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Carbon isotope composition of plant photosynthetic tissues reflects a Crassulacean Acid Metabolism (CAM) continuum in the majority of CAM lineages

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ABSTRACT

The stable carbon isotope composition of plant tissues, commonly expressed as $\delta^{13}C$, holds a wealth of information about photosynthetic pathway, water relations and stress physiology. Crassulacean acid metabolism (CAM) is a derived form of photosynthesis that allows plants to fix carbon at a higher water-use efficiency compared to the ancestral C_3 photosynthesis. While the central carbon-fixing enzyme of C_3 plants, Rubisco, strongly discriminates against the heavy ^{13}C isotope, CAM is characterized by a dual use of Rubisco and the much less discriminating PEP carboxylase as carbon-fixing enzymes, causing the $\delta^{13}C$ values of CAM plant tissues to be generally less negative than those found in C_3 plants. Past studies of $\delta^{13}C$ variation in CAM plant lineages have repeatedly found a bimodal distribution with very few samples representative of the range around -20‰ that is intermediate between C_3- and CAM-like values. Although $\delta^{13}C$ values of facultative CAM plants have long been known to extend well into the range below -20‰, this value is often tentatively used as threshold for character coding to distinguish C_3 from CAM species in studies of CAM evolution. Compiling 6623 $\delta^{13}C$ values reported in the literature for CAM/C_3 vascular plant lineages and presenting new data for 581 accessions mainly of the succulent Mesembryanthemoideae (Aizoaceae) and Aeonieae (Crassulaceae), we here investigate the diverse patterns of $\delta^{13}C$ distribution in different plant families and sub-familial taxa and demonstrate that a bimodal distribution is not universally present in all lineages. Moreover, we show by means of mixture modelling that the bimodal distribution of $\delta^{13}C$ values in the full dataset as well as in the very well-sampled Bromeliaceae is best described by a combination of three rather than two Gaussian distributions with one intermediary cluster between the more evident clusters of C_3- and CAM-like values. In view of these results and the furthermore emerging unimodal distribution of $\delta^{13}C$ values in Mesembryanthemoideae with mean close to -20‰, we conclude that the evident continuum between CAM and C_3 photosynthesis cautions against the usage of a $\delta^{13}C$ threshold in macroevolutionary studies. Finally, the observed diversity of $\delta^{13}C$ distribution patterns between monophyletic lineages urges for lineage-specific reconstructions rather than a unifying model of CAM evolution.

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1. Introduction

The stable isotope composition of biotic and abiotic materials has been utilized as valuable source of information on processes in the natural environment (West et al., 2006). The ratio of carbon isotopes in plants, described as $\delta^{13}C$ value (see definition below), can serve as powerful indicator of photosynthetic processes in plants and how they respond to environmental forcing (Farquhar et al., 1989a; Ehleringer and Monson, 1993). $\delta^{13}C$ values in tree rings (Loader et al., 2003; McCarroll and Loader, 2004; Sarris et al., 2013) or cacti spines (Hultine et al., 2018) can, for example, be used to inform about intrinsic water-use efficiency or available moisture, respectively, and thereby to track environmental stress limiting photosynthesis. The $\delta^{13}C$ values in plant tissue can also be employed to distinguish among different photosynthetic pathways in plants such as the C_3, C_4 or Crassulacean Acid Metabolism (CAM) pathway (Cernusak et al., 2013). Building on this distinction, another fascinating example comes from the analysis of $\delta^{13}C$ values in mammalian teeth (Cerling et al., 1997) and sediment samples (Quade et al., 1989) that inform on the expansion of C_4 grasslands during the Miocene (Edwards et al., 2010).

Of all Earth's atmospheric CO_2 molecules, 98.9 % contain a ^{12}C isotope and only 1.1 % the heavier ^{13}C isotope. The ratio of both isotopes in a sample is related to the ratio of both isotopes in a standard and expressed as a delta value, i.e. $\delta^{13}C$

$$\delta^{13}C = \frac{\left(\frac{^{13}C}{^{12}C}\right)_{sample}}{\left(\frac{^{13}C}{^{12}C}\right)_{standard}} - 1$$

commonly using the Vienna Pee Dee Belemnite (VPDB) as standard and replacement for the used-up Pee Dee Belemnite (PDB) from South Carolina, USA (Coplen, 2011).

The photosynthetic tissue of plants is typically ^{13}C-depleted compared to the substrate of photosynthesis which is atmospheric CO_2 with a $\delta^{13}C$ value of around -8.5‰ (see Scripps CO_2 programme: http://scrippsco2.ucsd.edu/). Due to the slower diffusion of the $^{13}CO_2$ molecule compared to $^{12}CO_2$ from the atmosphere into the stomatal cavity, intercellular CO_2 is typically ^{13}C-depleted by a maximum diffusional fractionation of 4.4‰ (O'Leary, 1981; Farquhar, 1983). In addition, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) binds $^{13}CO_2$ less frequently than $^{12}CO_2$ resulting in maximum kinetic fractionation of 29‰ for this enzymatic process (Roeske and O'Leary, 1984). Roeske and O'Leary (1984) explain this pronounced discrimination of Rubisco against $^{13}CO_2$ with apparently high dissociation rates of the substrate-enzyme complex during the initial carboxylation steps. Thus, the $\delta^{13}C$ value of a C_3 plant where Rubisco is the only carboxylating enzyme within the photosynthetic cycle usually lies around -27.1‰ (O'Leary, 1988). The expression of the diffusional fractionation and the kinetic fractionation depend, albeit in opposite direction, on the leaf internal CO_2 concentration (c_i) (Farquhar et al., 1982, 1989a, 1989b; O'Leary, 1988). The observed variability of $\delta^{13}C$ values in C_3 plants ranges from -20 to -37.5‰ (Farquhar et al., 1989a; Winter and Holtum, 2002). This variability is well reflected, e.g., in the $\delta^{13}C$ values of C_3 halophytes experiencing salinity to varying degrees (Guy et al., 1980).

Plants that exhibit a carbon concentrating mechanism (CCM) through initial fixation of carbon by phosphoenolpyruvate carboxylase (PEPC) such as C_4 plants and plants engaging in CAM often differ significantly in their $\delta^{13}C$ value from C_3 plants. CAM plants separate carbon fixation through PEPC and carbon re-fixation through Rubisco (the first step of the Calvin-Benson cycle) temporally. When operating in CAM mode, the plants open their stomata at night when water loss by transpiration is low. CO_2 is diffusing into the leaf and is fixed by PEPC and the resulting malic acid is stored in sizeable vacuoles. At daytime when stomata are usually closed, malic acid is transported out of the vacuole and decarboxylated and CO_2 is released again for final fixation by Rubisco (Osmond, 1978; Winter and Smith, 1996). Depending on the overall leakiness of CO_2, the fractionations associated with the primary CO_2 fixation by PEPC and the secondary CO_2 fixation by Rubisco more or less cancel each other in magnitude (Farquhar et al., 1989a). The diffusional fractionation of 4.4‰ then primarily dictates the $\delta^{13}C$ values of plants that solely fix atmospheric CO_2 via PEPC, such as C_4 plants and full CAM plants in the sense of Winter (2019). Assuming a $\delta^{13}C$ value of -8.5‰ for the atmospheric source CO_2, their tissues theoretically should reach a $\delta^{13}C$ value of -12.9‰.

However, in addition to nocturnal CO_2 fixation via PEPC, CAM photosynthesis usually involves more or less extended diurnal phases of primary CO_2 fixation via Rubisco at dawn and dusk, i.e., phases II and IV, respectively, of the model defined by Osmond (1978). Moreover, not all CAM plants express the CAM pathway constitutively, but instead many of them are able to switch between C_3 photosynthesis and CAM according to developmental (e.g., Holthe et al., 1992) and/or environmental triggers such as drought stress (e.g., Winter et al., 1978; Bloom and Troughton, 1979) or salinity (Winter and von Willert, 1972). This ability to operate in either a C_3 or CAM mode is usually called facultative CAM in contrast to obligate or constitutive CAM where a plant cannot abandon its rhythm of nocturnal CO_2 fixation and the diurnal, more or less pronounced closure of stomata (Winter, 1985; Griffiths, 1989; Kluge and Brulfert, 1996; Winter et al., 2008; Winter, 2019). It is important to note that the distinction between facultative and obligate CAM is independent of the distinction between full, strong and weak CAM, all three of which referring to the rate of nocturnal CO_2 assimilation and its relative contribution to overall assimilation compared to the contribution of diurnal CO_2 fixation (Silvera et al., 2010a; Males and Griffiths, 2017; Winter, 2019). For example, in the course of a previously published drought experiment on *Calandrinia* Kunth (Montiaceae; Hancock et al., 2019) nearly half of the sampled species were identified as constitutive but low-level CAM plants with significant but exiguous nocturnal acid accumulation. This picture is furthermore complicated by the occurrence of CAM cycling and CAM idling (Sipes and Ting, 1985). Both strategies entail nocturnal fixation of respiratory CO_2 via PEPC behind closed stomata, but they are differentiated by diurnal opening of stomata in CAM cycling and fulltime stomatal closure in CAM idling. All these types of CAM expression were well defined by Winter (2019; however, see Lüttge (2004) for an alternative definition of "full CAM"). Nevertheless, we here choose the broader definition of the term "CAM plant" sensu Gilman and Edwards (2020), i.e., not in the sense that a CAM plant is defined by gaining most of its carbon by nocturnal CO_2 fixation and thus by a $\delta^{13}C$ value less negative than -20‰, but that it expresses the CAM cycle in whatever way and irrespective of the proportion and magnitude of nocturnal carbon assimilation.

With respect to this high diversity in the expression of CAM and the above-mentioned differential isotopic fractionations associated with PEPC and Rubisco, $\delta^{13}C$ values of CAM plants become more negative when more primary CO_2 assimilation takes place by Rubisco. Moreover, there are additional factors that influence the $\delta^{13}C$ value such as the developmental stage of the plant or leaf, water-use efficiency (O'Leary, 1988; Farquhar et al., 1989b; Winter et al., 2005), mesophyll conductance (Griffiths et al., 2007; Seibt et al., 2008), the isotopic composition of source CO_2 (Farquhar et al., 1989a) and environmental factors that influence the diffusional limitation of CO_2 uptake, most importantly light and air humidity (Cernusak et al., 2013), but also salinity (Farquhar et al., 1989a) and elevation (Crayn et al., 2015). Therefore, CAM plants feature a wide array of $\delta^{13}C$ values that span the whole range from typical C_3-like to typical C_4-like values.

As for C_4 plants, $\delta^{13}C$ values have been used to predict the ability of a plant species to perform CAM (Bender et al., 1973; Smith and Winter, 1996 and references therein). Experimentally testing the linear relationship between plant $\delta^{13}C$ and the proportion of nocturnal relative to diurnal carbon assimilation that had been proposed theoretically by O'Leary (1988), Winter and Holtum (2002) showed an increase of 1.8‰ in $\delta^{13}C$ for every 10 % increase of dark fixated carbon in unstressed

plants of various species. However, the study also showed that the $\delta^{13}C$ value cannot differentiate low to intermediate CAM activity (less than 33 % dark fixation) from C_3 photosynthesis. Therefore, $\delta^{13}C$ values are useful to indicate strong CAM, but might fail to detect facultative and certainly cannot detect weak CAM. Accordingly, Winter et al. (2015) and Winter (2019) used the $\delta^{13}C$ values to tentatively classify the various CAM types and proposed partly overlapping ranges, with -8 to -20‰ to be indicative of strong CAM and values more negative than -20‰ indicative of C_3 photosynthesis, but with the possibility of CAM activity to some degree in species within the C_3 cluster. Such species are defined as C_3-CAM species, provided they are proven to express the CAM cycle by means of 24-h gas exchange or titratable acidity measurements (Winter, 2019), both of which are common methods with which to assess and quantify the operation of a CAM cycle. Looking at an important model plant for facultative CAM, *Mesembryanthemum crystallinum* L., the distinction between (strong) CAM and C_3-CAM intermediacy is challenged as salinity, photosynthetic mode and age were found to influence its $\delta^{13}C$ (Winter and Holtum, 2005), and as this species spans a range from -16 to -30‰ which covers all photosynthetic types (Winter et al., 1978; Bloom and Troughton, 1979; Gilman and Edwards, 2020).

