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Forty years of research into crassulacean acid metabolism in the genus *Clusia*: anatomy, ecophysiology and evolution

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Clusia is the only genus containing dicotyledonous trees with a capacity to perform crassulacean acid metabolism (CAM). Since the discovery of CAM in *Clusia* 40 years ago, several studies have highlighted the extraordinary plasticity and diversity of life forms, morphology and photosynthetic physiology of this genus. In this review, we revisit aspects of CAM photosynthesis in *Clusia* and hypothesize about the timing, the environmental conditions and potential anatomical characteristics that led to the evolution of CAM in the group. We discuss the role of physiological plasticity in influencing species distribution and ecological amplitude in the group. We also explore patterns of allometry of leaf anatomical traits and their correlations with CAM activity. Finally, we identify opportunities for further research on CAM in *Clusia*, such as the role of elevated nocturnal accumulation of citric acid, and gene expression in C₃–CAM intermediate phenotypes.

Key words: CAM photosynthesis, trees, plasticity, carbon, Neotropics.

INTRODUCTION

Crassulacean acid metabolism (CAM) is a photosynthetic mode found in ~7 % of all vascular land plants, commonly as an adaptation to water-limited environments. Unlike C₃ species, typical CAM plants fix atmospheric CO₂ predominantly at night, leading to the synthesis of malate and its overnight storage as malic acid in the vacuoles of photosynthetic cells. The following day, malic acid is decarboxylated, thereby regenerating CO₂, which is then refixed and reduced in the Calvin–Benson–Bassham cycle (Osmond, 1978). Thus, during the day (when the air is hottest and driest), CAM allows photosynthesis to occur behind closed stomata, resulting in less water being lost to the atmosphere, in comparison to C₃ plants, over a 24 h period. CAM has evolved convergently in ≥37 families of vascular plants, with multiple independent origins within most families (Winter and Smith, 1996; Winter *et al.*, 2021). However, despite CAM having evolved so many times, the Neotropical genus *Clusia* is thus far considered the only group of dicotyledonous trees in which some species show CAM (Lüttge, 2008).

Clusia is composed of ~321 species of terrestrial and hemiepiphytic woody plants distributed across the Neotropics (POWO, 2022). The plants also occur as epiphytes, although it is often not clear whether epiphytic individuals observed in the field eventually become hemiepiphytes. The plants occupy a wide range of habitats, from lowland forests to montane forests, in addition to high-elevation páramos (Fig. 1). Furthermore, *Clusia* is found in dry scrubs in interandean valleys, sandstone and granite rock outcrops, and open coastal sands (restingas), where they can often be pioneer species and function as nurse plants (Dias and Scarano, 2007; Gustafsson *et al.*, 2007). The terrestrial habit of *Clusia* species is most common in high-elevation

montane forests and in seasonally dry scrubland, where some species can have relatively high abundance [e.g. *Clusia trochiformis* Vesque in the Venezuelan Andes (Kelly *et al.*, 1994) and *Clusia flava* Jacq in some areas of the Yucatán peninsula (Fig. 2)]. In contrast, the hemiepiphytic habit of *Clusia* is common in lower montane forests and lowland wet forests, where *Clusia* species grow mostly as woody shrubs on top of the mid to high branches of phorophytes [e.g. *Clusia uvitana* Pittier (previously described as *Clusia odorata* Seem.) and *Clusia flavida* (Benth.) Pipoly on Barro Colorado Island in Panama (Todzia, 1986); *Clusia* spp. in rainforests in Jamaica (Kelly, 1985)].

The earliest physiological observations on *Clusia* were probably made by Alexander von Humboldt during his travels in Venezuela, where he noticed that leaves of *Clusia rosea* Jacq., unlike those of other species, did not produce bubbles when submerged in water under direct sunlight (Lüttge, 2007). The lack of bubbles probably occurred because this CAM plant had closed its stomata during the day. However, Humboldt could not interpret his observations further owing to the technical limitations of his time, and it was not until the late 1940s when the CAM cycle was described formally (Bonner and Bonner, 1948; Thomas and Beevers, 1949). The presence of CAM activity in *Clusia* was reported properly, for the first time, by Tinoco-Ojanguren and Vázquez-Yanes (1983) and Ting *et al.* (1985), who measured titratable acidity and gas exchange in *Clusia lundellii* Standl and *Clusia rosea*, respectively. Subsequent physiological studies found that some *Clusia* species can shift between C₃ and CAM depending on environmental conditions. This photosynthetic flexibility might be part of the reason why species of *Clusia* occupy such a wide range of habitats across the Neotropics (Franco *et al.*, 1990; Lüttge, 1996).



FIG. 1. Habitats of selected *Clusia* species. (A) *Clusia multiflora* Kunth in cloud forest in the Central Cordillera in Colombia. (B) *Clusia uvitana* growing as a hemiepiphytic shrub in lowland wet forest in Panama. (C) *Clusia pringlei* Lundell growing in dry scrublands in central Mexico. (D) *Clusia grandiflora* Splitg. growing in swamp forests in the Orinoco Delta in Venezuela.

PECULIARITIES OF THE CRASSULACEAN ACID METABOLISM CYCLE BIOCHEMISTRY IN *CLUSIA*

The CAM plants can be divided into two major groups regarding their mode of malate decarboxylation during the diurnal cycle: those that use mainly NADP- and NAD-malic enzyme (NAD-ME), and those that use predominantly phosphoenolpyruvate carboxykinase (PEPCK). In CAM-performing *Clusia*, PEPCK appears to be the predominant enzyme catalysing the decarboxylation reaction (Borland *et al.*, 1998). However, in one of the few attempts to study gene expression in *Clusia* thus far, RNA-seq analysis found that both PEPCK and NAD-ME were upregulated when *Clusia pratensis* Seem. switched from C_3 to CAM, suggesting that NAD-ME might also be contributing to malate decarboxylation (A. Leverett, unpublished data).

A peculiarity of *Clusia* species with CAM is the nocturnal accumulation of citric acid. When nocturnal H^+ accumulation is measured across phylogenetically diverse CAM taxa, *Clusia* often exhibits the greatest dawn–dusk difference in titratable

acidity (Winter and Smith, 2022). This is largely attributable to the accumulation of citric acid, which adds to the proton pool in the vacuole. Citric acid accumulation also occurs in other CAM species, such as *Talinum triangulare* (Jacq.) Willd., *Ananas comosus* (L.) Merr. and *Nidularium billbergioides* (Schult. and Schult.f.) L.B.Sm. However, the magnitude of citric acid accumulation in *Clusia* appears to be much greater than in any other taxa (Osmond *et al.*, 1996). It is unclear whether citric acid accumulation is a direct consequence of CO_2 assimilation. Citrate can be formed by converting malate to oxaloacetate, then adding a carbon atom via citrate synthase. However, this reaction requires acetyl-CoA, which is generated by the decarboxylation of pyruvate. Consequently, the net carbon balance of this reaction is neutral, meaning that nocturnal citrate accumulation would have no impact on CO_2 assimilation (Lüttge, 1988). In contrast, if mitochondrial isocitrate dehydrogenase were to carboxylate α -ketoglutarate, in the reverse direction to its typical function, the product of this reaction, D-isocitrate, could be converted to citrate. If this were the case, citric acid would be the direct consequence of

