Integrated Multi-Trait Analysis of Photosynthetic Carbon Assimilation Pathways and Adaptive Patterns in Dendrobium Species

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Research Article

Keywords: Dendrobium species, carbon assimilation pathways, crassulacean acid metabolism, ecological adaptability, leaf anatomy

Posted Date: December 22nd, 2025

DOI: https://doi.org/10.21203/rs.3.rs-8304845/v1

Additional Declarations: No competing interests reported.

Abstract

To analyze the diversity of photosynthetic carbon assimilation pathways and ecological adaptation characteristics in *Dendrobium* species, this study selected nine native Chinese *Dendrobium* species. A comprehensive approach was employed, integrating diurnal dynamics of net CO_2 exchange, carbon isotope ratios ($\delta^{13}C$), diurnal titratable acid accumulation (ΔH^+), leaf anatomical structure, and mesophyll succulent index (Sm). The results indicate: that *Dendrobium parishii*, *Dendrobium aphyllum*, and *Dendrobium anosmum* maintain positive net CO_2 exchange at night, with $\delta^{13}C$ values exceeding −16‰ and high nocturnal acid accumulation. Combined with their substantial leaf thickness (LT) and Sm, these species are classified as typical CAM plants; *Dendrobium cariniferum*, *Dendrobium gibsonii*, and *Dendrobium hancockii* exhibited no positive CO_2 exchange throughout the night, with $\delta^{13}C$ values below −26‰ and low ΔH^+ levels, demonstrating physiological characteristics consistent with the C_3 pathway; Additionally, *Dendrobium linawianum*, *Dendrobium moschatum*, and *Dendrobium crystallinum* exhibited weak nocturnal CO_2 exchange and moderate to low acid accumulation. However, their $\delta^{13}C$ values remained within the C_3 range, indicating they are not stable CAM types but may exhibit plastic expression under specific environmental conditions, displaying characteristics of intermediate C_3/CAM photosynthetic plants. The LT and SM exhibited partial overlap between C_3/CAM and strictly C_3 species, highlighting the limitations of using morphological traits as sole diagnostic criteria. Correlation analyses further revealed that although LT was significantly associated with Nighttime Net CO_2 Exchange (NNEE) and $\delta^{13}C$, the Sm displayed only weak correlations with key physiological indicators. In contrast, NNEE, $\delta^{13}C$, and ΔH^+ were strongly aligned with one another, underscoring that structural characteristics can serve only as supplementary references, while reliable classification must depend on the coordinated variation of physiological metrics. Furthermore, the photosynthetic types of the examined *Dendrobium* species closely corresponded to moisture availability and light conditions in their native habitats, reflecting the ecological plasticity of their carbon-assimilation strategies. In summary, *Dendrobium* species exhibit remarkable diversity and environmental plasticity in their photosynthetic carbon assimilation strategies. This study, through multi-indicator integrated assessment, not only clarifies the photosynthetic types of different species but also provides important references for identifying photosynthetic carbon assimilation pathways and cultivating *Dendrobium* species.

Introduction

Photosynthesis is the fundamental metabolic process through which plants convert light energy into chemical energy (Blankenship 2021; Nelson and Yocum 2006). Throughout evolution, plants have developed several distinct pathways for photosynthetic carbon assimilation, including the C_3, C_4 and crassulacean acid metabolism (CAM) pathways, to adapt to diverse ecological environments (Ehleringer and Monson 1993; Sage 2004). These pathways differ significantly in their in carbon-fixation efficiency, water-use characteristics and ecological adaptability. The C_3 pathway, being the most ancient and widespread form and predominates in temperate and humid regions; however, C_3 plants experience

pronounced photorespiration under high temperatures, drought, and intense light. In contrast, C_4 pathway evolved as an adaptation to such stress conditions. This pathway relies on Kranz anatomy, a specialized leaf structure and a spatial CO_2-concentrating mechanism that suppresses photorespiration, allowing plants to maintain efficient carbon fixation in hot and high-light habitats (Sage and Zhu 2011). CAM represents another specialized adaptation that shifts CO_2 exchange to the night, storing it as organic acids. By closing their stomata during the day and relying on nocturnally accumulated malate for CO_2 release, CAM plants minimize water loss and can survive in extremely arid environments (Winter and Smith 1996; Borland et al. 2009). Importantly, the C_3, C_4 and CAM pathways are not entirely isolated from one another. C_3/C_4 intermediate species are present in several plant groups. These intermediate forms reflect the intrinsic plasticity of photosynthetic carbon assimilation and provide valuable model systems for investigating how plants adjust their carbon-fixation strategies in response to environmental variations. The long-term evolution and distribution of these pathways have been influenced by changes in atmospheric CO_2 concentration, temperature and water availability. Since the Cenozoic era, global aridification, coupled with declining atmospheric CO_2 levels is believed to have promoted multiple independent origins of both C_4 and CAM photosynthesis (Sage et al. 2011; Winter and Holtum 2014). The interplay of these biochemical mechanisms, along with their varying modes of spatio-temporal CO_2 concentration, has resulted in a diverse array of adaptive carbon assimilation strategies. This diversity forms the theoretical foundation for understanding the ecological drivers of photosynthetic evolution.