In comparison to the evolution of C_4 photosynthesis, little is yet known about the evolution of CAM, and efforts in this direction are complicated by the inherently flexible nature of CAM that makes coding of CAM and non-CAM difficult (Edwards, 2019). Despite these challenges, $\delta^{13}C$ values may serve this purpose in phylogenetic studies of CAM evolution (e.g., Crayn et al., 2004; Silvera et al., 2009; Heyduk et al., 2016; Hancock et al., 2019; Li et al., 2019; De la Harpe et al., 2020). However, the coding used in these studies reduces the continuum of CAM activity to a threshold of CAM versus weak CAM or C_3 at -19‰ (De la Harpe et al., 2020), -22‰ (Silvera et al., 2009) or -20‰ (all other studies) informed by studies that suggest a bimodal distribution of the $\delta^{13}C$ value indicating that species with $\delta^{13}C$ less negative than -20‰ perform strong CAM. A bimodal distribution of the $\delta^{13}C$ value has first been shown by Winter and Holtum (2002: figure 7) summarizing $\delta^{13}C$ values of 506 species from nine plant families that have evolved CAM, and was supported several times since then (in Bromeliaceae: Crayn et al., 2004, 2015; in Euphorbiaceae: Horn et al., 2014; in Orchidaceae: Silvera et al., 2010b). Also, Edwards (2019) in a recent review stresses the bimodal pattern of $\delta^{13}C$ in photosynthetic metabolism occurring in multiple CAM-evolving lineages as striking and proposes evolutionary trajectories that cause the majority of species to use either mostly C_3 photosynthesis or mostly CAM. Edwards (2019) argues that CAM evolution might not be a continuum of phenotypes in which the CAM pathway is gradually upregulated (as proposed by Bräutigam et al., 2017) but evolves from C_3 + weak-CAM ancestors at different rates according to lineage-specific constraining or enhancing traits and as a result of limited C_3 performance with increased succulence as a driving selective force. Evidence for a more continuous model of CAM evolution from C_3 photosynthesis primarily comes from metabolic flux analysis (Bräutigam et al., 2017) and would be advocated for by a high proportion of C_3-CAM intermediate $\delta^{13}C$ values (i.e., around -20‰) in respective CAM lineages.

This study reviews available $\delta^{13}C$ data of the major CAM families, i.e., Aizoaceae, Asparagaceae, Asphodelaceae, Apocynaceae, Bromeliaceae, Cactaceae, Clusiaceae, Crassulaceae, Didiereaceae, Euphorbiaceae and Orchidaceae, as well as other families of (C_3 and) CAM plants, and adds new data for Crassulaceae tribe Aeonieae and Aizoaceae subf. Mesembryanthemoideae. Combining all data, we investigate the distribution of $\delta^{13}C$ values at a finer systematic scale with a dense sampling within lineages, testing the general existence of a bimodal distribution of $\delta^{13}C$. We set out the hypotheses (1) that the frequently found bimodal distribution of $\delta^{13}C$ values is not evident in all CAM plant lineages and (2) that the overall distribution of $\delta^{13}C$ values across all lineages - despite its bimodality - is best described by a mixture of three clusters, given the high frequencies of intermediate values around -20‰ in certain taxa of CAM plants. Jointly, our results demonstrate that the patterns of distribution of $\delta^{13}C$ values are diverse among different monophyletic lineages and that a simple classification of CAM presence based on any $\delta^{13}C$ threshold understates the actual diversity of species' photosynthetic phenotypes.

2. Material & methods

2.1. New $\delta^{13}C$ measurements

Samples for isotope analysis were taken from leaf or photosynthetically active stem tissue. The samples were collected from plants in their natural habitats and silica-dried or obtained from herbarium specimens (online supplementary Table S1). We sampled 358 accessions from 100 species of *Mesembryanthemum* L. (the only genus in subf. Mesembryanthemoideae of Aizoaceae sensu Klak et al., 2007), 43 accessions from 39 species of other Aizoaceae genera and 180 accessions from 40 species of the Crassulaceae genera *Aeonium* Webb & Berthel., *Aichryson* Webb & Berthel., *Monanthes* Haw. and *Sedum* L. The aim of our relatively deep sampling strategy (several samples per taxon) was to represent as much of the $\delta^{13}C$ variation in each sampled species as possible. Moreover, the accessions of *Mesembryanthemum* were selected to represent a high diversity of species with different life- and growth-forms (i.e., annuals, perennials, geophytes, stem- and leaf-succulents) that had never before been sampled for $\delta^{13}C$ analysis (previous studies mainly focused on *M. crystallinum*), and thus our sample includes almost all species of subfamily Mesembryanthemoideae (± 105 spp.; Klak et al., 2007). To cover the range of seasons throughout the vegetation period, most species of *Mesembryanthemum* were sampled thrice and samples had been collected at different times of the year and from different localities. The vast majority of accessions of Aeonieae were collected by two of the authors in spring 2018 and were sampled to cover a broad range of populations and the distribution area rather than seasonal variation.

Samples were ground for one minute at 20 Hz in a Retsch MM301 vibrating mill (Retsch GmbH, Haan, Germany) using steel balls. 200−500 µg of pulverized sample material were weighed into tin capsules on a high precision scale (Mettler-Toledo XP 6, Mettler-Toledo Int. Inc., Columbus, Ohio, USA) and sealed tightly. Total carbon content and $\delta^{13}C$ values were analyzed at the lab of the Applied and Analytical Palaeontology group at the Institute for Geoscience, JGU Mainz, and at the stable isotope ecology lab at the Department of Environmental Sciences, University of Basel. In both laboratories, $\delta^{13}C$ values were normalized to the VPDB scale using laboratory standards with known isotopic compositions, which were calibrated against international reference materials and standards. In Basel, analyses were performed with a Flash 2000 elemental analyser (EA) coupled to a Thermo Finnigan Delta Plus XP isotope ratio mass spectrometer via a Conflo IV interface (all Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). In Mainz, a Flash 2000 EA coupled to a MAT253 isotope ratio mass spectrometer via a Conflo IV interface (all Thermo Scientific, Wilmington, Delaware, USA) was used analogously. Analytical precision was assessed as the standard deviation of repeated analyses of a spirulina powder used as a quality control standard. The long-term analytical precision of $\delta^{13}C$ values from Basel is 0.2‰, for this study it was 0.1‰.

2.2. $\delta^{13}C$ values taken from literature

Only values measured against PDB or VPDB from samples of individuals growing in natural habitats were considered. These were extracted from the following 53 studies: Arroyo et al. (1990); Bloom and Troughton (1979); Bonal et al. (2000); Boutton et al. (1999); Buss (2020); Chomicki and Renner (2016); Crayn et al. (2015); dos Santos et al. (2015); Earnshaw et al. (1987); Ehleringer et al. (1987); Griffiths and Smith (1983); Guralnick et al. (2008); Herrera (2009); Heyduk (personal conversation based on Heyduk et al., 2016); Holtum and

Winter (2005); Holtum et al. (2004); Horn et al. (2014); Kluge (1977); Kluge et al. (1991, 1993, 1995); Martin et al. (1990); Mooney et al. (1977, 1989); Mort et al. (2007); Osmond et al. (1975); Oyungerel et al. (2004); Pate et al. (1998); Popp et al. (1987); Ramírez and Herrera (2017); Rao et al. (1979); Rundel and Dillon (1998); Rundel et al. (1979, 1999, 2003); Sanaiotti (1996); Silvera et al. (2010b); Skrzypek et al. (2013); Szpak et al. (2013); Teeri et al. (1981); Tenhunen et al. (1982); Ting et al. (1987); Torres-Morales et al. (2020); Troughton et al. (1977); Vargas-Soto et al. (2009); Wester et al. (2011); Winter (1979); Winter et al. (1981, 1983); Ziegler (1996); Ziegler et al. (1981); Zotz (2004), and Zotz and Ziegler (1997). Because Δ instead of $\delta^{13}C$ values were published by Rundel and Dillon (1998), and Rundel et al. (1999, 2003), the values were converted into $\delta^{13}C$ according to the equation provided in these references. A total of 7204 $\delta^{13}C$ values from 24 plant families were compiled in the final dataset (Table 1; online supplementary Table S2).

Table 1
Number of samples (*n*) and statistical test results for the 24 families and 27 sub-familial taxa sampled in this study. As a result of Hartigans' Dip-Test, the *p* value, indicating the probability that the test statistic is observed if the true distribution of $\delta^{13}C$ values were unimodal, is given for each listed taxon along with the resulting modality (uni = unimodal; multi = multimodal). The number of clusters (K) and difference in BIC (Bayesian Information Criterion) score (dBIC) compared to three clusters as inferred by mixture modelling is given along with the mean (± standard deviation) of $\delta^{13}C$ values and area under the curve (AUC) of each of up to three inferred clusters. Only lineages with > 45 samples (in boldface) were included in the mixture modelling analyses. For results thereof, see also Figs. 1 and 2.

family	sub-familial taxon	*n*	*p* (inferred result)	K	dBIC	K_1 mean (± s.d.) [‰]; AUC [%]	K_2 mean (± s.d.) [‰]; AUC [%]	K_3 mean (± s.d.) [‰]; AUC [%]
Aizoaceae		471 (total)	0.756 (uni)	2	−15.21	−24.02 (± 2.92); 41.8	−16.87 (± 3.27); 58.2	–
	Acrosanthoideae	3	1 (uni)					
	Aizooideae	31	0.876 (uni)					
	Mesembryanthemoideae	390	0.699 (uni)	1	−25.27	−19.07 (± 4.34); 100	–	–
	Ruschioideae	46	0.199 (uni)	3	0	−24.07 (± 3.29); 65.4	−18.25 (± 0.05); 10.4	−15.58 (± 1.23); 24.2
	Sesuvioideae	1	1 (uni)					
Anacampserotaceae		27	0.317 (uni)					
Apocynaceae		51	0.002 (multi)	2	−7.50	−27.37 (± 2.16); 52.5	−15.70 (± 2.24); 47.5	–
Asparagaceae		127 (total)	0.029 (multi)	2	−6.62	−25.59 (± 3.99); 38.9	−13.66 (± 1.28); 61.1	–
	Agavoideae	123	0.023 (multi)	2	−12.41	−25.51 (± 3.88); 39.1	−13.69 (± 1.31); 60.9	–
	Nolinoideae	4	0.782 (uni)					
Asphodelaceae		15	0.001 (multi)					
Asteraceae		101	0.371 (uni)	2	−8.65	−28.04 (± 2.33); 98.0	−17.11 (± 0.41); 2.0	–
Bromeliaceae		2266 (total)	0 (multi)	3	0	−26.22 (± 3.26); 58.1	−15.59 (± 2.06); 13.5	−13.18 (± 1.43); 28.4
	Brocchinioideae	19	0.891 (uni)					
	Bromelioideae	574	0.681 (uni)	2	−1.12	−25.24 (± 4.57); 12.1	−14.21 (± 1.91); 87.9	–
	Hechtioideae	35	0.504 (uni)					
	Lindmanioideae	34	0.691 (uni)					
	Navioideae	91	0.566 (uni)	1	−18.38	−27.77 (± 2.70); 100	–	–
	Pitcairnioideae	393	0 (multi)	2	−3.57	−27.95 (± 3.00); 72.3	−12.08 (± 1.26); 27.7	–
	Puyoideae	161	0.786 (uni)	3	0	−23.92 (± 1.52); 47.2	−22.14 (± 4.10); 41.8	−13.54 (± 0.82); 11.0
	Tillandsioideae	959	0 (multi)	2	−12.67	−25.68 (± 3.26); 74.4	−13.85 (± 1.50); 25.6	–
Cactaceae		79	0.764 (uni)	2	−6.66	−14.89 (± 1.90); 21.1	−12.60 (± 0.89); 78.9	–
Clusiaceae		379	0.011 (multi)	3	0	−27.40 (± 1.88); 65.2	−23.53 (± 4.53); 21.5	−15.33 (± 0.93); 13.3
Commelinaceae		6	0.214 (uni)					
Crassulaceae		519 (total)	0 (multi)	2	−14.90	−25.88 (± 3.92); 63.0	−14.59 (± 2.07); 37.0	–
	Aeonieae	268	0.736 (uni)	2	−3.53	−27.12 (± 3.76); 77.0	−16.48 (± 2.49); 23.0	–
	Crassuloideae	27	0.966 (uni)					
	Kalanchoideae	122	0.992 (uni)	2	−3.28	−20.73 (± 4.49); 39.8	−13.45 (± 1.55); 60.2	–
	Petrosedum Grulich	8	0.149 (uni)					
	Sedeae	74	0.029 (multi)	2	−9.78	−25.23 (± 1.88); 70.3	−14.10 (± 1.72); 29.7	–
	Semperviveae	11	0.906 (uni)					
	Telephieae	4	0.232 (uni)					
	Umbiliceae	5	0.214 (uni)					
Didiereaceae		27	0.085 (uni)					
Euphorbiaceae		180	0.008 (multi)	2	−12.75	−28.19 (± 2.54); 66.9	−15.61 (± 2.59); 33.1	–
Gesneriaceae		18	0.866 (uni)					
Montiaceae		31	0.871 (uni)					
Orchidaceae		2700 (total)	0 (multi)	2	−16.84	−28.67 (± 2.83); 87.0	−15.35 (± 2.16); 13.0	–
	Cypripedioideae	10	0.055 (uni)					
	Epidendroideae	2516	0 (multi)	3	0	−28.53 (± 2.70); 86.2	−17.57 (± 2.12); 5.6	−14.14 (± 1.39); 8.2
	Orchidoideae	138	0.788 (uni)	1	−19.62	−31.43 (± 2.75); 100	–	–
	Vanilloideae	30	0 (multi)					
Oxalidaceae		6	0.334 (uni)					
Passifloraceae		12	0.990 (uni)					
Piperaceae		35	0.644 (uni)					
Polypodiaceae		70	0.650 (uni)	1	−13.63	−27.48 (± 4.59); 100	–	–
Portulacaceae		2	1 (uni)					
Rubiaceae		64	0.928 (uni)	1	−18.60	−27.76 (± 4.21); 100	–	–
Vitaceae		7	0.037 (multi)					
Vittariaceae		11	0.091 (uni)					
All samples		7204	0 (multi)	3	0	−27.64 (± 3.16); 68.8	−17.11 (± 2.54); 12.8	−13.53 (± 1.49); 18.4

2.3. Statistical tests of multimodality and clusters in the distribution of $\delta^{13}C$ values

Statistical analysis of $\delta^{13}C$ values was carried out in R (R Core Team, 2020), on the full dataset of all 7204 data points, and on subsets thereof for plant families and sub-familial taxa with a total of more than 45 samples (see boldface taxa in Table 1). For each dataset, we first established whether it conformed to a normal distribution, using Shapiro-Wilk tests with the packages ggplot2 (Wickham, 2016), moments (Komsta and Novomestky, 2015), plyr (Wickham, 2011) and tibble (Müller et al., 2020).