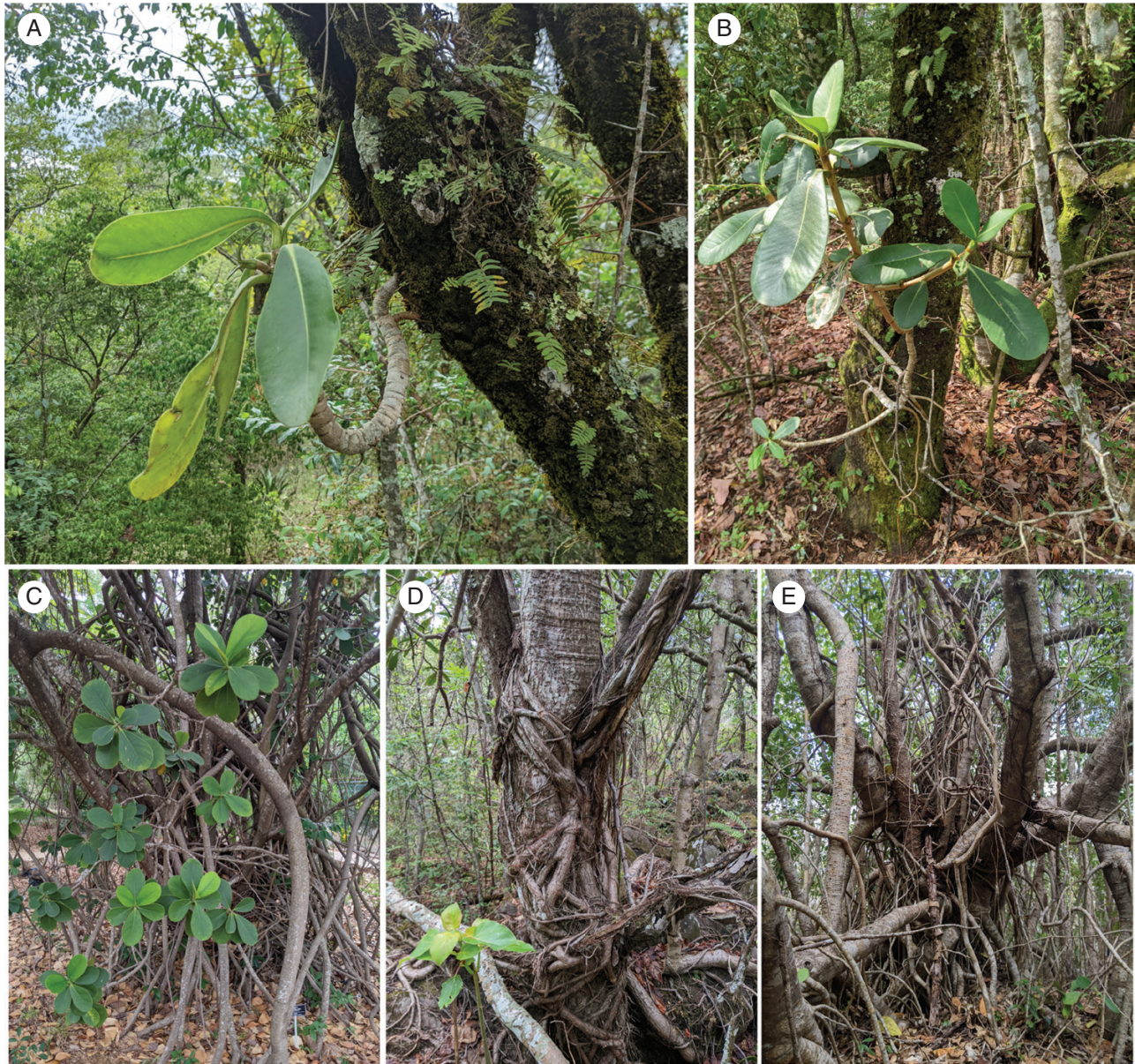


FIG. 2. Habit of selected *Clusia* species, showing epiphytic and terrestrial plants with mature adventitious roots. (A, B) Epiphytic forms of *Clusia massoniana* Lundell growing on a phorophyte (oak trees). (C) *Clusia rosea*. (D) *Clusia pringlei*. (E) *Clusia flava*. Most mature roots in C and D are apparently developed from the same terrestrial individual plant, which produces adventitious roots that auto-strangle the main parental stem.

CO₂ assimilation. Although this scenario has been simulated using flux balance analysis modelling, experimental evidence is lacking (Töpfer et al., 2020; Winter and Smith, 2022). *Clusia* is an ideal model in which to test the metabolic origin of citric acid, because this genus accumulates this molecule in abundance during the night.

Finally, CAM plants in the genus *Clusia* appear to exhibit elevated nocturnal respiratory rates, in comparison to C₃ species. A comparison of four Venezuelan species of *Clusia* reported nocturnal oxygen uptake rates that were more than twice as large in constitutive CAM species than in weak CAM species (Ting et al., 1987). Likewise, the drought-induced switch from C₃ to CAM in *Clusia pratensis* is accompanied by a 1.6-fold

increase in oxygen consumption. In contrast, the same drought treatment applied to obligate C₃ *Clusia* species does not cause a change in nocturnal oxygen consumption, suggesting that this switch in respiratory physiology is a requirement of CAM and not simply a more general drought response (A. Leverett, unpublished data). Recent flux balance analysis modelling of the CAM cycle (Shameer et al., 2018) has suggested that CAM requires elevated nocturnal respiratory rates, in large part to drive the ATP-dependent import of malate into the vacuole. *Clusia* appears to corroborate the predictions from this model. However, it remains unclear whether elevated nocturnal respiratory rates are a peculiarity, unique to *Clusia*, or if other CAM taxa share this trait.

LARGE PHOTOSYNTHETIC CELLS AND CRASSULACEAN ACID METABOLISM: A FUNCTIONAL RELATIONSHIP

In most leaf-succulent lineages that perform CAM, such as *Kalanchoë*, *Crassula* and *Yucca*, the mesophyll is composed of relatively uniform, spherical chlorenchyma cells (Abdel-Raouf, 2012; Heyduk et al., 2016; Fradera-Soler et al., 2021). In contrast, *Clusia* leaves contain well-defined palisade and spongy mesophyll layers, the latter with large intercellular spaces (Fig. 3; Popp et al., 1987; Borland et al., 1998). Relationships between CAM and anatomy have probably been studied more comprehensively in *Clusia* than in any other genus (Popp et al., 1987; Borland et al., 1998, 2018; Barrera-Zambrano et al., 2014; Luján et al., 2022; Leverett et al., 2023a). Investigations into both glasshouse- and field-grown *Clusia* species have demonstrated that CAM species have larger palisade cells and thicker palisade tissue than C₃ relatives (Barrera-Zambrano et al., 2014; Luján et al., 2022). Development of large photosynthetic tissues within leaves of CAM species is believed to provide overnight storage space for malic acid. Although interspecific comparative studies show that CAM is associated with large photosynthetic cells, these relationships remain correlative (Barrera-Zambrano et al., 2014; Males, 2018). This is problematic, because it is unclear whether greater volume in the palisade truly exists to aid the storage of nocturnal malic acid or whether large photosynthetic cells are, in fact, an indirect consequence of maximizing leaf water-storage capabilities, which would also be beneficial in water-limited niches (Edwards, 2019). Large cells are often thought to increase hydraulic capacitance, which allows leaves to mitigate reductions in water potential during dehydration, thereby protecting the mesophyll and vascular tissue from mechanical damage (Ogburn and Edwards, 2010, 2012). However, recent work has shown that interspecific variation

in hydraulic capacitance is not driven by differences in palisade cell size or thickness in *Clusia* (Leverett et al., 2023a). Instead, variation in the size of specialized water-storage hypodermis tissue (which is itself independent of CAM) determines hydraulic capacitance. Therefore, the relationship between palisade cell size and CAM is not complicated by the confounding variable, hydraulic capacitance. Eliminating this potentially confounding relationship increases the confidence that large palisade cells have evolved specifically for the purpose of malic acid storage, rather than to buffer leaf water potentials (Leverett et al., 2023a). Therefore, rather than being merely correlative, there appears to be a truly functional relationship between CAM and large photosynthetic cells in *Clusia*.

