalieae in Australia, suggesting that adaptations to these conditions may have arisen several times across the evolutionary history of the family. As expected of plants found in challenging, dry and hot environments, many species of Compositae present specialized morphological features such as leaves with lobed margins (Vogel, 1968), the presence of a dense cover of light-reflecting or hygroscopic trichomes (Werker, 2000; Lusa & al., 2015), an abundance of wax, or glaucescence, on the leaves (Mulroy, 1979), or compact growth. Yet while such features have been indicated to play a part in the successful evolutionary history of the family, no specific character has been tested that would link diversification to particular environmental pressures.

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One further physiological adaptation that is often considered to be associated with adaptation to a number of challenging environmental conditions is $C_4$ and CAM photosynthesis, as plants using these pathways are able to reduce water loss via transpiration and operate more efficiently in hot and dry conditions (Ehleringer & Monson, 1993; Sage & al., 2018). Acquisition of $C_4$ photosynthesis is one of the defining characteristics in the evolution of grasses, being considered an important reason for the diversification of the group and formation of extensive grasslands during the Miocene-Pliocene, also coincident with the diversification of succulent plants that use the CAM pathway (Arakaki & al., 2011). The physiological and anatomical changes leading to the development of $C_4$ and CAM photosynthesis have also been identified in several families of angiosperms, including Compositae (e.g., Schöch & Kramer, 1971; B.N. Smith & Turner, 1975; Szarec & Ting, 1977). Initial studies have identified a handful of taxa that present $C_4$ photosynthesis, including *Flaveria* Juss., *Pectis* L. (tribe Tageteae), and *Chrysantellum* Rich. (tribe Coreopsideae), and CAM photosynthesis in a few succulent taxa belonging to tribe Senecioneae. While *Flaveria* has become a model system for studying $C_4$ metabolism and intermediate metabolisms between $C_3$ and $C_4$ (Keeley & Rundel, 2003), little further work has been done to quantify the extent of photosynthesis metabolism adaptations across the family. However, a comprehensive review of species in which $C_4$ and CAM photosynthesis have been identified is still lacking (although, see Funk & al., 2009 for brief discussions in specific tribes).

Slow progress in discriminating between $C_3$ and $C_4$ and CAM evolution is due in no small part to the need for specialized equipment and associated time, labor, and reagent costs. Since the discovery of $C_4$ photosynthesis in the 1960s (Hatch & Slack, 1966), several methods have been used to study photosynthesis metabolism, which have been reviewed elsewhere (e.g., Tregunna & al., 1970). Early work, including that on *Flaveria* (e.g., Bassüner & al., 1984), used $^{14}CO_2$-$^{12}CO_2$ pulse-chase experiments, with leaves sequentially fed radiolabeled and unlabeled $CO_2$ to verify what the initial photosynthetic products are; however, this method relies on access to live plants. Other methods involving live plants have widespread use as well, such as enzymatic activity assays targeting the different enzymes involved in each type of metabolism (e.g., rubisco for $C_3$ plants and PEPC for $C_4$ species; reviewed in Bauwe, 1984) and measures of $CO_2$ compensation point (high in $C_3$ plants, low in $C_4$ plants, reviewed in Tregunna & al., 1970, for an example with *Flaveria*, see Byrd & al., 1992).

Alternative methods include anatomic study to verify the occurrence of Kranz anatomy—a distinct sheath around the vascular bundles in the leaves—however, this does not always directly correlate with $C_4$ metabolism, as the anatomical changes may occur before the whole metabolic pathway is established. This is shown in several documented cases of plants with Kranz anatomy and intermediate $C_3$ and $C_4$ metabolism, currently called "$C_2$ photosynthesis" (see E.J. Edwards, 2019 for a thorough review). More elaborate anatomical studies can be conducted with transmission electron microscopy to discriminate the localization of the chloroplasts within the bundle sheath cells (e.g., Moore & al., 1987), which is especially useful when studying intermediate phenotypes between $C_3$ and $C_4$. In situ hybridization and immunolabeling in combination with anatomical sections have also been used to locate expression of $C_4$-linked proteins within cells, first with anti-rubisco antibodies (e.g., Bauwe, 1984) and later with mRNA (e.g., G.E. Edwards & al., 2001, 2004).

Another relatively simple method that does not depend on the availability of live plants is the discrimination of the abundance of $^{12}C$ and $^{13}C$ isotopes in leaf tissue through mass spectrometry (reviewed in Lerman, 1975). Rubisco discriminates against $^{13}C$, so $C_4$ plants present higher proportions of this isotope in relation to $^{12}C$. This method has the added advantage that it can be used on relatively small samples of dried material, including herbarium specimens.

The occurrence of nocturnal acidification in the leaves of some plants has been known since the 1800s (Heyne, 1815), but its description as a metabolic change related to carbon fixation only occurred in the 1940s (Bonner & Bonner, 1948). CAM plants store malate in vacuoles during the dark part of the cycle generating a need for larger cells, leading to succulent phenotypes (Heyduk & al., 2019), a tell-tale sign of CAM photosynthesis. Epiphytic plants, which may also suffer from drought stress, are largely associated with CAM metabolism, especially in Orchidaceae and Bromeliaceae (J.A.C. Smith & Winter, 1996). As with the range of intermediate metabolisms found between $C_3$ and $C_4$ pathways, there are examples of "facultative CAM" plants, which are mainly $C_3$, but use CAM occasionally (Winter & Holtum, 2014). Given the lack of other specific anatomical changes related to CAM besides succulence, it is hard to detect possible intermediate phenotypes (details in E.J. Edwards, 2019).