We then used two approaches to test whether the empirical distribution of $\delta^{13}C$ values among-species conforms to a bimodal distribution, where one peak represents CAM species and the other represents C_3 species. First, we used Hartigans' Dip-Statistic to attempt to reject the hypothesis that a distribution was characterized by a single mode (Hartigan and Hartigan, 1985), implemented in the R package diptest (Maechler, 2016). The alternative hypothesis is multimodality, i.e., at least two modes. Secondly, we used a mixture modelling approach implemented in the R package Rmixmod (Langrognet et al., 2020) to test whether two is the optimal number of clusters to describe an observed distribution of $\delta^{13}C$ values. Here, an empirical distribution is assumed to contain a combination of K Gaussian subpopulations that are each characterized by their mean and standard deviation. We determined the

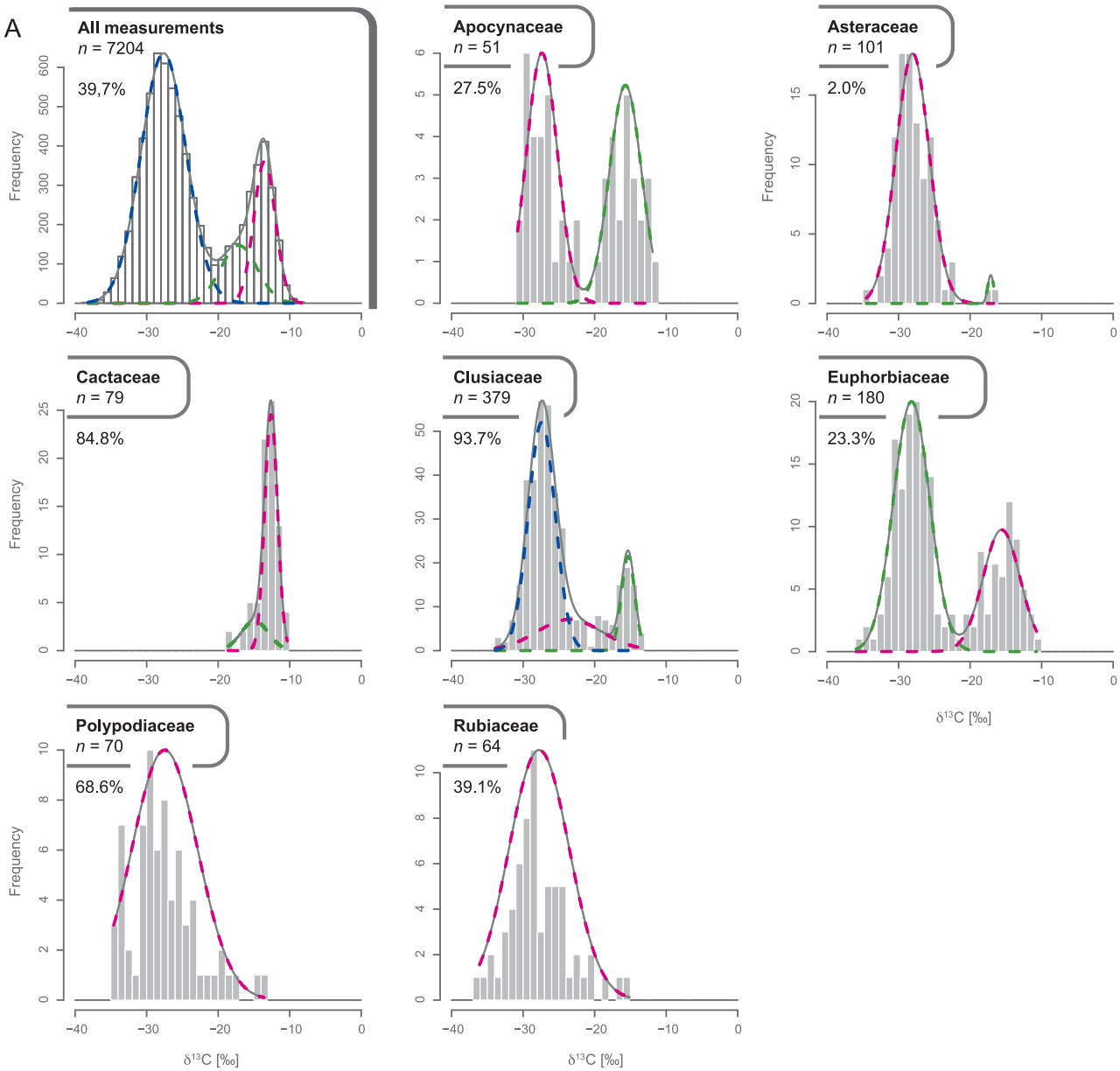

Fig. 1. Histograms illustrating the quantitative distribution of all leaf and stem $\delta^{13}C$ values inferred and compiled from the literature (All measurements) and as separated by individual plant families and infrafamilial taxa. Gaussian curves within the histograms correspond to the best-fitting model as inferred by mixture modelling analyses. Colours of the individual curves only serve an easier differentiation from other curves and do not have any particular meaning. Uniform colours of the bins signify plots of taxa belonging to the same family, but gray bins represent distinct single families that were not studied at the infrafamilial level. Note that the abscissa ($\delta^{13}C$ in ‰) is always in the same scale, whereas the ordinate scale (frequency) differs between individual plots. Frequency refers to the number of samples (not the number of sampled taxa), and the total number of samples contributing to each plot (*n*) is given below taxon names. To give a measure of how many species were sampled more than once for each plot, the percentage below the number of samples corresponds to the proportion of data points that were obtained from species sampled at least twice.

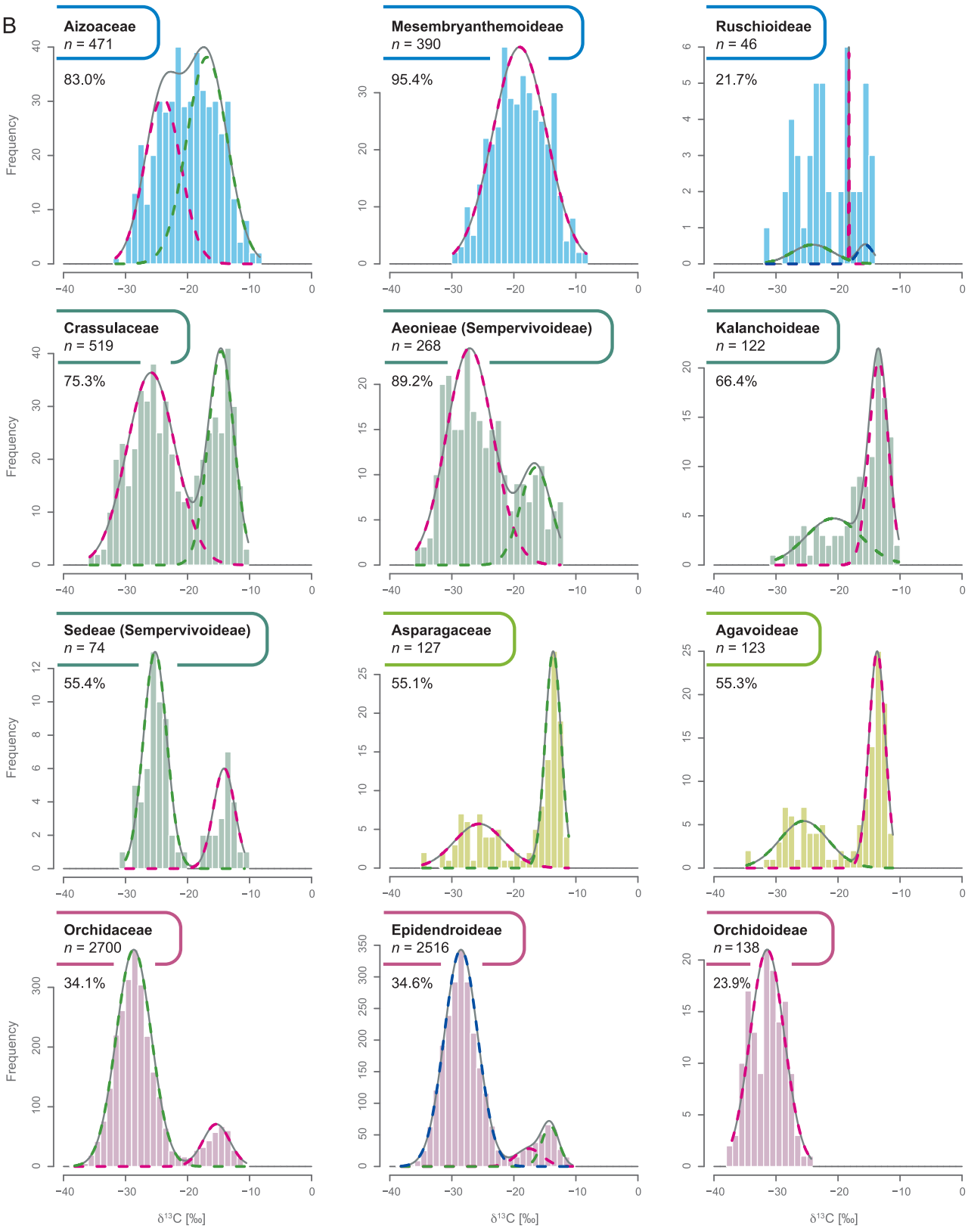

Fig. 1. Continued.

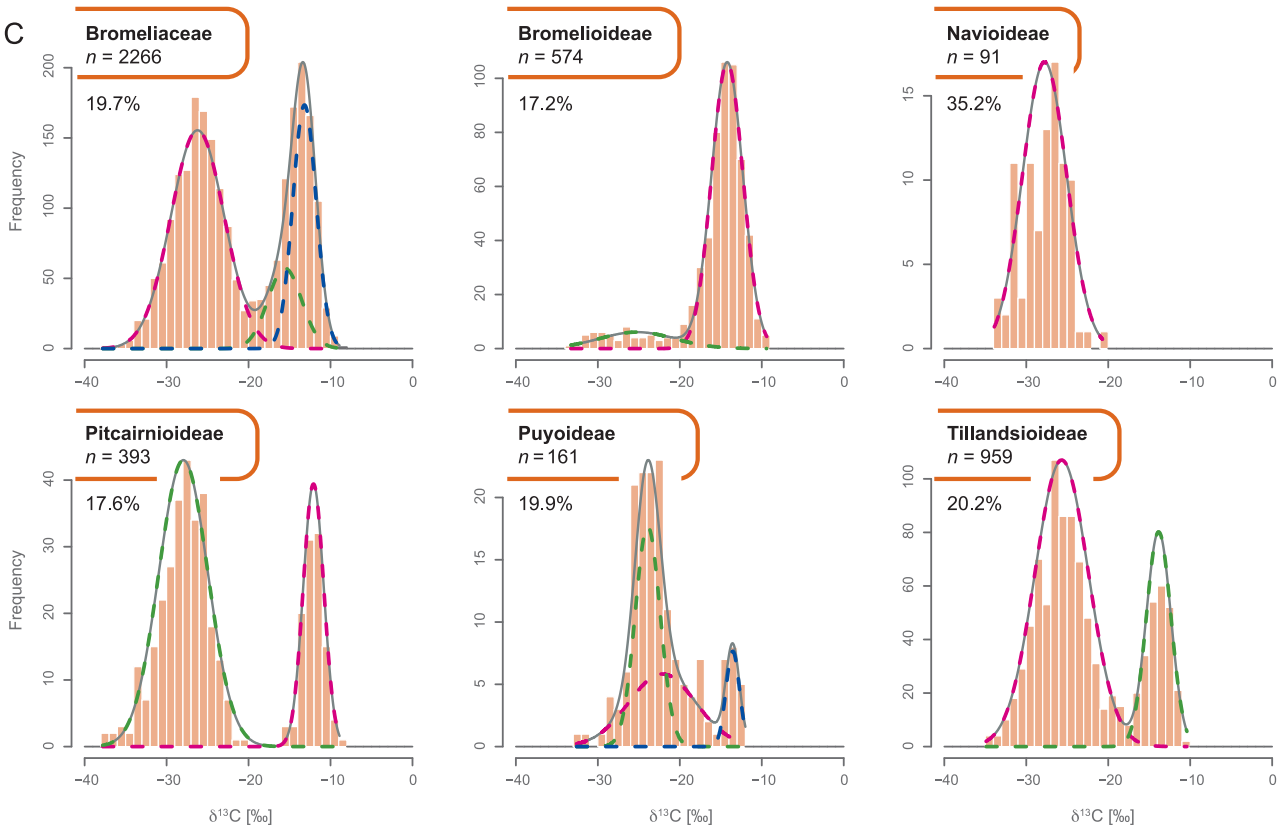

Fig. 1. Continued.

optimal number of clusters (K) using the Bayesian Information Criterion (BIC) after fitting Gaussian mixture models with K ranging from 1 to 4. To make sure the fitting algorithm converged to the optimal solution, we used 5 tries and a maximum of 100'000 iterations using the EM algorithm of Rmixmod, and re-ran each analysis 300 times to guarantee we compared the best BIC value for each value of K.