CRASSULACEAN ACID METABOLISM AFFECTS MESOPHYLL ALLOMETRY IN *CLUSIA* LEAVES

In *Clusia*, there is evidence that palisade cells, and not the spongy mesophyll cells, are the main location of the CAM cycle. The abundance of PEPC in the palisade is substantially higher in leaves of *Clusia* species with CAM than in those doing C₃, whereas abundance of this enzyme in the spongy mesophyll is comparable between species with contrasting photosynthetic modes (Barrera-Zambrano et al., 2014). Furthermore, anatomical studies have found that correlations between palisade cell size and CAM are far stronger than correlations between spongy mesophyll dimensions and CAM (Barrera-Zambrano et al., 2014; Borland et al., 2018; Luján et al., 2022). In contrast, *Clusia* species living in high-elevation cloud forests have thicker spongy mesophyll tissues. This might confer greater leaf longevity, as is seen in other taxa (Cordell et al., 1998). It is also possible that C₃ *Clusia* species living in moist, high-elevation montane

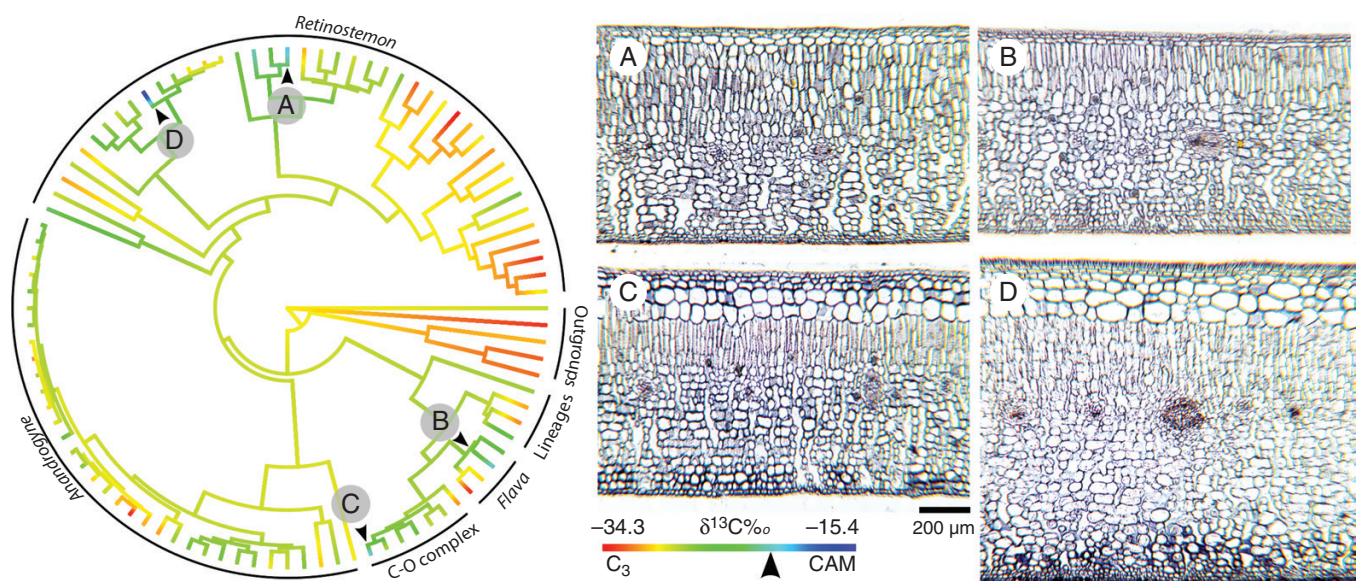


FIG. 3. Ancestral state reconstruction of $\delta^{13}\text{C}$ values on a maximum likelihood phylogenetic tree. Arrowheads indicate $\delta^{13}\text{C}$ less negative than -20‰ . (A–D) Transverse sections of leaves of plants with strong CAM. (A) *Clusia uvitana*. (B) *Clusia lundellii*. (C) *Clusia rosea*. (D) *Clusia firmifolia* Cuatrec. Modified from Luján et al. (2022).

forests develop thick spongy mesophyll layers to maximize absorption of light, which is often obscured by cloud cover and algal mats. Hence, different environmental stresses might affect the development of each tissue layer: low water availability has led to large palisade cells to facilitate CAM, and cloud cover might have led to thick spongy mesophyll to maximize C_3 photosynthesis in low-light conditions. A consequence of this anatomical specialization in *Clusia* leaves is that CAM affects the allometry of mesophyll layers. In other taxa, palisade and spongy mesophyll thickness and cell sizes scale tightly with each other, such that differences in palisade thickness will result in proportional differences in the spongy mesophyll (John et al., 2013). In *Clusia*, although palisade and spongy mesophyll thickness are correlated, the scaling factor that drives this relationship is affected by CAM. The contribution of CAM to mesophyll allometry can be observed, using published data (Luján et al., 2022) to build a multiple linear regression model, with palisade thickness as the dependent variable and both spongy mesophyll thickness and $\delta^{13}C$ as predictive independent variables. This model shows that spongy mesophyll thickness is a significant predictor of palisade thickness ($P = 0.02$). However, the predictive correlation between spongy mesophyll thickness and palisade thickness is different depending on $\delta^{13}C$, which has a significant interaction effect on this relationship ($P = 0.04$). Likewise, these same anatomical data can be split into three groups (in which $\delta^{13}C$ is < -30 , -30 to -25 or > -25 ‰) and linear regressions built for each subset of data (Fig. 4). When this is done, the slope of the line describing the correlation between palisade and spongy mesophyll thickness differs; steeper slopes are seen when $\delta^{13}C$ values are less negative (i.e. when there is a greater investment in CAM). Put simply, the presence of CAM affects the scaling relationship between spongy and palisade mesophyll thickness, such that CAM is associated with thicker palisade forming without proportional spongy mesophyll thickness. Interestingly, a recent study on the genus *Cymbidium* (Orchidaceae) suggests CAM might have had a similar effect on anatomical allometry to that observed in

Clusia (Yamaga-Hatakeyama et al., 2022). In *Cymbidium*, CAM is associated with larger adaxial palisade cells, whereas the relationship between CAM and spongy mesophyll size is less pronounced. It would be intriguing to compare any climatic factors that have led to allometric differences between C_3 and CAM plants in both *Clusia* and *Cymbidium*, to determine whether the anatomy of these genera has undergone parallel evolutionary trajectories.

INTERNAL AIR SPACE IN *CLUSIA* LEAVES

In addition to large photosynthetic cells, the leaves of CAM plants are often characterized by low fractional internal air space (% IAS), because the development of tightly packed cells will maximize the space available for the storage of malic acid within a given volume of leaf (Nelson et al., 2005; Males, 2018). However, the relationship between CAM and % IAS is called into question in *Clusia*. Analysis of 64 field-grown species found that $\delta^{13}C$ was negatively correlated with % IAS, although the effect size was weak (Fig. 5A). However, this trend did not hold true when these data were recalculated to estimate the % IAS in the spongy mesophyll. If porosity in the epidermis, hypodermis and palisade are assumed to be negligible, then the % IAS of the spongy mesophyll can be estimated by dividing the total % IAS by the fraction of leaf thickness composed of spongy mesophyll. This approach found no correlation between spongy mesophyll % IAS and $\delta^{13}C$ (Fig. 5B). Hence, correlations between total % IAS and CAM in *Clusia* are likely to be the consequence of thicker palisade tissues, rather than developmental differences in the spongy mesophyll. It seems that, in *Clusia*, the requisite anatomical configurations required by CAM can evolve in the palisade without affecting % IAS in the spongy mesophyll. Being able to evolve CAM without decreasing spongy mesophyll % IAS could be beneficial, because it would allow relatively high rates of mesophyll conductance, which would be likely to aid assimilation rates during phases I, II and IV of the CAM cycle (Owen and Griffiths, 2013).