As with $C_4$ plants, many methods have been used to investigate the occurrence of CAM in different taxa (briefly explained in Szarek & Ting, 1977; J.A.C. Smith & Winter, 1996). Methods that require living plants include measuring malic acid content, which can be achieved by determining the pH in leaf extracts (for an example in Compositae, see Jiménez & Morales, 1987), enzymatic activity assays (e.g., Ranson & Thomas, 1960; Dittrich & al., 1973), and nocturnal assimilation of $CO_2$, which occurs in much higher levels than during the day (reviewed in Neales, 1975). Carbon isotope ratios can help discriminate plants that use CAM metabolism, as they also use PEPC as the main carbon fixating enzyme. Despite numerous studies conducted in Compositae species, especially in *Kleinia* Mill. (e.g., Thoday & Mairgretta Jones, 1939; Thoday & Richards, 1944; Gowdridge & Thoday, 1952), to date, no Compositae has been identified as a model for CAM studies.

In the long history and tradition of physiological studies involving photosynthesis metabolism, experts have compiled extensive and thorough reviews on different facets of the subject. These include the methods used in these studies (e.g., Tregunna & al., 1970; Farquhar & al., 1989); the underlying genetic (e.g., Heyduk & al., 2019), metabolic and anatomical (e.g., Monson, 1989; E.J. Edwards, 2019) changes involved in the assembly of those pathways; the different types of Kranz anatomy and intermediate phenotypes (e.g., Ku & al., 1991; G.E. Edwards & Voznesenskaya, 2011; Sage & al., 2014); the ecological roles plants with different metabolisms play (e.g., E.J. Edwards & al., 2010; Gibson & Rundel, 2012; Sage & Stata, 2015); the evolutionary origins of both metabolisms (e.g., Kellog, 1999; Keeley & Rundel, 2003; Silvera & al., 2010; Bräutigam & al., 2017; E.J. Edwards, 2019; Heyduk & al., 2019); and the distribution of different metabolisms across angiosperms (e.g., J.A.C. Smith & Winter, 1996; Sage & al., 1999; Sage, 2017; E.J. Edwards, 2019).

Our goal in the present paper is to appraise the state of photosynthesis metabolism studies in the Compositae in order to provide the synantherology community with data gathered from previous studies and identify gaps in our knowledge. In doing so, we honor the memory of Vicki Funk by addressing broad-scale questions on the evolution of the Compositae, while proposing future directions that will continue her legacy. For this, we survey the literature to compile existing data on the presence/absence of $C_4$ and CAM metabolism across the Compositae, add new data on additional 66 species using mass spectrometry, focusing on tribes for which no data is available, and assess the evolutionary history of the trait in tribe Tageteae in a phylogenetic framework, as a case study of how physiological and evolutionary data can be combined in future studies.

## ■ MATERIALS AND METHODS

**Literature review. —** We conducted a literature search in Google Scholar using the search terms "$C_4$ photosynthesis", "CAM photosynthesis", "Kranz anatomy", "Compositae" and "carbon isotopes". The articles obtained were subsequently searched for the terms "sheath", "Kranz", "photosynthesis", "$C_4$", "$C_3$", and "CAM". Papers containing one or more of these terms were then read in full. Data obtained from these articles, such as carbon isotope values, types of studies conducted with each species and type of metabolism, are summarized in tables in the supplemental material.

**Carbon isotope analysis. —** We used the proportion of $^{12}C$ and $^{13}C$ isotopes in leaf samples as an indirect method for inferring photosynthesis metabolism (B.N. Smith & Epstein, 1971). Leaf samples were collected from herbarium specimens deposited at US and MEM, from personal silica-gel collections, or from leaves extracted from plants cultivated in a greenhouse at the University of Memphis (voucher list in suppl. Table S1). When possible, samples were extracted from the voucher specimens used in the phylogenetic study by Mandel & al. (2019), and priority was given for species from tribes that had not been previously studied, according to the literature review, or plants that are known to grow in environments that favor $C_4$ metabolism, such as deserts and montane areas.

The dry leaf samples were ground using a bead mill (Fisherbrand Bead Mill 24 Homogenizer), then weighed and packed in tin foil capsules (Costech Analytical). Mass spectrometry analysis was conducted at the Stable Isotope Laboratory in the Department of Earth and Planetary Sciences at the University of Tennessee, Knoxville. $^{13}C/^{12}C$ ratios are shown as delta in parts per thousand, using Pee Dee Belemnite (PDB) as the calibration point. The range of values typically considered indicative of $C_4$ plants is −15% to −10% and for $C_3$ plants −35% to −21%. Plants with CAM metabolism usually present intermediary values between −16% and −26%, although some variation has been found, typically in plants that display facultative CAM (B.N. Smith & Epstein, 1971).