Note that these tests simply characterize the distribution and are agnostic to the evolutionary process that gave rise to the distribution and should thus be interpreted with caution. Although phylogenetic modelling approaches that test for the presence of multiple classes without a-priori assigning a species to a class have recently been developed (Mitov et al., 2019), full implementations are not yet available. Moreover, we did not deem such an approach necessary for testing our hypotheses.

Finally, to illustrate the intraspecific variation of $\delta^{13}C$ values, boxplots were drawn comprising the 24 species (in five families) for which at least ten values were available.

3. Results

Several different patterns emerged when $\delta^{13}C$ values were plotted as histograms (see Fig. 1). The distribution of all 7204 $\delta^{13}C$ values covers a continuum from -8.3 to -38.2‰ with two distinct modes that reach their peak at around -14 and -28‰, as expected for typical CAM and C_3 species, respectively. Yet, numerous samples represented the local minimum between the two modes (216 samples fell between -21 and -19‰). With regard to the individual families, the overall pattern most closely resembles the well-sampled Bromeliaceae ($n = 2266$) and Crassulaceae ($n = 519$). Consequently, Hartigans' Dip-Test supported multimodality of $\delta^{13}C$ values across all samples (meaning > 1 mode was found in the distribution), as well as for the families Apocynaceae, Asparagaceae, Asphodelaceae, Bromeliaceae, Clusiaceae, Crassulaceae, Euphorbiaceae, Orchidaceae and Vitaceae (Table 1). From among these families, those that had several infrafamilial taxa with adequate sample size ($n > 45$) only displayed multimodality in a few subordinate taxa (Pitcairnioideae and Tillandsioideae of Bromeliaceae, Sedeae of Crassulaceae, Epidendroideae of Orchidaceae), indicating phylogenetic structure to the distribution of $\delta^{13}C$ values.

Results based on Gaussian mixture modelling (Figs. 1 and 2) were congruent with the Dip-Test results, but richer in detail and with additional lineages recovered as being composed of more than one cluster (Table 1), i.e., Aizoaceae, Asteraceae and Cactaceae as well as Ruschioideae of Aizoaceae, Bromelioideae and Puyoideae of Bromeliaceae, and Aeonieae and Kalanchoideae of Crassulaceae. With the exception of Cactaceae, all lineages for which at least two clusters were inferred featured distributions with at least one mean above and below -20‰ (Fig. 2, Table 1). Surprisingly, across all sampled taxa, the optimal number of clusters was 3, with means at -27.6, -17.1, and -13.5‰, diverging from the expectation of one cluster corresponding to CAM and another one corresponding to C_3 photosynthesis. Instead, a broad intermediate cluster was found in addition, characterized by a higher standard deviation than that of the rather narrow CAM-cluster (2.5 and 1.5‰, respectively; see Fig. 1A "All measurements" and Table 1). Put simply, the 'dip' between the modes of C_3 photosynthesis and CAM is too high to be explained under a mixture model with two Gaussian distributions. Evidence for species belonging to such an intermediate group (when defining it as groups with at least one cluster having a mean within one standard deviation of -20‰, the classic threshold to distinguish CAM plants from C_3 plants) was particularly pronounced in Mesembryanthemoideae of Aizoaceae, Puyoideae of Bromeliaceae, and Kalanchoideae of Crassulaceae (Fig. 2).

Among the mixture modelling analyses that inferred a single underlying Gaussian distribution, subfamily Mesembryanthemoideae of Aizoaceae ($n = 390$) stood out, as their inferred mean $\delta^{13}C$ value (i.e., -19.1‰) lay within the range of the intermediate cluster. All other lineages with distributions inferred as unimodal (i.e., Polypodiaceae and Rubiaceae as well as Navioideae of Bromeliaceae and Orchidoideae of Orchidaceae) feature $\delta^{13}C$ values well below -20‰ (Fig. 2). Shapiro-

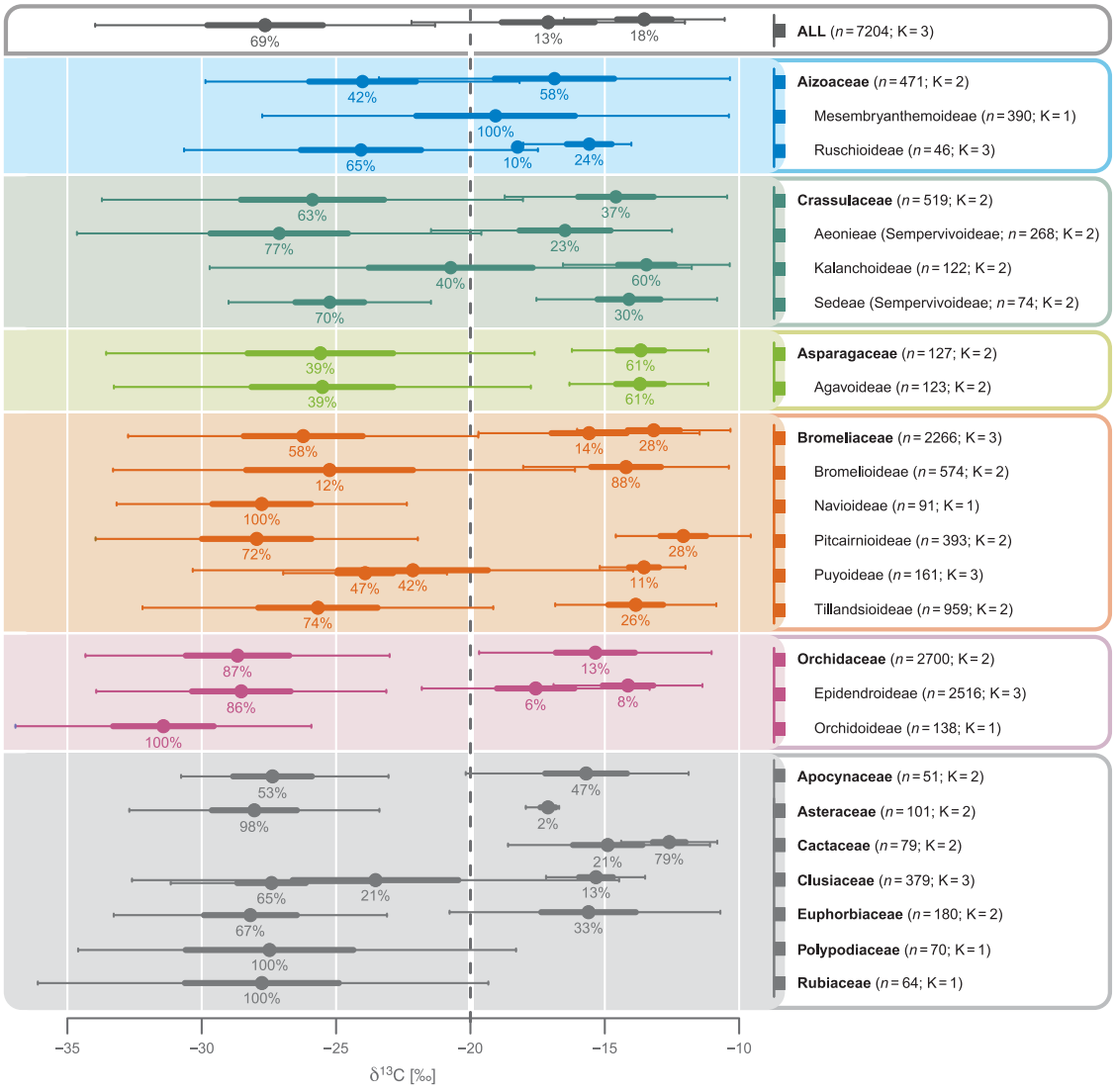

Fig. 2. Best fitting models of $\delta^{13}C$ distribution identified by mixture modelling analysis for the full dataset and each well-sampled lineage ($n > 45$). The scale corresponds to $\delta^{13}C$ [‰], and -20‰, the local minimum in the distribution between C_3- and CAM-like $\delta^{13}C$ values commonly used as threshold is indicated by a vertical dashed line. The number of samples (n) and number of inferred clusters (K) is given in brackets for each taxon. Thick bars indicate 1.35-fold standard deviation (covering 50 % of observations around the mean), while thin bars and whiskers extend to 4-fold standard deviation (95 % of observations).

Table 2
Shapiro-Wilk test results for $\delta^{13}C$ data. H_0 assumed normal distribution. Only results with $p > 0.05$ (i.e., where H_0 cannot be rejected) are shown. s.d: standard deviation.

taxon	*n*	mean	median	s.d.	skew	α	*p*-value
Ruschioideae (Aizoaceae)	46	−21.41	−22.27	4.67	−0.11	0.05	0.11
Navioideae (Bromeliaceae)	91	−27.77	−27.4	2.71	−0.24		0.11
Orchidoideae (Orchidaceae)	138	−31.43	−31.26	2.76	0.01		0.11
Rubiaceae	64	−27.76	−28.45	4.25	0.69		0.06

Wilk tests were congruent with unimodality of $\delta^{13}C$ values in Navioideae (Bromeliaceae), Orchidoideae (Orchidaceae) and Rubiaceae (Table 2), but rejected unimodality in the data for Mesembryanthemoideae ($p < 0.05$). Instead, here, a normal distribution of $\delta^{13}C$ values in Ruschioideae (also Aizoaceae) was not refuted ($p = 0.11$; Table 2).

Variability in $\delta^{13}C$ values of single species reveals the variation in amount of nighttime CO_2 fixation. Boxplots in Fig. 3 demonstrate the $\delta^{13}C$ values of all 24 species in our dataset that were represented by at least ten distinct samples. Notably, 21 out of these species (87.5 %) belonged to only two plant families (i.e., Clusiaceae and Crassulaceae) demonstrating that in the broadly sampled Bromeliaceae and Orchidaceae, where maximum numbers of samples per species were six and seven, respectively (see online supplementary Table S2), sampling strategies usually aimed at covering a wide range of species rather than a high number of samples per species. A notable exception to this is an in-depth study of juvenile individuals of three epiphytic bromeliad species (Beltrán et al., 2013). Given the overlap of whiskers from all species represented in Fig. 3, a continuous distribution of $\delta^{13}C$ values from -34‰ in *Aichryson laxum* (Haw.) Bramwell to -10‰ in *Opuntia engelmannii* Salm-Dyck ex Engelm. is apparent. Some species only vary slightly, most

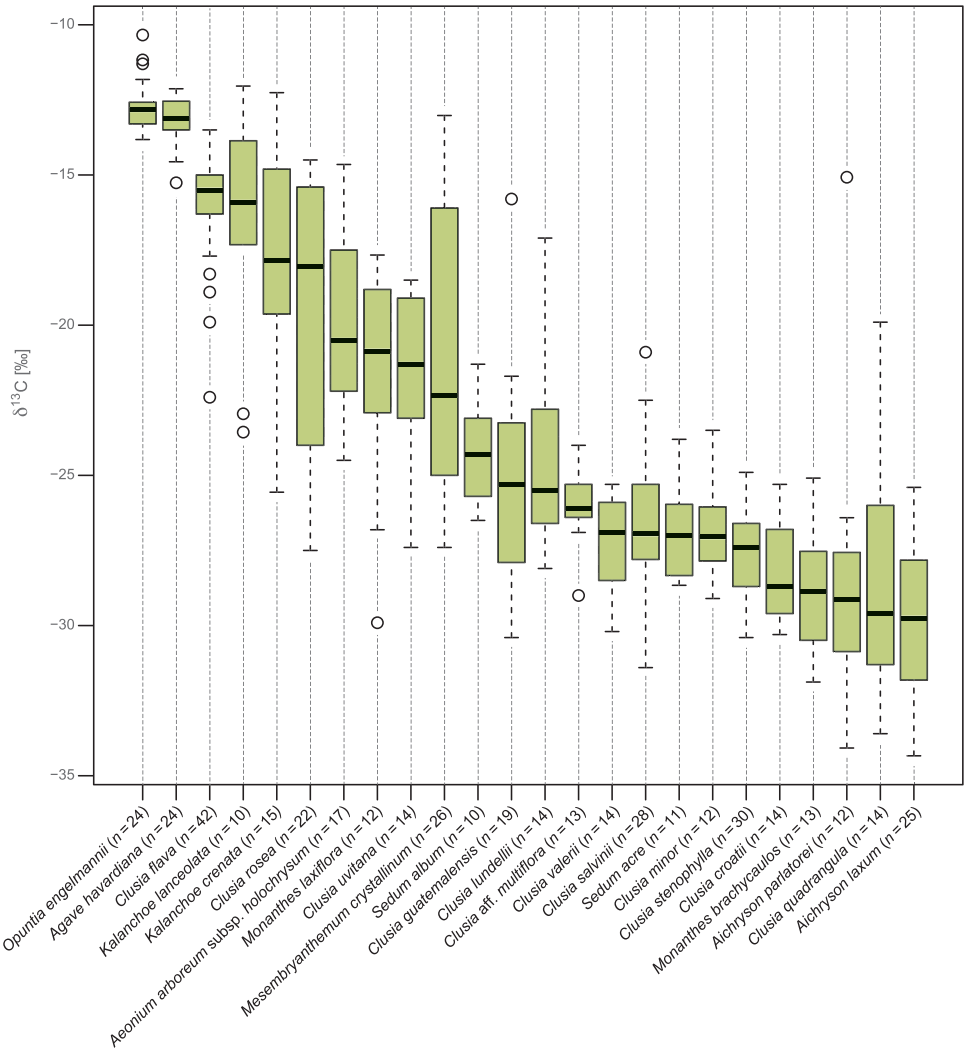

Fig. 3. Boxplots of $\delta^{13}C$ values for all species with at least ten distinct samples. Boxes contain the third and first quartile and the median as a thick horizontal line. Whiskers extend to a maximum of 1.5 the length of the box. Outliers are marked by hollow dots. Data pertaining to the names *Clusia stenophylla* Standl. and *Clusia* aff. *stenophylla* (Holtum et al., 2004) were united in one plot under *C. stenophylla*. The names *Aichryson parlatorei* Bolle and *Aichryson* cf. *parlatorei* were treated in the same way.

notably *O. engelmannii* and *Agave havardiana* Trel. in the CAM range between -10 and -15‰. Others such as *Clusia rosea* Jacq., *Mesembryanthemum crystallinum* and *Kalanchoe crenata* (Andrews) Haw. cover a wide range of $\delta^{13}C$ values that suggests a high variation in the expression of the CAM and C_3 pathways for these species.