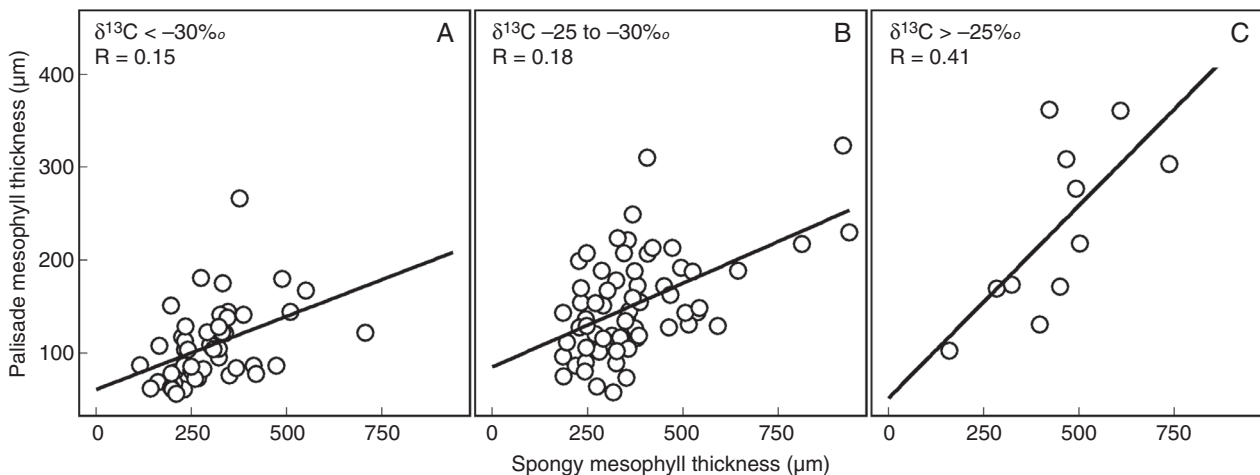


FIG. 4. CAM affects the allometry of mesophyll tissues in *Clusia* leaves. Anatomical data were grouped into three bins: (A) $\delta^{13}C < -30$ ‰; (B) $\delta^{13}C$ values between -30 and -25 ‰; and (C) $\delta^{13}C > -25$ ‰. The correlation between palisade and spongy mesophyll tissue thickness was estimated.

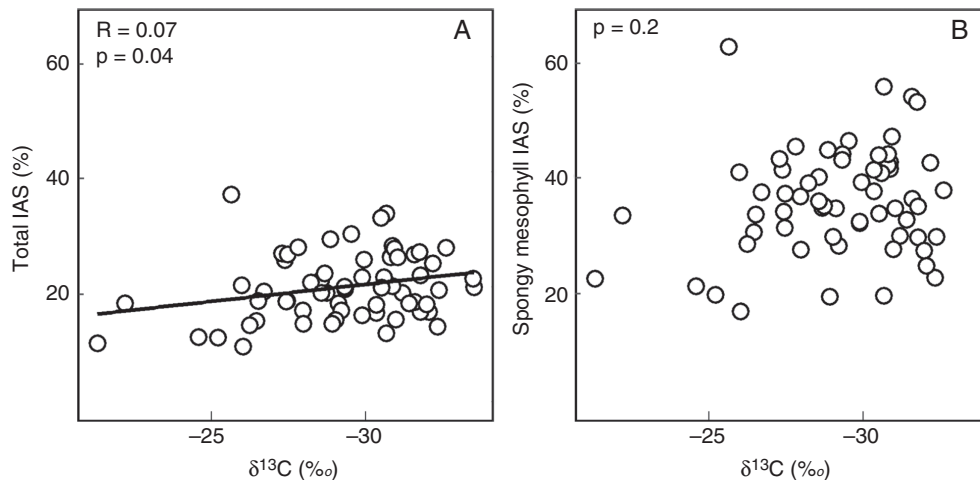


Fig. 5. Linear regression between $\delta^{13}\text{C}$ and the percentage of total intercellular air space (A) and the percentage intercellular air space of the spongy mesophyll (B).

LEAF VASCULAR ARCHITECTURE IS COORDINATED WITH CRASSULACEAN ACID METABOLISM

In addition to studies on mesophyll anatomy, the relationship between CAM and leaf vascular anatomy has recently been analysed in *Clusia* (Leverett et al., 2023b). Across ten photosynthetically diverse species, leaves with constitutive CAM phenotypes developed lower vein length per area (VLA) and a lower vein termini density (VTD) compared with facultative CAM or obligate C_3 species (Leverett et al., 2023b). The reduced transpiration rates in constitutive CAM species result in lower fluxes of water across the leaf (Winter et al., 2005; Leverett, et al., 2023a). Hence, constitutive CAM species can stay hydrated with lower leaf vein densities, because less water needs to flow through the xylem. In contrast, in facultative CAM species, VLA and VTD values closely resemble those seen in obligate C_3 species (Leverett et al., 2023b). In *Clusia* species in Panama, outside the dry season, facultative CAM species predominantly assimilate CO_2 via C_3 photosynthesis, which is associated with higher transpiration rates than those seen in CAM leaves. Given that facultative CAM species spend most of the year doing C_3 , they appear to optimize their VLA and VTD to allow elevated hydraulic conductance when plants are engaged in C_3 photosynthesis, rather than when plants are performing CAM.

In addition to VLA, CAM is linked to differences in vascular allometry in *Clusia* leaves (Leverett et al., 2023b). In almost all angiosperm species, intervein distance (IVD) is coordinated with the distance from veins to the lower epidermis (VED), such that $\text{IVD} \approx \text{VED}$ (Zwieniecki and Boyce, 2014). If IVD:VED ratios are higher than one, insufficient veins are present to maintain mesophyll hydration, whereas IVD:VED ratios less than one would often result in superfluous veins that do not add substantial hydraulic benefit to the leaf (Noblin et al., 2008). However, in some CAM taxa, the allometry between vein and mesophyll dimensions varies from that seen in most angiosperms (Males, 2017). In *Clusia*, C_3 species have IVD:VED ratios of approximately one. However, species that engage more in CAM have thicker leaves, without equivalently

lower vein densities, meaning that these species have IVD:VED ratios less than one. It is likely that the thick mesophyll tissue of CAM species is associated with greater mesophyll resistance, because water must travel a greater distance to move from the xylem conduits to stomata. The apparent ‘overinvestment’ in veins (i.e. IVD:VED ratios less than one) could act as an adaptation to overcome this apoplastic hydraulic resistance, thereby allowing the thick palisade tissue needed for CAM to remain hydrated when leaves are transpiring (de Boer et al., 2016). Overall, VLA, VTD and IVD:VED ratios all appear to be coordinated with CAM across *Clusia*, highlighting that this metabolic adaptation does not only affect photosynthetic cells but also has a considerable impact on the hydraulic architecture of leaves (Males and Griffiths, 2018).