**Phylogenetic analysis. —** A phylogeny for the tribe Tageteae was constructed using a combined synthetic tree from the Open Tree of Life (v.10.3, https://tree.opentreeoflife.org/about/synthesis-release/v10.3) with GenBank data (release 218), using the methods of S.A. Smith & Brown (2018). Trait values were extracted from the available literature for the sampled taxa (a full listing of articles is available in supplementary Table S2), comprising four states: $C_3$, $C_4$, $C_4$-like and $C_2$. Taxa for which the photosynthesis metabolism state was unknown were classified as NA. Ancestral trait reconstruction was carried out using the function "ace" in the R package ape (Paradis & al., 2004), treating character states as discrete, and using an equal-rates model for maximum likelihood estimation. Polytomies in the tree were randomly resolved using the function "multi2di" in the R package phytools (Revell, 2012), which results in branches with zero length. These were posteriorly changed to an infinitesimal value due to requirements of the ancestral character reconstruction analysis.

## ■ RESULTS

Our search of Google Scholar yielded 171 articles and book chapters that fulfilled the defined conditions. From these, 66 provided data about different Compositae species, with the majority of studies that focused specifically on Compositae concerned with *Flaveria* and the use of a range of different analysis methods. All relevant papers are listed in supplementary Table S2. From this set, we extracted information about photosynthesis metabolism for 348 Compositae taxa. Of these, 282 had carbon isotope data available, 72 had anatomical data, and 46 had data from other types of studies.

Carbon isotope data came from 23 studies and represented 19 tribes, with 172 species having ratio values that correspond to $C_3$ metabolism, including six species of *Flaveria* that are known to present intermediate $C_2$ metabolism. Three *Senecio* L. species had intermediate values between the $C_3$ and $C_4$ range that typically correspond to plants that present CAM metabolism, either exclusively or facultative. These were classified in our dataset based on the original studies. The remaining 95 species presented values that indicate $C_4$ metabolism. Representation across tribes was patchy, with Tageteae represented by 138 taxa (out of the ca. 270 species comprised in the tribe), Anthemideae, Astereae, Coreopsideae, Heliantheae, and Senecioneae each having ratios for 10 to 40 species, and the remaining tribes having values for fewer than 10 species. From the new mass spectrometry data generated for this study, we obtained $^{13}C/^{12}C$ ratio values for 66 species from 26 tribes, including 17 tribes that had not been previously investigated in other studies (Table 1 and suppl. Table S2). All results from these samples presented values corresponding to $C_3$ metabolism, varying from −23% to −34%. A full list of species and carbon isotope ratios recovered both from the literature and newly generated data is presented in supplementary Table S2.

Across all methods and including new data generated here, 258 taxa were identified as having $C_3$ metabolism, 15 as $C_2$ intermediates, 109 as $C_4$ photosynthesizers and 33 as having CAM or $C_3$ + CAM intermediate metabolism. Summarized results are presented in Table 1.

Those taxa found to have $C_4$ pathways are distributed in two tribes: Coreopsideae with 23 taxa, and Tageteae with 86 taxa. All intermediates between $C_3$ and $C_4$ belong to *Flaveria* (Tageteae), except for *Parthenium hysterophorus* L. (Heliantheae). Most of the CAM or CAM intermediate taxa belong to Senecioneae and are succulent *Senecio* species, with two further CAM species being identified in Astereae and Eupatorieae. Again, these results are detailed in supplementary Table S2 and summarized in Fig. 1.

The phylogenetic tree we constructed for tribe Tageteae contains 113 species from 28 genera; species of *Flaveria*, *Pectis* and *Porophyllum* Adans. comprise 58% of the sampling. The literature search yielded photosynthesis metabolism information for 82 out of the 113 taxa. Ancestral character reconstruction for Tageteae shows two independent origins for $C_4$ metabolism in the tribe, one in the clade containing all species of *Pectis* and another nested within *Flaveria* (Fig. 2). *Flaveria* emerges in a clade with *Sartwellia* A.Gray and *Haploësthes* A.Gray, and the ancestral state in the genus is $C_3$ metabolism. *Flaveria* species with $C_2$ metabolism emerge as a single clade within *Flaveria*, subsequently split into two clades, one of them containing all true $C_4$ species and another containing only intermediate species. The two $C_4$-like species emerge separately, with *F. palmeri* J.R.Johnst. located in the true $C_4$ clade and *F. brownii* A.M.Powell emerging in the $C_2$ clade. *Pectis* is highly nested within a large $C_3$ clade that contains most of the sampled genera, corresponding to the “core Tageteae” as identified by Loockerman & al. (2003).

## ■ DISCUSSION

**Current knowledge about photosynthesis metabolism in Compositae. —** In this review of photosynthesis metabolism in Compositae, we gathered studies that ranged from those focused on single species and using multiple methods (e.g., Bauwe, 1984) to floristic-level screenings using simple methods at large geographic scales (e.g., Körner & al., 1988). For the first time, a database of photosynthesis studies for ca. 400 species in Compositae is presented, with carbon isotope values, the methods used for each species, and the original studies that generated the data (see suppl. Table S2).

One of the main observations from our literature review is that data on photosynthesis metabolism is still greatly lacking in Compositae. We recovered information for only ~1% of the species in the family, with most of these being restricted to a single tribe and its genera (Tageteae). However, it should be noted that this review likely under-represents the number of taxa studied, especially from literature containing only anatomical information that did not explicitly mention Kranz anatomy.

As currently understood, $C_4$ metabolism is restricted to two tribes: Tageteae and Coreopsideae, both members of the Heliantheae alliance, but relatively distantly related, with an MRCA around 19 million years ago (Panero & Crozier, 2016; Mandel & al., 2019). The data generated in the present study added sampling across 17 tribes previously untested for metabolism type, for a total of 36 tribes. However, we did not find $C_4$ pathways in taxa beyond these two tribes. Photosynthesis metabolism has not been studied in an evolutionary framework at the family level, with the latest family-wide phylogeny of Compositae (Mandel & al., 2019) sampling two species of Tageteae that present $C_2$ metabolism and one species of Coreopsideae for which no information about photosynthesis metabolism is available.