4. Discussion

4.1. General existence of a bimodal distribution of $\delta^{13}C$ in CAM lineages? The phylogenetic scale matters

Our data compilation of more than 7000 $\delta^{13}C$ values from 24 plant families considering their distribution at sub-familial phylogenetic scale clearly shows the multi-facetted distribution patterns in different plant lineages. While the results of our mixture modelling analyses suggest three clusters with means at -27.6, -17.1, and -13.5‰, next to the clearly bimodal distribution of $\delta^{13}C$ values across all CAM/C_3 lineages (Fig. 1A"All measurements" and Table 1), it is apparent that this pattern is not necessarily reproduced by individual lineages. Even within families, several patterns can occur, such as in Orchidaceae, where three clusters were found for Epidendroideae and a single cluster for Orchidoideae (Fig. 1B). Similarly, for the particularly well-sampled, species-rich Bromeliaceae (2266 samples; > 4100 extant species), contrasting patterns among subfamilies were revealed: from truly discontinuous double peaks in Pitcairnioideae, double peaks with a connecting continuum in Tillandsioideae, and single CAM peak with a broad and flat distribution towards the C_3 range in Bromelioideae, to a fully C_3-like normal distribution in Navioideae (Fig. 1C). These results corroborate previous findings by Crayn et al. (2004) based on fewer data points for Bromelioideae, Tillandsioideae and other clades of the Bromeliaceae phylogeny. This indicates strong phylogenetic structure in the diversity of $\delta^{13}C$ values within lineages.

The results for Mesembryanthemoideae (contributing 83 % of all Aizoaceae samples) were unique as their $\delta^{13}C$ values displayed a continuous, unimodal distribution across a wide range (between -8 and -30‰) with mean at -19.1‰ (Fig. 1B). However, other subfamilies of Aizoaceae were relatively underrepresented. This broad distribution likely relates to the presence of predominantly ephemeral/caducous leaves in the species' varying use of CAM to extend growth into the dry season of their natural southern African habitat, as Winter et al. (1978) and Bloom and Troughton (1979) showed even within a single species, *Mesembryanthemum crystallinum*, in Israel and California, respectively (see the wide range of $\delta^{13}C$ values for this species in Fig. 3). On top of this intra-specific plasticity, species within lineages are highly diverse in morphology, anatomy and enzymatic variation, as well as leaf longevity and ecology, which will all greatly influence the overall degree of ^{13}C discrimination (Winter and Smith, 1996; Cernusak et al., 2013). This multi-tiered variation in $\delta^{13}C$ phenotypes cautions against interpreting summary-histograms of a diverse range of species, as characteristics of phylogenetic lineages with fewer species may be swamped by the few, particularly species-rich groups, e.g., Bromeliaceae or Orchidaceae. Here, it is particularly relevant to note that the bimodal

overall-histogram of all data (Fig. 1A "All measurements") is best characterized as a mixture of three rather than two Gaussian distributions. Thus, if a study's aim is to understand evolution of CAM, fine-scale phylogenetic structure to such multi-tiered variation, including in species-poor lineages, is particularly important.

4.2. Implications for macroevolutionary studies of CAM evolution using $\delta^{13}C$ values as proxy for photosynthetic type

Although the plasticity of CAM and its continuous transition towards C_3 photosynthesis have been duly acknowledged (Ting, 1985; Cushman and Borland, 2002; Dodd et al., 2002; Silvera et al., 2010a; Winter and Holtum, 2017; Hancock et al., 2019), the seemingly universal bimodal distribution of $\delta^{13}C$ values in lineages of CAM and C_3 plants has often led to the notion of a more or less clear distinction between these two photosynthetic pathways with a $\delta^{13}C$ threshold at approximately -20‰, the local minimum between the two peaks of the distribution (see Fig. 1A "All measurements"; Winter et al., 2015). However, the vast variation of leaf $\delta^{13}C$ values that may prevail within a single species, as exemplified in Fig. 3, demonstrates the inaccuracy with which the photosynthetic pathway of a whole species is deduced from just a single measurement of carbon isotope ratio: If, e.g., a relatively high $\delta^{13}C$ value within its range was inferred for *Clusia rosea*, it would be considered a CAM species, but if a more negative value within the usual variation of $\delta^{13}C$ values (see Fig. 3) were measured, the same species would be considered a C_3 species.

Importantly, $\delta^{13}C$ values represent the phenotype of a particular individual plant, not the overall characteristics of a species. Measurements of circadian titratable acidity as well as gas exchange in Aeonieae (Crassulaceae) have, for instance, confirmed that even a $\delta^{13}C$ value more negative than -30‰ may stem from leaves of species that otherwise engage in CAM facultatively (unpublished results), as had been shown for other facultative CAM plants (Bender et al., 1973; Silvera et al., 2005). In this case, the plants used for carbon isotope analysis might just have never been exposed to CAM-inducing environmental conditions although potentially being able to perform CAM, or CAM was only expressed in short bursts (Males, 2018). Deducing from a very low $\delta^{13}C$ that such a species is purely C_3 would obviously be wrong and would have consequences for a macroevolutionary study of CAM evolution. This is also reflected in our results showing more than two, overlapping clusters for multiple plant families (Fig. 2), implying that any $\delta^{13}C$ threshold used to distinguish between full C_3 (i.e., the absence of a CAM cycle) and CAM is error-prone, with consequences for phylogenetic studies inferring the number of CAM origins and reversals to C_3 photosynthesis.

Clearly, the fact that facultative or weak-CAM plants may have a much more negative $\delta^{13}C$ value than the usually consulted threshold of -20‰ has repeatedly been acknowledged (e.g., Bender et al., 1973; Silvera et al., 2005; Heyduk et al., 2016; Hancock et al., 2019). Thus, we hypothesize that studies of CAM evolution in individual plant lineages may have overestimated the number of CAM origins whenever photosynthetic pathway was only coded according to $\delta^{13}C$, because in these cases many facultative CAM species have been likely coded as C_3 species. Therefore, a number of the inferred transgressions of the -20‰ threshold value in an ancestral character state reconstruction will actually not represent transgressions from C_3 to CAM or the other way around, but instead from, e.g., facultative CAM to full CAM or the other way around. Instead of using a $\delta^{13}C$ threshold or searching for morphological or anatomical features such as leaf thickness and succulence which are poorly correlated with weak CAM activity (Silvera et al., 2005; Herrera, 2020), the distinction between C_3 and facultative or weak CAM can accurately be evidenced by means of physiological experiments using living plants and testing their diurnal acid rhythmicity and 24-h gas exchange under varying environmental conditions. Although this procedure is much more time-consuming and laborious than measuring leaf carbon isotope ratios and although it necessitates the availability of living plant material, it allows for a comprehensive characterization of CAM physiology in the studied plant accessions and will therefore be much more accurate for the study of CAM evolution than defining an artificial and potentially misleading $\delta^{13}C$ threshold between C_3 and CAM that is essentially founded on the assumption of a bimodal distribution. Hancock et al. (2019) who compared the usage of $\delta^{13}C$ values and measurements of experimentally induced nocturnal acid accumulation as the basis for coding CAM activity in an ancestral character state reconstruction came to similar conclusions.

4.3. Implications for the model of CAM evolution through continuous phenotypes

The simplified distinction between CAM and non-CAM based on the bimodal distribution of $\delta^{13}C$ values has not only influenced the number of CAM origins and reversals to C_3 that we may deduce from phylogenies, but it has also shaped the ongoing discussion concerning a model of CAM evolution. In this context, the most important hypotheses have been a continuous model on the one hand (Silvera et al., 2010a; Bräutigam et al., 2017) and a threshold evolutionary model on the other (Edwards, 2019). The continuous model emphasizes the plastic nature of CAM and states that this complex trait evolved by gradually upregulating the biochemical pathway of a full CAM cycle from C_3 ancestors in which exactly the same pathway is already present but serving amino acid metabolism (Bräutigam et al., 2017). This model assumes a true continuum from C_3 to CAM plants essentially meaning that few changes in the genotype are needed to turn a C_3 plant into a weak CAM plant and that the conversion from weak to strong CAM at a later point in CAM evolution is similarly simple but necessitating anatomical and biochemical optimization. Edwards (2019) on the other hand emphasized the bimodal distribution in the $\delta^{13}C$ values of large CAM/C_3 plant lineages (especially Bromeliaceae, Crassulaceae, *Euphorbia* L., and Orchidaceae) and argued that a continuous model of CAM evolution cannot satisfactorily explain the so often observed bimodal distribution. By correlating $\delta^{13}C$ with also bimodally distributed leaf thickness data from earlier publications for Bromeliaceae (Earles et al., 2018; Males, 2018), Crassulaceae (Teeri et al., 1981) and Orchidaceae (Silvera et al., 2005), she put forward convincing evidence for a "threshold evolutionary model" in which strong CAM is not readily and continuously evolved from a C_3 and weak-CAM ancestry but instead requires rather extensive anatomical restructuring towards high succulence and thereby low mesophyll conductance. This discrepancy between a more or less mesomorphic leaf anatomy with optimal properties for C_3 photosynthesis and weak CAM on the one hand, and pronounced succulence optimal for strong CAM on the other hand should cause anatomical changes to be the "rate-limiting step" in the evolution of strong CAM from a C_3 or weak-CAM background (Edwards, 2019).

Our own $\delta^{13}C$ data for Mesembryanthemoideae (Aizoaceae) and recently published $\delta^{13}C$ values in the genus *Drosanthemum* Schwantes of the same family (Schweiger et al., 2021) could be explained by a continuous model of CAM evolution since they both feature a unimodal distribution of carbon isotope ratios (Figs. 1B, 2; Fig. 1B in Schweiger et al., 2021). In these cases, each representing a lineage of approximately 100 species (Hartmann, 2017), facultative CAM or C_3-CAM intermediacy are likely to be the predominant variants of CAM expression, as clearly evidenced by the distribution of $\delta^{13}C$ values. Therefore, natural selection seems to have stabilized this mode of CAM expression rather than acting disruptively and favouring more distinct C_3 and CAM phenotypes. This, however, does not mean that there are no species with a strong CAM phenotype. For example, all $\delta^{13}C$ values of *Mesembryanthemum tetragonum* Thunb. and *M. pseudoschlichtianum* (S.M.Pierce & Gerbaulet) Klak were less negative than -20‰ whereas all $\delta^{13}C$ values of, e.g., *M. clandestinum* Haw. were more negative than -20‰ ($n = 5$ for all three species, samples collected at different seasons throughout the vegetation cycle), indicating that species at the extremes of the C_3-CAM spectrum are relatively rare but existent. A very different trajectory of

CAM evolution may be deduced for Pitcairnioideae (Bromeliaceae; Fig. 1C) which feature a strongly bimodal histogram with CAM- and C_3-like $\delta^{13}C$ values. There, the $\delta^{13}C$ values pertaining to the genera of Pitcairnioideae (see online supplementary Table S2) are clearly subdivided into C_3-like values in the genera *Fosterella* L.B.Sm. and *Pitcairnia* L'Hér. on the one hand and CAM-like values in the remaining three genera, i.e., *Deuterocohnia* Mez, *Dyckia* Schult.f. and *Encholirium* Mart. ex Schult. & Schult.f. (Crayn et al., 2004, 2015). Thanks to a well-resolved phylogeny (Givnish et al., 2014), we know in this particular case that this observation exactly marks one origin of CAM in the evolution of Pitcairnioideae, since the former two genera are earliest diverging in this clade. Thus, the clear bimodal distribution of $\delta^{13}C$ values supports the above-cited threshold evolutionary model for this plant lineage and backs its significance for CAM evolution in the Bromeliaceae as a whole.