PHOTOSYNTHETIC PATHWAY DIVERSITY IN *CLUSIA*: C_3 VERSUS CRASSULACEAN ACID METABOLISM PHOTOSYNTHESIS

According to the survey by Pachon et al. (2022), photosynthetic pathway information is available for 156 of the 321 currently accepted species of *Clusia* (POWO, 2022). For ≥ 35 species, there is evidence of CAM, although the nature and magnitude of CAM expression vary widely between species (Lüttge, 2006; Barrera-Zambrano et al., 2014). *Clusia* species with CAM typically occur in lowland habitats and belong to section *Retinostemon* (which corresponds, in part, to the *Clusia minor* group sensu Hammel, 1986) and to a clade that includes the *Clusia flava* group and the *Omphalanthera–Chlamydoclusia* complex (Luján et al., 2022). In contrast, C_3 *Clusia* species are predominantly found at higher elevations and typically belong to the species-rich section *Anandrogynae* (similar to the *Clusia multiflora* group in the study by Hammel, 1986).

Examples of species in which the CAM pathway is the principal contributor to carbon gain are the drought-tolerant *Clusia hilariana* Schldl. and *Clusia flava* Jacq. *Clusia hilariana* grows in the edaphically and climatically harsh restingas of eastern Brazil (Franco et al., 1996), and *Clusia flava* is well known

from the seasonally dry Yucatan Peninsula in Mexico. Thirty-eight of 39 specimens of *Clusia flava* collected at various sites in Mexico had $\delta^{13}\text{C}$ values less negative than -20‰ , indicative of strong CAM ($-15.6 \pm 2.9\text{‰}$, mean \pm s.d., $n = 39$; Vargas-Soto et al., 2009). At the other end of the CAM spectrum are species with low-level CAM, such as those with only small nocturnal increases in leaf tissue acidity. In these species, C_3 -photosynthetic CO_2 uptake during the daytime remains the main route of carbon acquisition during the annual cycle, as indicated by their C_3 -type $\delta^{13}\text{C}$ values (Holtum et al., 2004). In addition, some species display pronounced periodic, facultative CAM, such as the closely related *Clusia minor* L. and *Clusia pratensis*. Potted plants of these species, when well watered, fix CO_2 mainly through the C_3 pathway, although some weak CAM can also occur. When irrigation is withheld, C_3 photosynthetic CO_2 uptake during the daytime declines, and nocturnal net CO_2 via CAM is induced or strongly upregulated. Plants revert to a largely C_3 -type pattern of diel net CO_2 exchange upon rewatering (e.g. Borland et al., 1998; Winter et al., 2008; Winter and Holtum, 2014). Individual attached leaves of *Clusia pratensis* have the capacity to switch between C_3 and CAM reversibly, at least four times during successive wet–dry–wet cycles (K. Winter, unpublished data).

Consistent with field studies by Borland et al. (1992) on *Clusia minor* in Trinidad during the transition from the wet season to the dry season and consistent with facultative CAM, nocturnal increases in leaf-tissue acidity were restricted to the 4-month-long dry season, from mid-December to mid-April, in 3-m-tall plants of *Clusia minor* and *Clusia pratensis* growing at the forest edge at the Smithsonian Tropical Research Institute research facilities in Gamboa, Panama (K. Winter, unpublished data). Nocturnal acidification was not detectable during the remaining wet months of the year. In contrast, *Clusia rosea* at the same site exhibited nocturnal acidification throughout the seasons. The iconic *Clusia rosea* is one of the best-known and best-studied species of *Clusia* and generally considered a species with strong obligate CAM. Indeed, $\delta^{13}\text{C}$ values as high as -14.5‰ have been recorded for plants from Mexico, northern Venezuela and southern Florida (Popp et al., 1987; Sternberg et al., 1987; Vargas-Soto et al., 2009), although $\delta^{13}\text{C}$ values in the C_3 range were also noted. For example, in a Panamanian field study of *Clusia rosea*, $\delta^{13}\text{C}$ values ranged from -16.7 to -27.5‰ (Holtum et al., 2004), and in a recent Colombian survey, $\delta^{13}\text{C}$ values were entirely within the C_3 range (-22.2 to -26.5‰ ; Pachon et al., 2022), suggesting that *Clusia rosea* has greater photosynthetic pathway plasticity than previously thought.

One of the most remarkable studies on C_3 –CAM physiology in *Clusia* is probably the detailed work by Gerhard Zotz on epiphytic and hemiepiphytic individuals of *Clusia uvitana* Pittier in the crown of a 47-m-tall kapok tree [*Ceiba pentandra* (L.) Gaertn.] on Barro Colorado Island, Panama. In agreement with laboratory studies of potted *Clusia uvitana* exhibiting clear features of facultative CAM on top of a low- to medium-level obligate CAM background (Winter et al., 1992), Zotz's *in situ* gas-exchange measurements revealed increased dark CO_2 fixation and greater nocturnal acidification of *Clusia uvitana* during the dry season compared with the wet season (Zotz and Winter, 1993, 1994a, 1994b, 1996; Zotz et al., 1995). Besides

these long-term seasonal trends, extremely rapid alterations in CAM activity were also evident *in situ*. On days without precipitation, *Clusia uvitana* showed uptake of atmospheric CO_2 at night, a feature of CAM, and the early morning and late afternoon, whereas during 36 h of almost continuous rainfall the nocturnal net CO_2 uptake stopped, and the diel pattern of net CO_2 exchange became like that of a C_3 plant (Zotz and Winter, 1993). The rapid switching between CAM- and C_3 -type carbon fixation occurred because decarboxylation was repressed in low-light conditions, thereby saving malic acid reserves until environmental conditions warranted the use of CAM.

Despite the extraordinary photosynthetic flexibility of some species of *Clusia*, gene-expression studies are lacking that track the up- and downregulation of enzymes during transitions from C_3 to CAM and vice versa. We suggest this to be a top priority of future *Clusia* research.

CARBON ISOTOPE RATIOS IN *CLUSIA*

Lineages with large numbers of C_3 and CAM species, such as Bromeliaceae, Orchidaceae or the genus *Euphorbia* (Euphorbiaceae), show a distinct bimodal distribution of $\delta^{13}\text{C}$ values, with a pronounced frequency peak around -27‰ , typical of species with C_3 photosynthesis, and a second peak around -15‰ , typical of species with strong CAM (Silvera et al., 2010; Horn et al., 2014; Crayn et al., 2015). In contrast, only a few *Clusia* species, such as *Clusia flava*, are known to exhibit $\delta^{13}\text{C}$ values consistently within the strong CAM range (Vargas-Soto et al., 2009), and in frequency histograms of *Clusia* isotope surveys, the CAM peak, if one exists, is very small (Holtum et al., 2004; Pachon et al., 2022). The $\delta^{13}\text{C}$ values of the majority of *Clusia* species, including many isotope values of *Clusia* species with facultative CAM, contribute to a large cluster of values consistent with predominantly C_3 photosynthetic CO_2 fixation (Winter and Holtum, 2002). In other lineages containing annual facultative CAM species, such as *Mesembryanthemum crystallinum* L., CAM is induced in the middle of the lifespan of the plant, with onset of the dry season. At the end of the lifespan, leaves from this species will exhibit $\delta^{13}\text{C}$ values common to CAM (Winter et al., 1978). In contrast, the situation in *Clusia* species with facultative CAM is completely different, because leaf longevity significantly exceeds 1 year. Although species such as *Clusia minor* and *Clusia pratensis* can show substantial CAM activity during a 3-month dry season in Panama (K. Winter, unpublished data), for most of the year the leaves perform predominantly C_3 photosynthesis, which strongly dilutes the isotopic signal of periodically occurring CAM. In addition, it is possible that growth rates are substantially slower in the dry season, when water availability becomes limiting. Such a scenario would mean that even if a quarter of the year was spent doing CAM, this would be disproportionately underrepresented in the $\delta^{13}\text{C}$ value of leaves. Consequently, in *Clusia*, the value of $\delta^{13}\text{C}$ in characterizing photosynthetic pathway diversity and predicting the ability of a species to perform CAM is clearly limited, unless $\delta^{13}\text{C}$ values are in the -15‰ range consistent with strong sustained CAM.