Ancestral state reconstruction on our phylogeny confirms that within Tageteae, there have been at least two independent evolutions of the $C_4$ state in *Flaveria* and *Pectis*, as previously discussed by other authors (Loockerman & al., 2003; McKown & al., 2005; Hansen & al., 2016). A previously published molecular phylogeny for *Flaveria* shows at least three origins for the true $C_4$ metabolism and intermediate states, although topologies from different markers are highly conflicting (McKown & al., 2005). While the phylogeny we present lacks $C_4$ taxa that would confirm these multiple origins in *Flaveria*, we confirm that *Pectis* represents an independent origin of $C_4$ within the tribe. Even though Tageteae has been more widely sampled for photosynthesis metabolism than any other tribe in Compositae, there is still a large gap in our knowledge, especially regarding species in the “core Tageteae” clade sensu Loockerman & al. (2003), where *Pectis* is inserted.

Previous studies have dated the crown ages of *Pectis* and *Flaveria* to 9.2 Ma and 4.2 Ma, respectively (Hansen & al., 2016; Christin & al., 2011). While we cannot be sure where along evolutionary stems transitions may have occurred, it is estimated that the origin of metabolism in *Pectis* is at youngest 9 Ma (Hansen & al., 2016), while the appearance of $C_2$ metabolism in *Flaveria* is around 3 Ma, with true $C_4$ appearing at less than 1 Ma (Christin & al., 2011). These age estimates both fit with the scenario of global warming and drying coincident with the expansion of $C_4$ grasslands, bursts of diversification in $C_4$ monocots and eudicots, and increased diversification of several succulent lineages often associated with the decrease in atmospheric $CO_2$ levels that occurred during the late Miocene-early Pliocene (E.J. Edwards & al., 2010; Arakaki & al., 2011).

These differences in clade and transition ages also fit the hypothesis that smaller, more recently radiated clades (*Flaveria*) will exhibit intermediate metabolism in a small number of species, whereas older and larger radiations (*Pectis*) have been allowed more time for the transition through or extinction of, intermediate phenotypes with subsequent diversification (E.J. Edwards, 2019). Given that phylogenies represent a certain point

**Table 1.** Number of taxa presenting $C_3$, $C_4$, $C_2$, and CAM metabolism, summarized by tribe and including results from the literature review and new additions from the present study.

| Tribe | Total no. species | Taxa with information about metabolism | No. of $C_3$ | No. of $C_4$ | No. of $C_2$ | No. of CAM |
|---|---|---|---|---|---|---|
| Anthemideae | 1800 | 14 | 14 | 0 | 0 | 0 |
| Arctotideae | 200 | 7 | 7 | 0 | 0 | 0 |
| Astereae | 3080 | 34 | 33 | 0 | 0 | 1 |
| Bahieae | 83 | 3 | 3 | 0 | 0 | 0 |
| Barnadesieae | 91 | 5 | 5 | 0 | 0 | 0 |
| Calenduleae | 120 | 3 | 3 | 0 | 0 | 0 |
| Cardueae | 2500 | 13 | 13 | 0 | 0 | 0 |
| Cichorieae | 1100 | 6 | 6 | 0 | 0 | 0 |
| Coreopsideae | 550 | 33 | 10 | 23 | 0 | 0 |
| Corymbieae | 9 | 1 | 1 | 0 | 0 | 0 |
| Dicomeae | 100 | 2 | 2 | 0 | 0 | 0 |
| Eremothamneae | 3 | 1 | 1 | 0 | 0 | 0 |
| Eupatorieae | 2200 | 4 | 3 | 0 | 0 | 1 |
| Gnaphalieae | 1240 | 6 | 6 | 0 | 0 | 0 |
| Gochnatieae | 70 | 3 | 3 | 0 | 0 | 0 |
| Hecastocleideae | 1 | 1 | 1 | 0 | 0 | 0 |
| Helenieae | 120 | 6 | 6 | 0 | 0 | 0 |
| Heliantheae | 1461 | 21 | 20 | 0 | 1 | 0 |
| Hyalideae | 6 | 1 | 1 | 0 | 0 | 0 |
| *Cyclolepis* | 1 | 1 | 1 | 0 | 0 | 0 |
| Inuleae | 687 | 1 | 1 | 0 | 0 | 0 |
| Liabeae | 174 | 2 | 2 | 0 | 0 | 0 |
| Madieae | 203 | 2 | 2 | 0 | 0 | 0 |
| Millerieae | 380 | 2 | 2 | 0 | 0 | 0 |
| Moquinieae | 2 | 1 | 1 | 0 | 0 | 0 |
| Nassauvieae | 313 | 2 | 2 | 0 | 0 | 0 |
| Neurolaeneae | 153 | 1 | 1 | 0 | 0 | 0 |
| Onoserideae | 52 | 1 | 1 | 0 | 0 | 0 |
| Perityleae | 84 | 1 | 1 | 0 | 0 | 0 |
| Pertyeae | 70 | 2 | 2 | 0 | 0 | 0 |
| Platycarpheae | 3 | 1 | 1 | 0 | 0 | 0 |
| Senecioneae | 3500 | 48 | 17 | 0 | 0 | 31 |
| Stifftieae | 44 | 3 | 3 | 0 | 0 | 0 |
| Tageteae | 267 | 154 | 54 | 86 | 14 | 0 |
| Tarchonantheae | 13 | 1 | 1 | 0 | 0 | 0 |
| Vernonieae | 1540 | 27 | 27 | 0 | 0 | 0 |
| Wunderlichieae | 35 | 1 | 1 | 0 | 0 | 0 |
| **Total** | 22,255 | 415 | 258 | 109 | 15 | 33 |