While the Pitcairnioideae histogram of $\delta^{13}C$ values is virtually devoid of intermediate values around -20‰, these intermediate values are the most abundant ones in Mesembryanthemoideae and fairly numerous in Aeonieae, too, with the majority of data points here (77 %) belonging to the C_3-like cluster in our mixed-modelling analyses (Figs. 1B and 2, Table 1). These distributions of $\delta^{13}C$ values (together with other evidence from extensive physiological experiments in Aeonieae; Lösch, 1990) suggest that facultative CAM is not only the predominant mode of photosynthesis in the species of these clades but also that its evolution might happen in a continuous way with intermediate forms between C_3 photosynthesis and CAM being regularly expressed and stably selected for. However, the question if facultative CAM may be regarded as transitional state towards obligate or strong CAM or if it constitutes an independent evolutionary trajectory is a matter of ongoing discussion (Winter et al., 2015; Winter, 2019). The peculiar distribution of $\delta^{13}C$ values in Mesembryanthemoideae and Aeonieae may probably be explained by their ecology. In both taxa, the representative species feature a tremendous diversity of growth- and life-forms (see Bittrich (1987) and Klak et al. (2007) for Mesembryanthemoideae; Lösch (1990) and Mort et al. (2007) for Aeonieae), and all of these were sampled in our analysis. Furthermore, species of Mesembryanthemoideae and Aeonieae cover a wide range of different climate zones and habitats, the former occurring in both summer and winter rainfall regions as well as all-year rainfall regions of southern Africa (Bittrich, 1987), and the latter occurring throughout all different climate zones of the Macaronesian islands (Lösch, 1990) as defined by elevation and aspect, with only few species on the African continent (Liu, 1989). Giving credit to the ecophysiological advantage of CAM, Lüttge (2004) characterized it as a "strategy for variable, flexible and plastic niche occupation rather than lush productivity". Establishing links between CAM evolution and the ecology of specific plant lineages, however, requires much more detailed information than currently at hand, e.g., data derived from species distribution modelling and well-sampled phylogenetic dating analyses. Understanding the idiosyncrasies of CAM evolution in different plant lineages can be further complicated by the fact that habitats in which CAM evolved are not necessarily the same habitats that are ultimately preferred by the respective lineage. A similar example for this is the evolution of C_4 photosynthesis in grasses having coincided with pronounced habitat shifts from tropical moist forests into savannahs (Edwards and Smith, 2010), the vegetation type now dominated by C_4 grasses. It must therefore suffice at this point to underline the high ecological diversity of phylogenetically independent CAM plant lineages that brings about the necessity to evaluate the evolutionary pathway towards CAM case by case. Finally, CAM might in reality be much more widespread across the plant tree of life than currently assumed because broad surveys for CAM lineages typically involve a screening of $\delta^{13}C$ values. Since these, however, as opposed to relatively easily accessible measurements of titratable acidity, cannot detect weak CAM activity, a potentially high number of CAM species are likely to yet await discovery (Winter, 2019) and thereby add new evidence to the evolution of this complex trait.

4.4. Conclusion

The results of our statistical analyses of 7204 $\delta^{13}C$ values from 24 CAM/C_3 plant families demonstrate that the most frequently found bimodal distribution with a conspicuous dearth of samples in the C_3-CAM intermediate range around -20‰ is not universally applicable to well-sampled monophyletic groups. Instead, we identify a few lineages that have a clearly continuous and unimodal distribution of $\delta^{13}C$ values, most notably Mesembryanthemoideae (Aizoaceae) where the distribution is centred around -20‰. In other lineages such as Aeonieae (Crassulaceae) and Clusiaceae, the distribution of $\delta^{13}C$ values is best described by a combination of more than one Gaussian cluster, but there, values in the C_3-CAM intermediate range (approximately -27 to -19‰; Winter et al., 2015) contribute to more than one third of all data points, indicating a considerable proportion of facultative CAM. This frequent representation of intermediate values also causes the distribution of $\delta^{13}C$ values across all sampled plant families to be best modelled by three instead of two clusters with one C_3-CAM intermediate cluster. These findings have important consequences for our understanding of CAM evolution, underlining the frequently discussed but sometimes ignored absence of a clear $\delta^{13}C$ threshold with which to delineate CAM from C_3 plants, making CAM impossible to code as a character solely on the basis of $\delta^{13}C$ alone. As suggested by several authors before (e.g., Winter et al., 2015; Hancock et al., 2019), we conclude that coding the occurrence of the CAM or C_3 pathway by means of physiological experiments such as acid titration is much more appropriate.

Author contributions

TFEM, JW and GK conceived the project. NB, CK and JAL conducted field work collecting plant material. JW, NB, JAL, DBN and JMV performed the laboratory work. Data analysis was conducted by TFEM, JW and JMV. TFEM, JW and GK wrote an initial manuscript, and all authors contributed to the final version.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ppees.2021.125619.

References

Arroyo, M.K., Medina, E., Ziegler, H., 1990. Distribution and $\delta^{13}C$ values of Portulacaceae species of the High Andes in northern Chile. Bot. Acta 103 (3), 291–295. https://doi.org/10.1111/j.1438-8677.1990.tb00163.x.

Beltrán, J.D., Lasso, E., Madriñán, S., Virgo, A., Winter, K., 2013. Juvenile tank-bromeliads lacking tanks: do they engage in CAM photosynthesis? Photosynthetica 51 (1), 55–62. https://doi.org/10.1007/s11099-012-0077-8.

Bender, M.M., Rouhani, I., Vines, H.M., Black, C.C., 1973. $^{13}C/^{12}C$ ratio changes in Crassulacean acid metabolism plants. Plant Physiol. 52, 427–430. https://doi.org/10.1104/pp.52.5.427.

Bittrich, V., 1987. Untersuchungen zum Merkmalsbestand, Gliederung und Abgrenzung der Unterfamilie Mesembryanthemoideae (Mesembryanthemaceae Fenzl). Mitt. Inst. Allg. Bot. Hamburg 21, 5–116.

Bloom, A.J., Troughton, J.H., 1979. High productivity and photosynthetic flexibility in a CAM plant. Oecologia 38 (1), 35–43. https://doi.org/10.1007/BF00347822.

Bonal, D., Sabatier, D., Montpied, P., Tremeaux, D., Guehl, J.M., 2000. Interspecific variability of $\delta^{13}C$ among trees in rainforests of French Guiana: functional groups and canopy integration. Oecologia 124 (3), 454–468. https://doi.org/10.1007/PL00008871.

Boutton, T.W., Archer, S.R., Midwood, A.J., 1999. Stable isotopes in ecosystem science: structure, function and dynamics of a subtropical savanna. Rapid Commun. Mass Spectrom. 13 (13), 1263–1277. https://doi.org/10.1002/(SICI)1097-0231(19990715)13:13<1263::AID-RCM653>3.0.CO;2-J.

Bräutigam, A., Schlüter, U., Eisenhut, M., Gowik, U., 2017. On the evolutionary origin of CAM photosynthesis. Plant Physiol. 174, 473–477. https://doi.org/10.1104/pp.17.00195.

Buss, A., 2020. Como o gradiente altitudinal de luz no sub-bosque afeta as características estruturais e fisiológicas de hemiepífitas: o caso de *Vanilla bahiana* Hoehne (Orchidaceae). Post-graduate thesis. Federal University of Uberlândia.

Cerling, T.E., Harris, J.M., MacFadden, B.J., Leakey, M.G., Quade, J., Eisenmann, V., Ehleringer, J.R., 1997. Global vegetation change through the Miocene/Pliocene boundary. Nature 389, 153–158. https://doi.org/10.1038/38229.

Cernusak, L.A., Ubierna, N., Winter, K., Holtum, J.A.M., Marshall, J.D., Farquhar, G.D., 2013. Environmental and physiological determinants of carbon isotope discrimination in terrestrial plants. New Phytol. 200 (4), 950–965. https://doi.org/10.1111/nph.12423.

Chomicki, G., Renner, S.S., 2016. Evolutionary relationships and biogeography of the ant-epiphytic genus *Squamellaria* (Rubiaceae: Psychotrieae) and their taxonomic implications. PLoS One 11 (3). https://doi.org/10.1371/journal.pone.0151317 e0151317.

Coplen, T.B., 2011. Guidelines and recommended terms for expression of stable-isotope-ratio and gas-ratio measurement results. Rapid Commun. Mass Spectrom. 25 (17), 2538–2560. https://doi.org/10.1002/rcm.5129.

Crayn, D.M., Winter, K., Smith, J.A.C., 2004. Multiple origins of Crassulacean acid metabolism and the epiphytic habit in the Neotropical family Bromeliaceae. Proc. Natl. Acad. Sci. U. S. A. 101 (10), 3703–3708. https://doi.org/10.1073/pnas.0400366101.

Crayn, D.M., Winter, K., Schulte, K., Smith, J.A.C., 2015. Photosynthetic pathways in Bromeliaceae: phylogenetic and ecological significance of CAM and C_3 based on carbon isotope ratios for 1893 species. Bot. J. Linn. Soc. 178 (2), 169–221. https://doi.org/10.1111/boj.12275.

Cushman, J.C., Borland, A.M., 2002. Induction of Crassulacean acid metabolism by water limitation. Plant Cell Environ. 25 (2), 295–310. https://doi.org/10.1046/j.0016-8025.2001.00760.x.

De la Harpe, M., Paris, M., Hess, J., Barfuss, M.H.J., Serrano-Serrano, M.L., Ghatak, A., Chaturvedi, P., Weckwerth, W., Till, W., Salamin, N., Wai, C.M., Ming, R., Lexer, C., 2020. Genomic footprints of repeated evolution of CAM photosynthesis in a Neotropical species radiation. Plant Cell Environ. 1–15. https://doi.org/10.1111/pce.13847, 2020.

Dodd, A.N., Borland, A.M., Haslam, R.P., Griffiths, H., Maxwell, K., 2002. Crassulacean acid metabolism: plastic, fantastic. J. Exp. Bot. 53 (369), 569–580. https://doi.org/10.1093/jexbot/53.369.569.

dos Santos, C.P., Coe, H.H.G., Borrelli, N., da Silva, A.L.C., de Sousa, L.O.F., Ramos, Y.B. M., Silvestre, C.P., Seixas, A.P., 2015. Opal phytolith and isotopic studies of »Restinga« communities of Maricá, Brazil, as a modern reference for paleobiogeoclimatic reconstruction. Braz. J. Oceanogr. 63 (3), 255–270. https://doi.org/10.1590/S1679-87592015086606303.

Earles, J.M., Théroux-Rancourt, G., Roddy, A.B., Gilbert, M.E., McElrone, A.J., Brodersen, C.R., 2018. Beyond porosity: 3D leaf intercellular airspace traits that impact mesophyll conductance. Plant Physiol. 178 (1), 148–162. https://doi.org/10.1104/pp.18.00550.

Earnshaw, M.J., Winter, K., Ziegler, H., Stichler, W., Cruttwell, N.E.G., Kerenga, K., Cribb, P.J., Wood, J., Croft, J.R., Carver, K.A., Gunn, T.C., 1987. Altitudinal changes in the incidence of Crassulacean acid metabolism in vascular epiphytes and related life forms in Papua New Guinea. Oecologia (Berl.) 73 (4), 566–572. https://doi.org/10.1007/BF00379417.

Edwards, E.J., 2019. Evolutionary trajectories, accessibility and other metaphors: the case of C4 and CAM photosynthesis. New Phytol. 223 (4), 1742–1755. https://doi.org/10.1111/nph.15851.

Edwards, E.J., Smith, S.A., 2010. Phylogenetic analyses reveal the shady history of C_4 grasses. Proc. Natl. Acad. Sci. U. S. A. 107 (6), 2532–2537. https://doi.org/10.1073/pnas.0909672107.

Edwards, E.J., Osborne, C.P., Strömberg, C.A.E., Smith, S.A., Bond, W.J., Christin, P.-A., Cousins, A.B., Duvall, M.R., Fox, D.L., Freckleton, R.P., Ghannoum, O., Hartwell, J., Huang, Y., Janis, C.M., Keeley, J.E., Kellogg, E.A., Knapp, A.K., Leakey, A.D.B., Nelson, D.M., Saarela, J.M., Sage, R.F., Sala, O.E., Salamin, N., Still, C.J., Tipple, B., 2010. The origins of C_4 grasslands: integrating evolutionary and ecosystem science. Science 328, 587–591. https://doi.org/10.1126/science.1177216.

Ehleringer, J.R., Monson, R.K., 1993. Evolutionary and ecological aspects of photosynthetic pathway variation. Annu. Rev. Ecol. Syst. 24, 411–439. https://doi.org/10.1146/annurev.es.24.110193.002211.

Ehleringer, J.R., Lin, Z.F., Field, C.B., Sun, G.C., Kuo, C.Y., 1987. Leaf carbon isotope ratios of plants from a subtropical monsoon forest. Oecologia 72 (1), 109–114. https://doi.org/10.1007/BF00385053.

Farquhar, G.D., 1983. On the nature of carbon isotope discrimination in C_4 species. Aust. J. Plant Physiol. 10, 205–226. https://doi.org/10.1071/PP9830205.

Farquhar, G.D., O'Leary, M.H., Berry, J.A., 1982. On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. Aust. J. Plant Physiol. 9 (2), 121–137. https://doi.org/10.1071/PP9820121.