EVOLUTIONARY TRAJECTORIES OF CRASSULACEAN ACID METABOLISM IN *CLUSIA*

It is unclear whether C_3 -CAM phenotypes are evolutionary transitional stages towards strong CAM or stable states that have been selected during the evolutionary history of various plant lineages (Edwards, 2019). The significant number of C_3 -CAM phenotypes in *Clusia* might indicate that these intermediate forms have been stably selected. There are multiple advantages of maintaining C_3 -CAM phenotypes, because the optional use of CAM during the dry season improves water-use efficiency (Winter and Holtum, 2014). It stands to reason that these plastic phenotypes might be favoured by stabilizing selection, although the role of phenotypic plasticity remains a controversial topic in evolutionary biology, because conflicting predictions exist about whether plasticity constrains or facilitates adaptive evolution (Paenke et al., 2007; Ghalambor et al., 2015). Describing the genetic mechanisms involved in upregulating CAM activity in C_3 -CAM phenotypes might allow testing for potential correlations between allele frequencies and environmental variables. Such studies might identify patterns of genomic variation that are indicative of natural selection. Furthermore, given that CAM has evolved multiple times independently within *Clusia* (Luján et al., 2022), it would be valuable to test whether the genetic mechanisms involved in the upregulation of CAM activity are comparable across different CAM lineages. In this context, the role of phenotypic plasticity, and in particular the patterns of how plasticity might be inherited over successive generations, deserves further attention (Charmantier et al., 2008; Fox et al., 2019). Cross-disciplinary approaches, including phylogenomic reconstructions based on transcriptomic data of CAM-related genes coupled with experimental physiological studies, are warranted to understand the role of photosynthetic plasticity in the evolution of *Clusia*.

EVOLUTIONARY HISTORY OF CRASSULACEAN ACID METABOLISM IN *CLUSIA*

Early phylogenetic reconstructions based on plastid (*rbcL*) and nuclear (ITS) markers found *Clusia* to be a well-supported monophyletic group (Gustafsson et al., 2002; Gustafsson and Bittrich, 2003). At least two independent origins of CAM photosynthesis have been suggested in the evolutionary history of *Clusia* (Vaasen et al., 2002; Gehrig et al., 2003); although multiple origins of CAM and no fewer than nine reversals have also been reconstructed (Gustafsson et al., 2007), indicating that CAM photosynthesis is a highly homoplasious trait. Recent multi-locus phylogenies have confirmed the monophyly of the genus and supported multiple independent origins of CAM in *Clusia* (Fig. 3). Although a dated phylogeny is still needed to describe the timing of the evolution of CAM in *Clusia*, preliminary time-calibrated phylogenetic analyses suggest that CAM is a relatively recent innovation in the group (Luján et al., 2022).

Reproductive morphology of the fossil species *Paleoclusia chevalieri* Crepet and Nixon suggest that Clusiaceae was well differentiated and a highly diversified group as early as the Turonian (i.e. 93.9–89.8 Ma; Crepet and Nixon, 1998; Schönenberger et al., 2020). Furthermore, *Clusia fossilia* Berry,

a fossil species from the later Miocene (23.03–5.3 Ma) from Trinidad, displays close morphological resemblance to *Clusia rosea* (Berry, 1925), a species capable of strong CAM, suggesting that CAM capacity was likely developed in *Clusia* by that time. Major changes in terrestrial ecosystems, including the reduction of closed forests and expansion of grasslands, marked the transition between late Oligocene and early Miocene (30 Ma). These changes can be linked to fluctuations in global atmospheric CO_2 . Atmospheric CO_2 directly affected tree cover by modifying water relationships in herbaceous vs. woody plants (Polley et al., 2002) and by altering plant growth rates (Bond et al., 2003). A significant drawdown of CO_2 might have influenced the proliferation of open grassland habitat and opening of the forest vegetation during the early Miocene (Kürschner et al., 2008). Although explicit ancestral state reconstruction modelling is still necessary, it is likely that CAM capacity in *Clusia* evolved ~30 Ma as a carbon-concentration mechanism in response to the abrupt decline in global atmospheric CO_2 that occurred in the Oligocene to Miocene transition.

The evolution of CAM in the clusioid clade (i.e. Clusiaceae and its allied families within Malpighiales; Ruhfel et al., 2011) seems to be exclusive to *Clusia*, because CAM has not been reported in any other clusioid genus. In the Neotropics, *Clusia* is the most species-rich lineage within the clusioid clade, followed by *Garcinia* (~105 Neotropical species). Given its relatively high species richness, it is tempting to propose that the presence of CAM might be correlated with increased speciation rates in *Clusia*. Nonetheless, CAM activity has not been found in the most species-rich clade within *Clusia* (section *Anandrogynae*; Fig. 3). Plants in this group are mostly trees from montane cloud forests in the Andes and Central America, habitats where terrestrial plants rarely experience severe soil water deficits (Bruijnzeel and Veneklaas, 1998). The increased species richness seen in the *Anandrogynae* clade might not be related to photosynthetic diversity but could instead have resulted from adaptive changes in reproductive biology (discussed in more detail below) linked to the Andean orogeny, a pattern seen in many other Neotropical plant lineages (Schwery et al., 2015; Lagomarsino et al., 2016). Relatively lower extinction rates in *Clusia* section *Anandrogynae* might also explain higher species richness observed in this group, although this remains to be tested.

THE ROLE OF LIFE HISTORY AND CLIMATE IN THE EVOLUTION OF CRASSULACEAN ACID METABOLISM IN *CLUSIA*

In some plant lineages, such as orchids, CAM is strongly associated with epiphytism. Species with a constitutive epiphytic life habit are considerably more likely to have CAM capacity, because the increased water-use efficiency conferred from this metabolic adaptation is advantageous to plants living with no access to soil water (Lüttge, 2004; Heyduk et al., 2019). However, in *Clusia*, the association between CAM and epiphytism is far less clear. No analysis has been able to show directly that epiphytic plants do more CAM. This might be, in part, because the life history of *Clusia* is highly plastic, given that many species can survive as epiphytes, hemiepiphytes or free-standing shrubs or trees (Figs 1 and 2). The plasticity in life history across

Clusia is so pronounced that it is unclear whether any species of *Clusia* live exclusively as epiphytic plants. Likewise, photosynthetic physiology is extremely plastic in *Clusia*, because some species can upregulate carbon flux facultatively through the CAM cycle when subjected to drought. High plasticity, both in the predisposition for epiphytism–hemiepiphytism and in the strength of CAM, makes it challenging to determine the character states of species clearly (i.e. epiphyte, hemiepiphyte or terrestrial; or strong CAM, C₃–CAM or C₃, etc.). Therefore, it is extremely difficult to determine whether these characters are correlated across the genus. For example, extensive sampling of 909 herbaria samples found no relationship between epiphytism and $\delta^{13}\text{C}$ ratios within *Clusia* (Pachon et al., 2022). It is also important to consider that although phenotypic plasticity might obscure any relationship between epiphytism and photosynthetic physiology, other traits, such as the predisposition to form adventitious aerial roots, might be more important than CAM in driving differences in growth habit. More comprehensive data on the extent of epiphytism across different species are required for a full understanding of the role (if any) that CAM plays in the life habit of *Clusia* species.