Total species numbers extracted from Mandel & al. (2019). A full listing of all species is available in suppl. Table S2.

in a continuum, upon which we interpret past history, the evolutionary potential of lineages in *Flaveria* that present intermediate metabolism is unknown. Properly testing this hypothesis relies upon understanding the fate of intermediate metabolisms. Further work using in-depth macroevolutionary analyses over a well-sampled, time-calibrated phylogeny could help untangle the role of extinction and diversification in Tageteae and could become a new avenue for photosynthesis studies in Compositae and other angiosperms.

Much less data is available for Coreopsideae, both in the physiological and phylogenetic ends. $C_4$ metabolism has been identified in 20 species belonging to five genera, three of each have been sampled in a phylogeny, with one species each (Kimball & Crawford, 2004; Crawford & al., 2009). Taxonomy in Coreopsideae is problematic, especially around *Bidens* L. and *Coreopsis* L., and one of the genera where $C_4$ is known to occur, *Glossogyne* Cass., has been proposed as synonym of other genera (Crawford & al., 2009). Even though the three species sampled in phylogenies form a clade in these analyses, possibly indicating a single transition of photosynthesis type, too few $C_4$ species from the total of ca. 60 have been studied either for metabolic type or in a phylogenetic context to confidently rule out parallel evolution of this pathway. No time-calibrated phylogeny is available for Coreopsideae, with the timing of the transition from $C_3$ to $C_4$ unknown, although the origin of the tribe itself is estimated sometime in the Miocene (Panero & Crozier, 2016; Mandel & al., 2019). Different types of Kranz anatomy are found across angiosperms, and three of them are found in Coreopsideae, with *Isostigma* presenting an exclusive type not found in other angiosperms (Peter & Katinas, 2003; Muhaidat & al., 2007).

Our results did not identify any new instances of CAM metabolism across the family. True CAM photosynthesis was