Farquhar, G.D., Ehleringer, J.R., Hubick, K.T., 1989a. Carbon isotope discrimination and photosynthesis. Annu. Rev. Plant Physiol. Plant Mol. Biol. 40, 503–537. https://doi.org/10.1146/annurev.pp.40.060189.002443.

Farquhar, G.D., Hubick, K.T., Condon, A.G., Richards, R.A., 1989b. Carbon isotope fractionation and plant water-use efficiency. In: Rundel, P.W., Ehleringer, J.R., Nagy, K.A. (Eds.), Eds.), Ecological Studies 68: Stable Isotopes in Ecological Research. Springer-Verlag, New York, Berlin, Heidelberg, London, Paris, Tokyo, pp. 21–40.

Gilman, I.S., Edwards, E.J., 2020. Crassulacean acid metabolism. Curr. Biol. 30 (2), R57–R62. https://doi.org/10.1016/j.cub.2019.11.073.

Givnish, T.J., Barfuss, M.H.J., Van Ee, B., Riina, R., Schulte, K., Horres, R., Gonsiska, P. A., Jabaily, R.S., Crayn, D.M., Smith, J.A.C., Winter, K., Brown, G.K., Evans, T.M., Holst, B.K., Luther, H., Till, W., Zizka, G., Berry, P.E., Sytsma, K.J., 2014. Adaptive radiation, correlated and contingent evolution, and net species diversification in Bromeliaceae. Mol. Phylogenet. Evol. 71, 55–78. https://doi.org/10.1016/j.ympev.2013.10.010.

Griffiths, H., 1989. Crassulacean acid metabolism: a re-appraisal of physiological plasticity in form and function. Adv. Bot. Res. 15, 43–92. https://doi.org/10.1016/S0065-2296(08)60044-0.

Griffiths, H., Smith, J.A.C., 1983. Photosynthetic pathways in the Bromeliaceae of Trinidad: relations between life-forms, habitat preference and the occurrence of CAM. Oecologia 60 (2), 176–184. https://doi.org/10.1007/BF00379519.

Griffiths, H., Cousins, A.B., Badger, M.R., von Caemmerer, S., 2007. Discrimination in the dark. Resolving the interplay between metabolic and physical constraints to phospho*enol*pyruvate carboxylase activity during the Crassulacean acid metabolism cycle. Plant Physiol. 143, 1055–1067. https://doi.org/10.1104/pp.106.088302.

Guralnick, L.J., Cline, A., Smith, M., Sage, R.F., 2008. Evolutionary physiology: the extent of C_4 and CAM photosynthesis in the genera *Anacampseros* and *Grahamia* of the Portulacaceae. J. Exp. Bot. 59 (7), 1735–1742. https://doi.org/10.1093/jxb/ern081.

Guy, R.D., Reid, D.M., Krouse, H.R., 1980. Shifts in carbon isotope ratios of two C_3 halophytes under natural and artificial conditions. Oecologia 44 (2), 241–247. https://doi.org/10.1007/BF00572686.

Hancock, L.P., Holtum, J.A.M., Edwards, E.J., 2019. The evolution of CAM photosynthesis in Australian *Calandrinia* reveals lability in C_3+CAM phenotypes and a possible constraint to the evolution of strong CAM. Integr. Comp. Biol. 59 (3), 517–534. https://doi.org/10.1093/icb/icz089.

Hartigan, J.A., Hartigan, P.M., 1985. The dip test of unimodality. Ann. Stat. 13 (1), 70–84.

Hartmann, H.E.K., 2017. Illustrated Handbook of Succulent Plants: Aizoaceae, 2nd ed. Springer, Berlin.

Herrera, A., 2009. Crassulacean acid metabolism and fitness under water deficit stress: if not for carbon gain, what is facultative CAM good for? Ann. Bot. 103 (4), 645–653. https://doi.org/10.1093/aob/mcn145.

Herrera, A., 2020. Are thick leaves, large mesophyll cells and small intercellular air spaces requisites for CAM? Ann. Bot. 125 (6), 859–868. https://doi.org/10.1093/aob/mcaa008.

Heyduk, K., McKain, M.R., Lalani, F., Leebens-Mack, J., 2016. Evolution of a CAM anatomy predates the origins of Crassulacean acid metabolism in the Agavoideae (Asparagaceae). Mol. Phylogenet. Evol. 105, 102–113. https://doi.org/10.1016/j.ympev.2016.08.018.

Holthe, P.A., Patel, A., Ting, I.P., 1992. The occurrence of CAM in Peperomia. Selbyana 13, 77–87.

Holtum, J.A.M., Winter, K., 2005. Carbon isotope composition of canopy leaves in a tropical forest in Panama throughout a seasonal cycle. Trees 19, 545–551. https://doi.org/10.1007/s00468-005-0413-8.

Holtum, J.A.M., Aranda, J., Virgo, A., Gehrig, H.H., Winter, K., 2004. $\delta^{13}C$ values and Crassulacean acid metabolism in *Clusia* species from Panama. Trees 18, 658–668. https://doi.org/10.1007/s00468-004-0342-y.

Horn, J.W., Xi, Z., Riina, R., Peirson, J.A., Yang, Y., Dorsey, B.L., Berry, P.E., Davis, C.C., Wurdack, K.J., 2014. Evolutionary bursts in *Euphorbia* (Euphorbiaceae) are linked with photosynthetic pathway. Evolution 68 (12), 3485–3504. https://doi.org/10.1111/evo.12534.

Hultine, K.R., Dettmann, D.L., Williams, D.G., Puente, R., English, N.B., Butterfield, B.J., Búrquez, A., 2018. Relationships among climate, stem growth, and biomass $\delta^{13}C$ in the giant saguaro cactus (*Carnegiea gigantea*). Ecosphere. 9 (11) https://doi.org/10.1002/ecs2.2498 e02498.

Klak, C., Bruyns, P.V., Hedderson, T.A.J., 2007. A phylogeny and new classification for Mesembryanthemoideae (Aizoaceae). Taxon 56 (3), 737–756. https://doi.org/10.2307/25065857.

Kluge, M., 1977. Is *Sedum acre* L. a CAM plant? Oecologia 29 (1), 77–83. https://doi.org/10.1007/BF00345364.

Kluge, M., Brulfert, J., 1996. Crassulacean acid metabolism in the genus *Kalanchoë*: ecological, physiological and biochemical aspects. In: Winter, K., Smith, J.A.C. (Eds.), Ecological Studies 114: Crassulacean Acid Metabolism - Biochemistry, Ecophysiology and Evolution. Springer-Verlag, Berlin, Heidelberg, pp. 324–335.

Kluge, M., Brulfert, J., Ravelomanana, D., Lipp, J., Ziegler, H., 1991. Crassulacean acid metabolism in *Kalanchoë* species collected in various climatic zones of Madagascar: a survey by $\delta^{13}C$ analysis. Oecologia 88 (3), 407–414. https://doi.org/10.1007/BF00317586.

Kluge, M., Brulfert, J., Lipp, J., Ravelomanana, D., Ziegler, H., 1993. A comparative study by $\delta^{13}C$-analysis of Crassulacean acid metabolism (CAM) in *Kalanchoë*