Although relationships between life habit and photosynthetic mode remain elusive, a clear pattern can be observed between CAM, climate and elevation within *Clusia*. In a Panamanian study, C₃–CAM species have not been reported in species living >1689 m above sea level, and species with strong CAM were not collected at >680 m above sea level (Holtum et al., 2004). It appears that C₃ species dominate montane cloud forests, possibly because these environments are less water limited and have less severe dry seasons than low-elevation ecosystems (Brujinzeel and Veneklaas, 1998). Phylogenetic comparative analyses have established a link between CAM and water availability across *Clusia* species. For example, a study of 64 species found that species with lower $\delta^{13}\text{C}$ values live in habitats characterized by a more severe dry season and greater precipitation seasonality, suggesting that constitutive CAM has evolved in response to precipitation deficits (Luján et al., 2022). In addition, species that can switch facultatively from C₃ to CAM were found to live in climatic niches characterized by relatively high annual precipitation, but strong acute dry seasons (Leverett et al., 2021). Therefore, facultative CAM appears to be an adaptation to fluctuating precipitation availability; this adaptation allows plants to make use of higher growth rates associated with C₃ when water is available, whilst also benefitting from the water-conserving nature of CAM during the dry season.

In addition to CAM and plant habit, reproductive biology might have also played an important role in the ecological distributions of some *Clusia* species. Although links between CAM and climate can be established over regional distributions (see Luján et al., 2022), these links are less clear at local scales. For example, no relationships were found between photosynthetic physiology and water availability at different local microsites in two locations in Southeast Brazil (Lüttge et al., 2015). The authors suggest that aspects of the reproductive biology of the species might be more relevant in explaining differences in plant distribution at local scales. Furthermore, two of the more widespread *Clusia* species (i.e. *Clusia flavida* and *Clusia minor*; Fig. 6), are terrestrial or hemiepiphytic shrubs with facultative CAM activity (Ting et al., 1987; Zotz et al., 1999). It would be intuitive to assume that CAM capacity might increase the

ability of species to occupy diverse habitats. However, *Clusia flavida* and *Clusia minor* have smaller fruits than most facultative CAM *Clusia* species, which have narrower geographical ranges and larger fruits (e.g. *Clusia lundellii* and *Clusia uvitana*; Fig. 6). A correlation between fruit size and species distribution has been observed in other tropical woody plant groups, in which smaller-fruited species have wider distribution ranges than species with larger fruits (e.g. Lauraceae; [Rossetto et al., 2015]). Therefore, although CAM has probably played a role determining the distribution of *Clusia* species, aspects of reproductive biology, particularly related to fruit morphology and seed dispersion, might also be important. Potential correlations between fruit size and geographical range are not straightforward in *Clusia*, because there are widespread species (Fig. 7) with relatively large fruits, such as *Clusia rosea* (a species capable of strong CAM). Phylogenetic analyses that explicitly test potential correlations between CAM activity and speciation rates, reproductive morphology and geographical distribution are now needed to further our understanding of the evolutionary history of *Clusia*.

CLUSIA: A GENUS WITH TREES CAPABLE OF CRASSULACEAN ACID METABOLISM

Carbon-concentrating mechanisms appear to be very rare in tree species. Although some attention has been given to explaining the scarcity of C₄ trees (Sage, 2014; Young et al., 2020, 2022), far less consideration has been given to understanding why there are so few trees capable of CAM (Borland et al., 2015). *Clusia* is the only known genus of trees (*sensu stricto*) with CAM, although woody arborescent CAM plants do exist; for example, the North American cactus *Carnegiea gigantea* (Engelm.) Britton and Rose and the African *Euphorbia tirucalli* L. (Hastilestari et al., 2013; Drezner, 2014). In addition, woody monocot CAM plants, such as *Yucca filifera* Chabaud and *Aloidendron dichotomum* (Masson) Klopper and Gideon F.Sm., can grow to many metres in height (González-Salvatierra, 2019; Grey et al., 2022). The existence of these species demonstrates that CAM is not incompatible with an arborescent life form, although CAM trees are the exception rather than the norm. The scarcity of the arborescent life form among CAM plants might have two explanations. Maximum rates of CO₂ uptake per unit surface area are generally lower in CAM plants than in C₃ and C₄ plants, but in typical CAM plants this is offset, at least in part, by the fact that nearly the entire shoot surface is photosynthetic, as in *Agaves* and cacti. This is not the case in a tree, where substantial quantities of carbon are needed to support a largely non-photosynthetic woody trunk. Moreover, the high construction cost of succulent CAM leaves with much higher leaf masses per area than those of C₃ tree leaves further limits overall plant growth rates compared with non-CAM trees, especially under a closed or partly closed canopy. It is therefore not surprising that although *Clusia* species with CAM can be important components of closed tropical forests, they are typically not found as free-standing trees on the ground of forests with a high leaf area index, but rather as epiphytes and hemiepiphytes high up in the canopy, where their foliage is closer to the sun. For example, in Panama, *Clusia* plants will often appear along forest edges and clearings, only to disappear