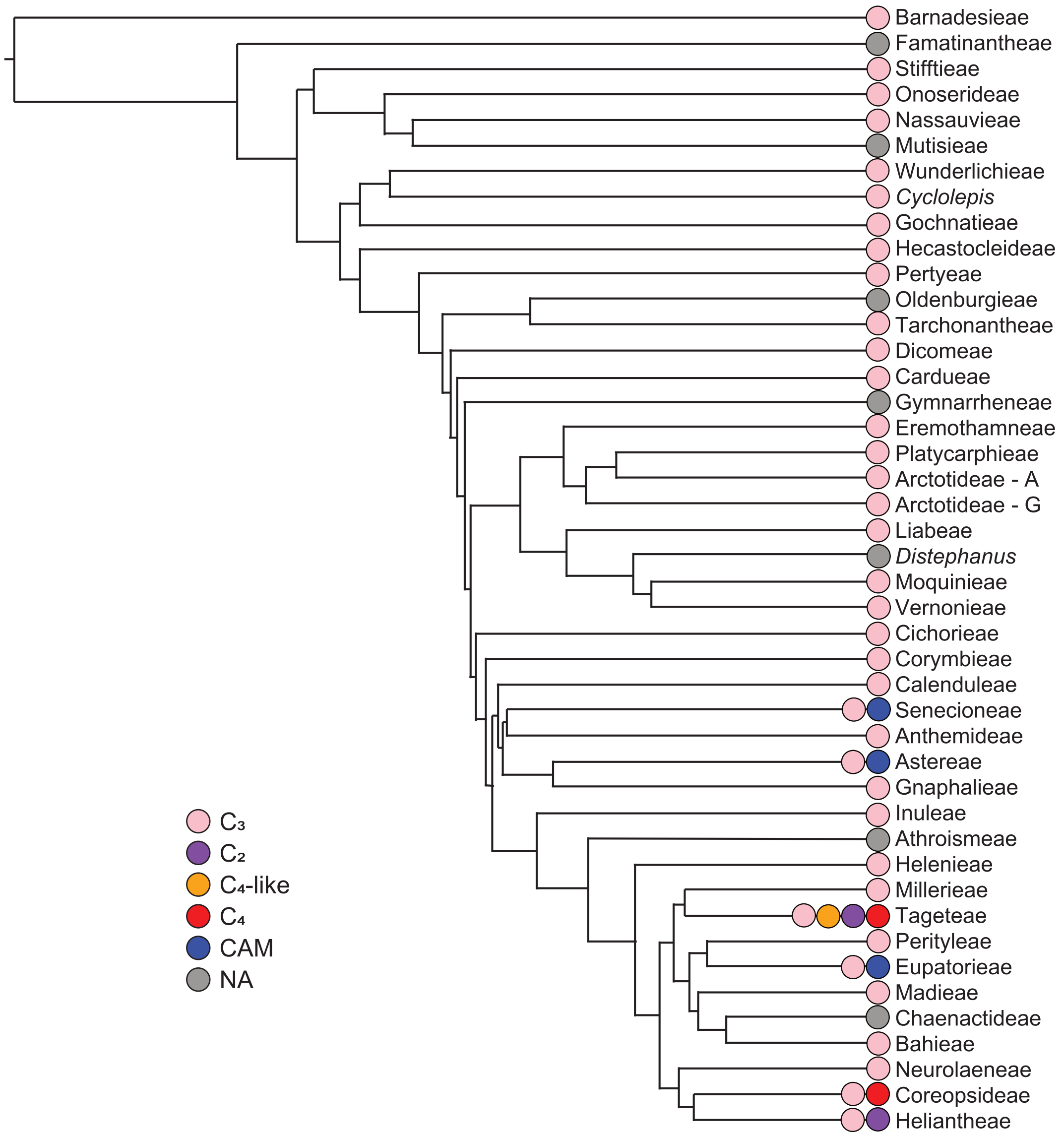

**Fig. 1.** Distribution of photosynthesis metabolism in Compositae: Summarized tribe-level tree of Compositae, based on Mandel & al. (2019). Colored dots indicate what types of metabolism have been identified in each branch.

previously recorded in the tribe Senecioneae, where it is associated with succulence (Szarec & Ting, 1977; Fioretto & Alfani, 1988), and in one species of Astereae, *Tripolium pannonicum* (Jacq.) Dobrocz. (Ganzmann & von Willert, 1972). The taxonomy of Senecioneae has been a long-standing problem, especially in relation to the large genus *Senecio*, with many genera being segregated and lumped back into it by different authors. Given the lack of well-resolved phylogenies for the tribe Senecioneae and its genera, it is hard to infer how many times this type of metabolism arose (Nordenstam & al., 2009). Genera that have been identified as presenting CAM photosynthesis, namely *Kleinia*, *Notonia* DC., and *Notoniopsis* B.Nord., emerge in a clade with other succulent species of *Senecio* usually referred as the *Curio* group, but away from the species in the *Senecio medley-woodii* group, in which CAM is also found, possibly indicating multiple origins of this type of metabolism in this tribe (Nordenstam & al., 2009).

The difficulties of identifying CAM metabolism and especially species that cycle between $C_3$ and CAM, due to lack of specialized anatomy and carbon isotope values similar to $C_3$ species, have been well reviewed by other authors (e.g., E.J. Edwards, 2019; Heyduk & al., 2019). One interesting approach