(Crassulaceae) species of Africa and Madagascar. Bot. Acta 106 (4), 320–324. https://doi.org/10.1111/j.1438-8677.1993.tb00755.x.
Kluge, M., Brulfert, J., Rauh, W., Ravelomanana, D., Ziegler, H., 1995. Ecophysiological studies on the vegetation of Madagascar: a $\delta^{13}C$ and δD survey for incidence of Crassulacean acid metabolism (CAM) among orchids from montane forests and succulents from the xerophytic thorn-bush. Isotopes Environ. Health Stud. 31 (2), 191–210. https://doi.org/10.1080/10256019508234018.
Komsta, L., Novomestky, F., 2015. Moments: Moments, cumulants, skewness, kurtosis and related tests. R Package Version 0.14. https://CRAN.R-project.org/package=moments.
Langrognet, F., Lebret, R., Poli, C., Iovleff, S., Auder, B., 2020. Rmixmod: classification with mixture modelling. R Package Version 2.1.5 (Accessed 13 October 2020). https://CRAN.R-project.org/package=Rmixmod.
Li, M.-H., Liu, D.-K., Zhang, G.-Q., Deng, H., Tu, X.-D., Wang, Y., Lan, S.-R., Liu, Z.-J., 2019. A perspective on Crassulacean acid metabolism photosynthesis evolution of orchids on different continents: *Dendrobium* as a case study. J. Exp. Bot. 70 (22), 6611–6619. https://doi.org/10.1093/jxb/erz461.
Liu, H.-Y., 1989. Systematics of *Aeonium* (Crassulaceae). Hsin Yi Press & Printing Co., Ltd., Taipeh.
Loader, N.J., Robertson, I., McCarroll, D., 2003. Comparison of stable carbon isotope ratios in the whole wood, cellulose and lignin of oak tree-rings. Palaeogeogr. Palaeoclimatol. Palaeoecol. 196, 395–407. https://doi.org/10.1016/S0031-0182(03)00466-8.
Lösch, R., 1990. Dissertationes Botanicae Band 146: Funktionelle Voraussetzungen der adaptiven Nischenbesetzung in der Evolution der makaronesischen Semperviven. J. Cramer in der Gebrüder Borntraeger Verlagsbuchhandlung. Berlin, Stuttgart.
Lüttge, U., 2004. Ecophysiology of Crassulacean acid metabolism (CAM). Ann. Bot. 93 (6), 629–652. https://doi.org/10.1093/aob/mch087.
Maechler, M., 2016. Diptest: Hartigan's dip test statistic for unimodality - corrected. R Package Version 0.75-7 (Accessed 13 October 2020). https://CRAN.R-project.org/package=diptest.
Males, J., 2018. Concerted anatomical change associated with Crassulacean acid metabolism in the Bromeliaceae. Funct. Plant Biol. 45 (7), 681–695. https://doi.org/10.1071/FP17071.
Males, J., Griffiths, H., 2017. Stomatal biology of CAM plants. Plant Physiol. 174, 550–560. https://doi.org/10.1104/pp.17.00114.
Martin, C.E., Loeschen, V.S., Coke, L.B., 1990. Crassulacean acid metabolism in selected terrestrial succulents in southeastern Jamaica, including two species in the Commelinaceae. Oecologia 84 (1), 99–102. https://doi.org/10.1007/BF00665601.
McCarroll, D., Loader, N.J., 2004. Stable isotopes in tree rings. Quat. Sci. Rev. 23, 771–801. https://doi.org/10.1016/j.quascirev.2003.06.017.
Mitov, V., Bartoszek, K., Stadler, T., 2019. Automatic generation of evolutionary hypotheses using mixed Gaussian phylogenetic models. Proc. Natl. Acad. Sci. U. S. A. 116 (34), 16921–16926. https://doi.org/10.1073/pnas.1813823116.
Mooney, H.A., Troughton, J.H., Berry, J.A., 1977. Carbon isotope ratio measurements of succulent plants in southern Africa. Oecologia 30 (4), 295–305. https://doi.org/10.1007/BF00399762.
Mooney, H.A., Bullock, S.H., Ehleringer, J.R., 1989. Carbon isotope ratios of plants of a tropical dry forest in Mexico. Funct. Ecol. 3 (2), 137–142. https://doi.org/10.2307/2389294.
Mort, M.E., Soltis, D.E., Soltis, P.S., Santos-Guerra, A., Francisco-Ortega, J., 2007. Physiological evolution and association between physiology and growth form in *Aeonium* (Crassulaceae). Taxon 56 (2), 453–464. https://doi.org/10.1002/tax.562016.
Müller, K., Wickham, H., Francois, R., Bryan, J., 2020. Tibble: simple data frames. R Package Version 3.0.3 (Accessed 09 September 2020). https://CRAN.R-project.org/package=tibble.
O'Leary, M.H., 1981. Carbon isotope fractionation in plants. Phytochemistry 20 (4), 553–567. https://doi.org/10.1016/0031-9422(81)85134-5.
O'Leary, M.H., 1988. Carbon isotopes in photosynthesis: fractionation techniques may reveal new aspects of carbon dynamics in plants. BioScience 38 (5), 328–336. https://doi.org/10.2307/1310735.
Osmond, C.B., 1978. Crassulacean acid metabolism: a curiosity in context. Ann. Rev. Plant Physiol. 29, 379–414. https://doi.org/10.1146/annurev.pp.29.060178.002115.
Osmond, C.B., Ziegler, H., Stichler, W., Trimborn, P., 1975. Carbon isotope discrimination in alpine succulent plants supposed to be capable of Crassulacean acid metabolism (CAM). Oecologia 18 (3), 209–217. https://doi.org/10.1007/BF00345423.
Oyungerel, S., Tsendeekhuu, T., Tserenkhand, G., 2004. A study to detect CAM plants in Mongolia. Mong. J. Biol. Sci. 2 (1), 29–37.
Pate, J.S., Unkovich, M.J., Erskine, P.D., Stewart, G.R., 1998. Australian mulga ecosystems – ^{13}C and ^{15}N natural abundances of biota components and their ecophysiological significance. Plant Cell Environ. 21 (12), 1231–1242. https://doi.org/10.1046/j.1365-3040.1998.00359.x.
Popp, M., Kramer, D., Lee, H., Diaz, M., Ziegler, H., Lüttge, U., 1987. Crassulacean acid metabolism in tropical dicotyledonous trees of the genus *Clusia*. Trees 1 (4), 238–247. https://doi.org/10.1007/BF01816822.
Quade, J., Cerling, T.E., Bowman, J.R., 1989. Development of Asian monsoon revealed by marked ecological shift during the latest Miocene in northern Pakistan. Nature 342, 163–166. https://doi.org/10.1038/342163a0.
R Core Team, 2020. The R Project for Statistical Computing (Accessed 09 September 2020). https://www.R-project.org/.
Ramírez, N., Herrera, A., 2017. Reproductive efficiency and photosynthetic pathway in seed plants. Perspect. Plant Ecol. Evol. Syst. 24, 48–60. https://doi.org/10.1016/j.ppees.2016.12.004.
Rao, I.M., Swamy, P.M., Das, V.S.R., 1979. Some characteristics of Crassulacean acid metabolism in five nonsucculent scrub species under natural semiarid conditions. Zeitschrift für Pflanzenphysiologie 94 (3), 201–210. https://doi.org/10.1016/S0044-328X(79)80159-2.
Roeske, C.A., O'Leary, M.H., 1984. Carbon isotope effects on the enzyme-catalyzed carboxylation of ribulose bisphosphate. Biochemistry 23, 6275–6284. https://doi.org/10.1021/bi00320a058.
Rundel, P.W., Dillon, M.O., 1998. Ecological patterns in the Bromeliaceae of the lomas formations of coastal Chile and Peru. Pl. Syst. Evol. 212 (3–4), 261–278. https://doi.org/10.1007/BF01089742.
Rundel, P.W., Rundel, J.A., Ziegler, H., Stichler, W., 1979. Carbon isotope ratios of central Mexican Crassulaceae in natural and greenhouse environments. Oecologia 38 (1), 45–50. https://doi.org/10.1007/BF00347823.
Rundel, P.W., Esler, K.J., Cowling, R.M., 1999. Ecological and phylogenetic patterns of carbon isotope discrimination in the winter-rainfall flora of the Richtersveld, South Africa. Plant Ecol. 142, 133–148. https://doi.org/10.1023/A:1009878429455.
Rundel, P.W., Gibson, A.C., Midgley, G.S., Wand, S.J.E., Palma, B., Kleier, C., Lambrinos, J., 2003. Ecological and ecophysiological patterns in a pre-altiplano shrubland of the Andean Cordillera in northern Chile. Plant Ecol. 169, 179–193. https://doi.org/10.1023/A:1026075721045.
Sanaiotti, T.M., 1996. The Woody Flora and Soils of Seven Brazilian Amazonian Dry Savanna Areas. PhD thesis. University of Stirling.
Sarris, D., Siegwolf, R., Körner, C., 2013. Inter- and intra-annual stable carbon and oxygen isotope signals in response to drought in Mediterranean pines. Agric. For. Meteorol. 168, 59–68. https://doi.org/10.1016/j.agrformet.2012.08.007.
Schweiger, A.H., Nürk, N.M., Beckett, H., Liede-Schumann, S., Midgley, G.F., Higgins, S. I., 2021. The eco-evolutionary significance of rainfall constancy for facultative CAM photosynthesis. New Phytol. 230 (4), 1653–1664. https://doi.org/10.1111/nph.17250.
Seibt, U., Rajabi, A., Griffiths, H., Berry, J.A., 2008. Carbon isotopes and water use efficiency: sense and sensitivity. Oecologia 155, 441–454. https://doi.org/10.1007/s00442-007-0932-7.
Silvera, K., Santiago, L.S., Winter, K., 2005. Distribution of Crassulacean acid metabolism in orchids of Panama: evidence of selection for weak and strong modes. Funct. Plant Biol. 32 (5), 397–407. https://doi.org/10.1071/FP04179.
Silvera, K., Santiago, L.S., Cushman, J.C., Winter, K., 2009. Crassulacean acid metabolism and epiphytism linked to adaptive radiaions in the Orchidaceae. Plant Physiol. 149, 1838–1847. https://doi.org/10.1104/pp.108.132555.
Silvera, K., Neubig, K.M., Whitten, W.M., Williams, N.H., Winter, K., Cushman, J.C., 2010a. Evolution along the Crassulacean acid metabolism continuum. Funct. Plant Biol. 37 (11), 995–1010. https://doi.org/10.1071/FP10084.
Silvera, K., Santiago, L.S., Cushman, J.C., Winter, K., 2010b. The incidence of Crassulacean acid metabolism in Orchidaceae derived from carbon isotope ratios: a checklist of the flora of Panama and Costa Rica. Bot. J. Linn. Soc. 163 (2), 194–222. https://doi.org/10.1111/j.1095-8339.2010.01058.x.
Sipes, D.L., Ting, I.P., 1985. Crassulacean acid metabolism and Crassulacean acid metabolism modifications in *Peperomia camptotricha*. Plant Physiol. 77, 59–63. https://doi.org/10.1104/pp.77.1.59.
Skrzypek, G., Paul, D., Wojtuń, B., 2013. The altitudinal climatic effect on the stable isotope compositions of *Agave* and *Opuntia* in arid environments – a case study at the Big Bend National Park, Texas, USA. J. Arid Environ. 92, 102–112. https://doi.org/10.1016/j.jaridenv.2013.02.002.
Smith, J.A.C., Winter, K., 1996. Taxonomic distribution of Crassulacean acid metabolism. In: Winter, K., Smith, J.A.C. (Eds.), Ecological Studies 114: Crassulacean Acid Metabolism - Biochemistry, Ecophysiology and Evolution. Springer-Verlag, Berlin, Heidelberg, pp. 427–436.
Szpak, P., White, C.D., Longstaffe, F.J., Millaire, J.-F., Sánchez, V.F.V., 2013. Carbon and nitrogen isotopic survey of northern Peruvian plants: baselines for paleodietary and paleoecological studies. PLoS One 8 (1). https://doi.org/10.1371/journal.pone.0053763 e53763.
Teeri, J.A., Tonsor, S.J., Turner, M., 1981. Leaf thickness and carbon isotope composition in the Crassulaceae. Oecologia 50 (3), 367–369. https://doi.org/10.1007/BF00344977.
Tenhunen, J.D., Tenhunen, L.C., Ziegler, H., Stichler, W., Lange, O.L., 1982. Variation in carbon isotope ratios of Sempervivoideae species from different habitats of Teneriffe in the spring. Oecologia 55 (2), 217–224. https://doi.org/10.1007/BF00384490.
Ting, I.P., 1985. Crassulacean acid metabolism. Ann. Rev. Plant Physiol. 36, 595–622. https://doi.org/10.1146/annurev.pp.36.060185.003115.
Ting, I.P., Hann, J., Holbrook, N.M., Putz, F.E., Sternberg, L.S.L., Price, D., Goldstein, G., 1987. Photosynthesis in hemiepiphytic species of *Clusia* and *Ficus*. Oecologia 74 (3), 339–346. https://doi.org/10.1007/BF00378927.
Torres-Morales, G., Lasso, E., Silvera, K., Turner, B.L., Winter, K., 2020. Occurrence of crassulacean acid metabolism in Colombian orchids determined by leaf carbon isotope ratios. Bot. J. Linn. Soc. 193 (4), 431–477. https://doi.org/10.1093/botlinnean/boaa027.
Troughton, J.H., Mooney, H.A., Berry, J.A., Verity, D., 1977. Variable carbon isotope ratios of *Dudleya* species growing in natural environments. Oecologia 30 (4), 307–311. https://doi.org/10.1007/BF00399763.
Vargas-Soto, J.G., Andrade, J.L., Winter, K., 2009. Carbon isotope composition and mode of photosynthesis in *Clusia* species from Mexico. Photosynthetica 47 (1), 33–40. https://doi.org/10.1007/s11099-009-0007-6.
West, J.B., Bowen, G.J., Cerling, T.E., Ehleringer, J.R., 2006. Stable isotopes as one of nature's ecological recorders. Trends Ecol. Evol. 21 (7), 408–414. https://doi.org/10.1016/j.tree.2006.04.002.

Wester, S., Mendieta-Leiva, G., Nauheimer, L., Wanek, W., Kreft, H., Zotz, G., 2011. Physiological diversity and biogeography of vascular epiphytes at Río Changuinola, Panama. Flora 206, 66–79. https://doi.org/10.1016/j.flora.2010.01.011.

Wickham, H., 2011. The split-apply-combine strategy for data analysis. J. Stat. Softw. 40 (1), 1–29. https://doi.org/10.18637/jss.v040.i01.

Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis, 2nd ed. Springer-Verlag, New York. ISBN 978-3-319-24277-4.

Winter, K., 1979. $\delta^{13}C$ values of some succulent plants from Madagascar. Oecologia 40 (1), 103–112. https://doi.org/10.1007/BF00388814.

Winter, K., 1985. Crassulacean acid metabolism. In: Barber, J., Baker, N.R. (Eds.), Topics in Photosynthesis Volume 6: Photosynthetic Mechanisms and the Environment. Elsevier, Amsterdam, New York, Oxford, pp. 329–387.

Winter, K., 2019. Ecophysiology of constitutive and facultative CAM photosynthesis. J. Exp. Bot. 70 (22), 6495–6508. https://doi.org/10.1093/jxb/erz002.

Winter, K., Holtum, J.A.M., 2002. How closely do the $\delta^{13}C$ values of Crassulacean acid metabolism plants reflect the proportion of CO_2 fixed during day and night? Plant Physiol. 129, 1843–1851. https://doi.org/10.1104/pp.002915.

Winter, K., Holtum, J.A.M., 2005. The effects of salinity, Crassulacean acid metabolism and plant age on the carbon isotope composition of *Mesembryanthemum crystallinum* L., a halophytic C_3-CAM species. Planta 222, 201–209. https://doi.org/10.1007/s00425-005-1516-6.

Winter, K., Holtum, J.A.M., 2017. Facultative Crassulacean acid metabolism (CAM) in four small C_3 and C_4 leaf-succulents. Aust. J. Bot. 65 (2), 103–108. https://doi.org/10.1071/BT16015.

Winter, K., Smith, J.A.C., 1996. An introduction to crassulacean acid metabolism. Biochemical principles and ecological diversity. In: Winter, K., Smith, J.A.C. (Eds.), Ecological Studies 114: Crassulacean Acid Metabolism - Biochemistry, Ecophysiology and Evolution. Springer-Verlag, Berlin, Heidelberg, pp. 1–13.

Winter, K., von Willert, D.J., 1972. NaCl-induzierter Crassulaceen-Säurestoffwechsel bei *Mesembryanthemum crystallinum*. Zeitschrift für Pflanzenphysiologie 67 (2), 166–170. https://doi.org/10.1016/S0044-328X(72)80131-4.

Winter, K., Lüttge, U., Winter, E., Troughton, J.H., 1978. Seasonal shift from C_3 photosynthesis to Crassulacean acid metabolism in *Mesembryanthemum crystallinum* growing in its natural environment. Oecolgia (Berl.) 34 (2), 225–237. https://doi.org/10.1007/BF00345168.

Winter, K., Osmond, C.B., Pate, J.S., 1981. Coping with salinity. In: Pate, J.S., McComb, A.J. (Eds.), The Biology of Australian Plants. University of Western Australia Press, Nedlands, pp. 88–113.

Winter, K., Wallace, B.J., Stocker, G.C., Roksandic, Z., 1983. Crassulacean acid metabolism in Australian vascular epiphytes and some related species. Oecologia 57 (1–2), 129–141. https://doi.org/10.1007/BF00379570.

Winter, K., Aranda, J., Holtum, J.A.M., 2005. Carbon isotope composition and water-use efficiency in plants with Crassulacean acid metabolism. Funct. Plant Biol. 32 (5), 381–388. https://doi.org/10.1071/FP04123.

Winter, K., Garcia, M., Holtum, J.A.M., 2008. On the nature of facultative and constitutive CAM: environmental and developmental control of CAM expression during early growth of *Clusia*, *Kalanchoë*, and *Opuntia*. J. Exp. Bot. 59 (7), 1829–1840. https://doi.org/10.1093/jxb/ern080.

Winter, K., Holtum, J.A.M., Smith, J.A.C., 2015. Crassulacean acid metabolism: a continuous or discrete trait? New Phytol. 208 (1), 73–78. https://doi.org/10.1111/nph.13446.

Ziegler, H., 1996. Carbon- and hydrogen-isotope discrimination in Crassulacean acid metabolism. In: Winter, K., Smith, J.A.C. (Eds.), Ecological Studies 114: Crassulacean Acid Metabolism - Biochemistry, Ecophysiology and Evolution. Springer-Verlag, Berlin, Heidelberg, pp. 336–348.

Ziegler, H., Batanouny, K.H., Sankhla, N., Vyas, O.P., Stichler, W., 1981. The photosynthetic pathway types of some desert plants from India, Saudi Arabia, Egypt, and Iraq. Oecologia 48 (1), 93–99. https://doi.org/10.1007/BF00346993.

Zotz, G., 2004. How prevalent is Crassulacean acid metabolism among vascular epiphytes? Oecologia 138 (2), 184–192. https://doi.org/10.1007/s00442-003-1418-x.

Zotz, G., Ziegler, H., 1997. The occurrence of Crassulacean acid metabolism among vascular epiphytes from central Panama. New Phytol. 137 (2), 223–229. https://doi.org/10.1046/j.1469-8137.1997.00800.x.