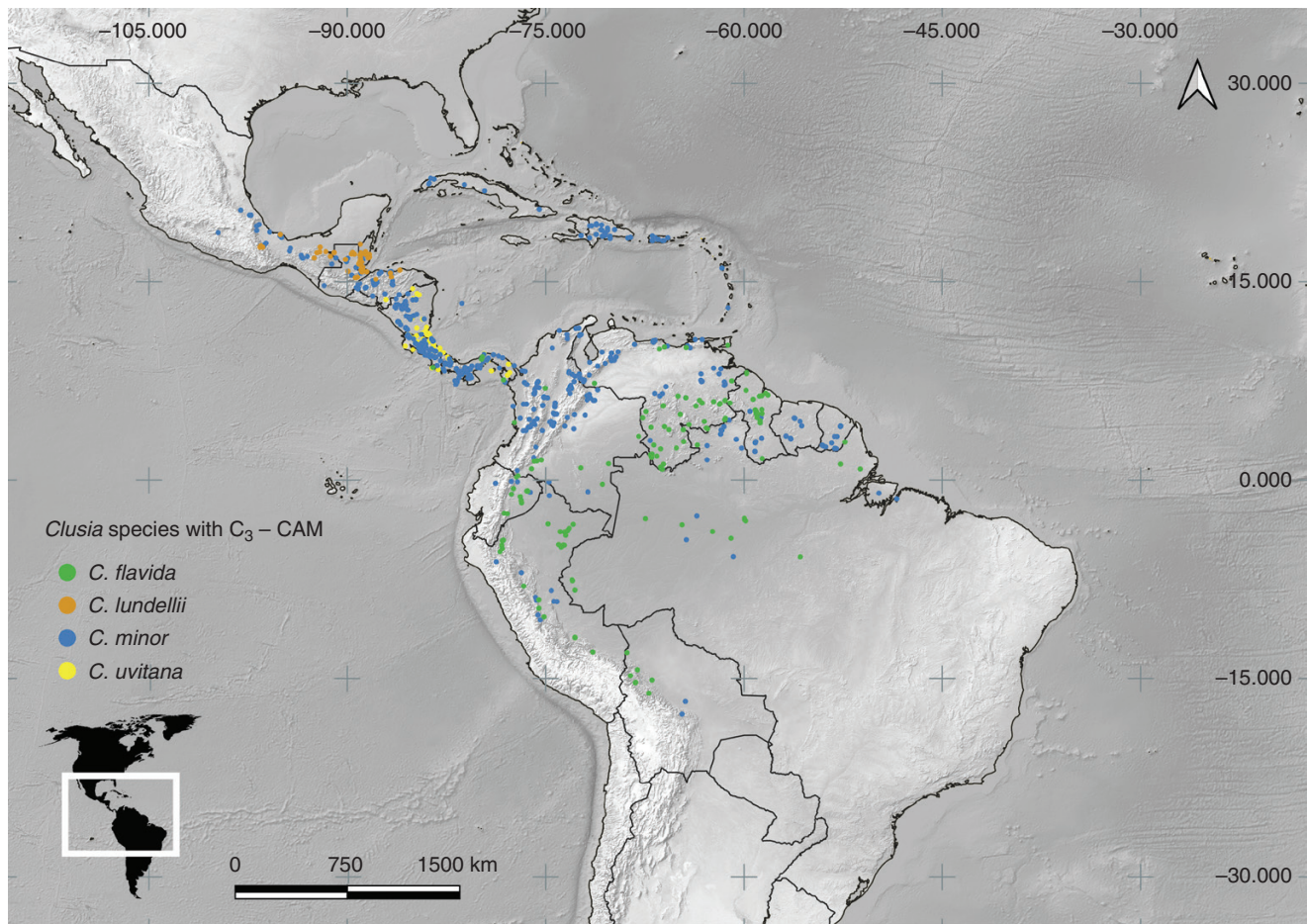


FIG. 6. Geographical distribution map of selected species with intermediate C_3 -CAM, including *Clusia flavida*, *C. minor*, *C. lundellii* and *C. uvitana*.

when the forest canopy closes. Terrestrial CAM trees in *Clusia* are common in fully exposed, high-light environments, such as *Clusia flava* in open dry forest in the Yucatan and *Clusia hilariana* in dry Southeast Brazil. It is likely that environments with high light exposure might be a requirement for CAM trees, because they cannot sustain sufficient growth rates to become trees in low-light conditions.

CONCLUSIONS AND FUTURE DIRECTIONS

The study of CAM photosynthesis in *Clusia* continues to be a fascinating topic even after four decades of research on this system. The fact that some members of this group of tropical trees display such an extraordinary photosynthetic plasticity in response to rapid environmental change is relevant more than ever in the face of current global anthropogenic climatic change and in the context of bioengineering efforts to increase drought tolerance of useful plants. For instance, there is evidence that presence of *Clusia* species with CAM activity positively affects soil conditions in restinga vegetation in Southeast Brazil (Dias and Scarano, 2007), in addition to seed dispersion in forest edges and fragments of moist lowland forests in Colombia (Aide and Cavelier, 1994). If CAM

capacity could be enhanced in C_3 species of *Clusia* and other groups of trees growing in different types of vegetation, it would potentially enable short-term survival for more severe or extended drought periods, a desirable adaptation in plants used in forestry and ecological restoration across tropical regions.

Given that *Clusia* includes species with leaf architecture typical of C_3 metabolism, in addition to species with relatively enlarged cells and tissues that allow strong CAM capacity, comparative analyses between C_3 and CAM species have the potential to identify the necessary changes in critical enzymes and transporters needed for establishment of CAM. A complex network of interactions between genotypes, phenotypes and environmental conditions influences the expression of CAM. Integrative approaches that consider anatomical constraints in addition to gene expression patterns have been useful in disentangling the biochemical photosynthetic pathways of CAM (e.g. Heyduk et al., 2022; Moreno-Villena et al., 2022). Having a clearer understanding of the biochemical and genetic factors governing photosynthetic plasticity in *Clusia* could be instrumental for engineering CAM into C_3 plants, allowing safer agriculture and forestry in a warming world with more unpredictable weather.

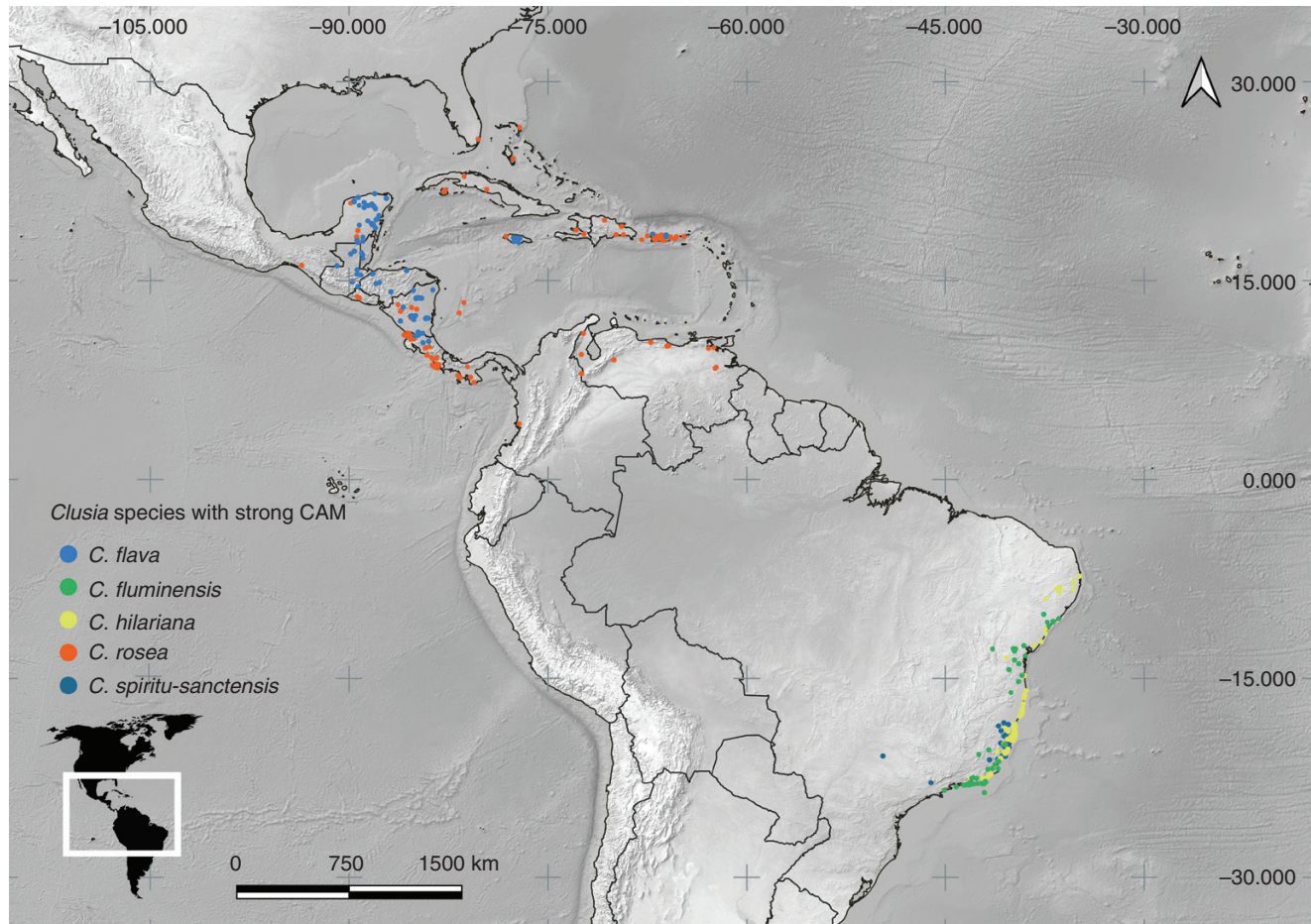


FIG. 7. Geographical distribution map of selected species with strong CAM, including *Clusia flava*, *C. fluminensis* Planch. & Triana, *C. hilariana*, *C. rosea* and *C. spiritu-sanctensis* G. Mariz & B. Weinberg.

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