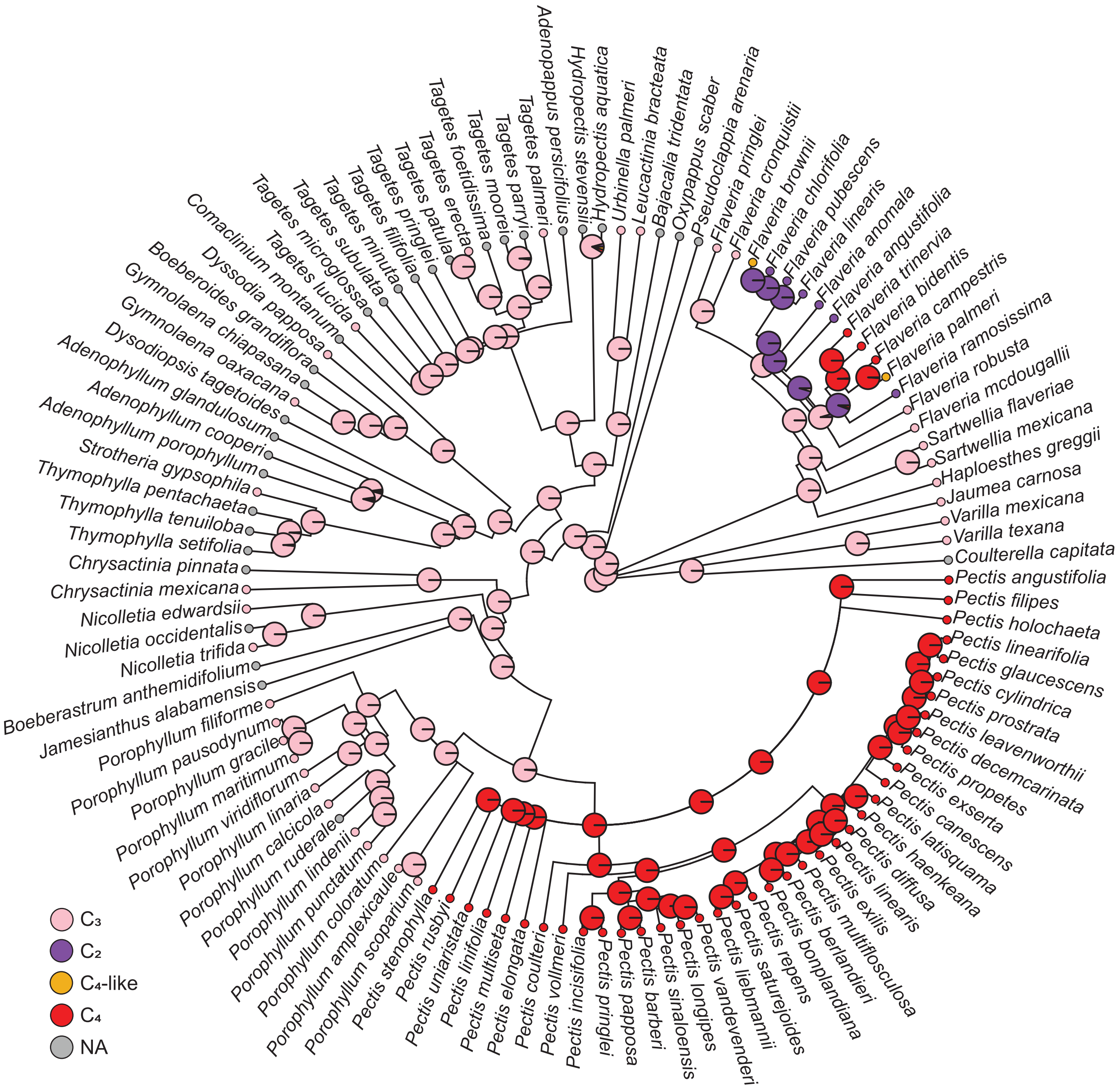

**Fig. 2.** Distribution of photosynthesis metabolism in Compositae: Ancestral character reconstruction of photosynthesis metabolism in Tageteae. Colored dots indicate what types of metabolism have been identified in each branch.

that could circumvent this issue was recently demonstrated with *Mikania micrantha* Kunth, a non-succulent species in the tribe Eupatorieae. The recently published genome of the species shows the presence of a high number of copies of genes involved with CAM photosynthesis, with complementary RNA-seq studies showing that the primary use of this pathway is likely to be carbon fixation (Liu & al., 2020). This workflow could be expanded to other Compositae, using the genomes that have been published for the family (e.g., sunflower, lettuce, *Mikania*), and the resources available in databases such as the Sequence Read Archive, and RNA-seq data from the 1kp project (One Thousand Plant Transcriptomes Initiative, 2019). Using bioinformatics-based approaches could help narrow down candidate species for dedicated studies in a cost-effective way.

**Future directions. —** Given the diversity and importance of Compositae in habitats across the world, a concerted and systematic effort to characterize metabolic pathways across the family would represent an important step in understanding the evolution and role of different photosynthesis types at large scales, and across a range of environments. This is particularly salient in a time of shifting global climate conditions, when acquiring and organizing biodiversity data is essential for understanding their impact and informing conservation strategies. This requires two streams of data: identifying the metabolic pathway for each species, and a systematic framework within which to contextualize these character states. The lack of knowledge of functional traits has been identified as a shortfall for large-scale biodiversity studies ("Raunkiæran shortfall", Hortal & al., 2015), and it is obvious how much it has impacted diversification studies in Compositae. High-throughput screening of photosynthesis metabolism types could be carried out through carbon isotope analysis, as it offers a relatively rapid method for screening between $C_3$, $C_4$ and CAM pathways in plants. The results can be verified using anatomical dissection of Kranz anatomy, with both methods allowing the use of older preserved material. Associating both these methods also allows identification of possible intermediate phenotypes, i.e., plants that show a Kranz-like anatomy but present a $C_3$ carbon isotope ratio.

These data would need to be complimented by a robust, well-sampled phylogeny at least across key tribes, but ideally across the entire family. The development (Mandel & al., 2014) and refinement (Mandel & al., 2019) of nuclear genomic markers across the family makes the generation of a species-level phylogeny technically feasible. Collaboration across research groups using this standard marker set and a community approach to funding and generating data would benefit the whole field of Compositae systematics and evolutionary study.

Deep sequencing could also be used as a tool for differentiating among photosynthesis syndromes. Comparison of transcriptomes across species known to have $C_4$ or CAM metabolisms could be used to identify genes linked to these processes, as previously done with several species of *Flaveria* (Gowik & al., 2011) and more recently with *Mikania micrantha* (Liu & al., 2020). Given the increase in publicly available transcriptome data, it would be possible to screen taxa of unknown state for genes related to photosynthesis metabolism.

Similar data could be used to investigate a hypothesized link between whole-genome duplications (WGDs) and increased anatomical and morphological innovation, including the development of $C_4$ pathways (Monson & al., 2003). WGDs have already been associated with increased diversification rates within the Compositae (Barker & al., 2008, 2016; Huang & al., 2016), including in the branch leading to the Heliantheae alliance (Huang & al., 2016), which corresponds to the highest increase in diversification rate in the family (Mandel & al., 2019). WGD has also been identified on the branch leading to *Flaveria* (One Thousand Plant Transcriptomes Initiative, 2019), one of the few Compositae genera known to possess $C_4$ metabolism so far. In the related genus *Pectis*, ~20% of species are polyploid (Hansen & al., 2016); however, there is no information on whether they share a WGD event with *Flaveria*.

From the multiple studies in *Flaveria*, using a range of different physiological methods, we know that $C_4$ photosynthesis metabolism and anatomy are not homogeneous, with different intermediate phenotypes. Studies with $C_2$ plants across Angiosperms have shown that the assembly of $C_4$ pathways can occur in multiple ways (Sage & al., 2014; Edwards, 2019). While studies in *Flaveria* gave an important contribution to our knowledge about photosynthesis, studies in other Compositae taxa have been scarce, with few examples on the physiology of *Pectis* (e.g., Muhaidat & al., 2007) and anatomical data in *Isostigma* (Peter & Katinas, 2003) and *Senecio* (Fioretto & Alfani, 1988). Extending physiological studies to other $C_4$ and CAM taxa in Compositae could expand this knowledge.

Since the pioneer work by B.N. Smith & Turner (1975), there has been no other wide-scale sampling of carbon isotope ratios across the Compositae, except for few studies in specific groups (e.g., Hansen & al., 2016). Even though modest, our contribution is the largest sampling in Compositae since the 1970s. We failed to identify any new instances of $C_4$ or CAM metabolism within the family, despite focusing on species that grow in hot and dry environments and tribes that were not sampled in previous studies. Wide-scale carbon isotope sampling in environments favored by $C_4$ plants, such as North American deserts, the Succulent Karoo in South Africa, and Old World deserts (Ehleringer & Cooper, 1988; Rundel & al., 1999; Ziegler & al., 1981), also failed to uncover additional $C_4$ and CAM Compositae. While further shotgun sampling may uncover groups that have not been previously identified as having either of these processes, the strong association of $C_4$ and CAM with the hot and arid environments of southern North America, which is identified as a $C_4$ hotspot (Sage & al., 2011), suggest that this area may be worth targeting for further examples. The development of ecological niche modeling methods and macroevolutionary analyses that allow modeling diversification rates in relation to climatic variables could also be used to estimate and project associated climatic conditions both in North America and globally to identify areas where non-$C_3$ metabolism may be more likely to be found, narrowing down the number of taxa to screen. It is likely that there are more Compositae species that present some type of intermediate phenotypes between $C_3$ and $C_4$ or CAM that are undiscovered. These species would not be evident in surveys using only carbon isotope analysis, thus requiring a combination of different techniques. As intermediate lineages are usually found in close relation to taxa that present $C_4$ photosynthesis (Sage & al., 2018), efforts could be made to increase photosynthesis studies in taxa close to the known $C_4$ species in Tageteae and Coreopsideae, for example.

The abundance of Compositae in harsher, dry and hot environments and the apparent lack of physiological changes to photosynthesis metabolisms also raises questions about what other possible strategies these plants use to thrive in these unfavorable environmental conditions. The annual/biennial growth form is common in the family, occurring in all tribes, which would allow for plants in seasonal environments to opportunistically complete their life cycle in the less hot and dry months, thus bypassing the need for extreme adaptations (examples in Downton & al., 1984; Ludwig & al., 1988). Nevertheless, this hypothesis does not hold when we look at growth forms in *Flaveria*, where $C_3$ and $C_4$ species that co-occur in the same regions in south-central Mexico present perennial and annual habits, respectively (Sudderth & al., 2009). Annual and perennial growth forms are also present in *Pectis*, where all species present $C_4$ metabolism (Hansen & al., 2016). The association of $C_3$ or $C_4$ metabolism with the annual habit seems to be linked with other natural history traits: In North American deserts, most $C_3$ plants are winter annuals, while $C_4$ metabolism is found in summer annuals (Ludwig & al., 1988). One of the effects of the Raunkiæran shortfall in Compositae is that there is no organized information about growth forms in the family, nor of where annual species occur, precluding the community from tackling these questions on a family-wide scale.

Other morphological features frequently hypothesized as adaptations to drought- and heat-stress are frequently seen across different tribes, such as deeply lobed leaves covered with reflective trichomes or wax, hard and spiny leaves, and a highly sculptured exine that keeps the integrity of pollen grains in case of desiccation. Functional studies including some of these traits in relation to extreme environments have been carried out with Compositae species, such as the spectral reflectance of leaf hairs in the desert-dweller *Encelia* Adans. (Ehleringer & Cook, 1987), the effect of leaf angle and orientation in photosynthesis in the prairie inhabitant *Silphium terebinthinaceum* Jacq. (M. Smith & Ullberg, 1989), regulation of pollen volume in Cichorieae, which are frequent in Mediterranean climates (Blackmore, 1982), and resource-use traits in the granite outcrop endemic *Helianthus porteri* (A.Gray) Pruski (Bowsher & al., 2016), but a full review is out of the scope of this paper.

The success of Compositae in extreme environments might be a combination of morphological, anatomical and physiological changes, without any one of them standing out as the main driver. Vicki Funk (2018) called the 21st century the "Age of Thinking Big". We are at a privileged point in time where massive collections of specimens have been gathered, public databases of genomic and morphological data assembled, with the potential for deeper than ever understanding about the evolutionary relationships among all organisms. The call for a collaborative effort in attacking large-scale questions about the drivers of evolution and diversification of Compositae perfectly represents Vicki Funk's spirit and legacy and should be motivation for all of us. As a community, we have identified gaps in our knowledge of the family that hinder biodiversity studies, such as the lack of information in traits like photosynthesis metabolism and growth form. Thus, we hope to renew the interest in the study of photosynthesis pathways in the family, not only from the physiological aspect, but combining information from new sources, such as phylogenies, niche modeling, and macroevolutionary analysis.

## ■ AUTHOR CONTRIBUTIONS

CMS, RDE and JRM designed the research, JLG and CMS conducted the literature review, ERM and CMS prepared material for carbon isotope analysis, JRM provided funding for laboratory procedures, RDE produced and provided the phylogenetic tree, CMS and RDE analyzed and interpreted the data, CMS and RDE wrote the manuscript; all authors read, commented and approved the final version. — CMS, https://orcid.org/0000-0003-3349-5081; RDE, https://orcid.org/0000-0002-4993-2453; JRM, https://orcid.org/0000-0003-3539-2991

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