In the crassulacean acid metabolism (CAM) pathway, plants exhibit distinctive adaptive advantages. Studies exhibit that the water-use efficiency of typical CAM species can be up to six times greater than that of C_3 plants and three times greater than that of C_4 plants (Nobel 1996). This highly efficient water-saving strategy enables CAM plants to maintain a competitive edge in arid and semi-arid environments. Additionally, CAM species demonstrate significant tolerance to intense light and high temperatures, which collectively define their ecological roles in dry and semi-dry ecosystems (Silvera et al. 2010). CAM is characterized by a unique CO_2 fixation mechanism and operates through four distinct phases. During Phase I, which occurs at night, stomata open and CO_2 is fixed by phosphoenolpyruvate carboxylase to form malate, which is stored in vacuoles. Phase II occurs around dawn, when stomata are partially open, allowing both C_3 and C_4 carbon fixation to occur simultaneously. Phase III takes place during the day, when stomata remain closed and malate is decarboxylated, releasing CO_2 for the Calvin cycle through the action of Rubisco. Finally, Phase IV occurs in the late afternoon, when stomata reopen briefly and CO_2 is fixed directly via the C_3 pathway (Osmond 1978; Ting 1985). CAM expression can be further categorized into obligate CAM, which is constitutively expressed throughout the plant's life cycle, facultative CAM, which is induced under drought or other stress conditions, CAM cycling, which involves nocturnal acid accumulation without net CO_2 exchange, and CAM idling, which occurs under extreme drought when stomata remain nearly completely closed and carbon recycling is maintained internally (Winter and Holtum 2014). This diversity reflects the high degree of plasticity with which plants adapt to their environment. Research on CAM and the broader spectrum of carbon assimilation pathways is therefore essential for understanding plant stress physiology and offers new strategies for crop genetic

improvement. Insights into the water-saving and heat-tolerant traits of CAM provide a foundation for breeding drought-resistant and water-efficient crops. Moreover, the carbon-fixation characteristics of CAM plants are enhancing for refining global carbon-cycle models and improving predictions of ecosystem function in the context of climate change.

Dendrobium is one of the largest genera within the Orchidaceae family, encompassing over 1,500 species that are widely distributed across tropical and subtropical regions of Asia (Hou et al. 2017). Species within this genus hold significant ecological and economic value. Many *Dendrobium* species are integral components of forest ecosystems and serve as important medicinal and horticultural resources. Their survival status and physiological adaptability are directly related to biodiversity conservation and sustainable utilization (Cheng et al. 2019). Recently, the diversity of photosynthetic carbon assimilation pathways and their environmental regulation have emerged as a major focus in plant physiological ecology (Sage et al. 2012). Prior studies have demonstrated that the Orchidaceae family exhibits a wide range of photosynthetic types, spanning from typical C_3 species to strong CAM species (Silvera et al. 2010). Evidence of plastic carbon assimilation in *Dendrobium* suggests that some species may modify their carbon-acquisition strategies in response to abiotic stress (Silvera et al. 2009; Zhang et al. 2020). This physiological plasticity may represent one of the key mechanisms that enable *Dendrobium* species to thrive in diverse habitats. Given the extensive distribution and varied ecological contexts of the genus, it is probable that a rich variety of photosynthetic types exists, particularly within the transitional range between CAM and C_3 pathways. Nonetheless, systematic research on photosynthetic carbon assimilation pathways in *Dendrobium* remains limited. Most existing studies concentrate on a few economically significant species, and comprehensive comparisons that integrate multiple indicators are still lacking.

To address these issues, this study investigates nine native Chinese *Dendrobium* species, including *D. parishii*, *D. aphyllum*, *D. ellipsophyllum*, *D. cariniferum*, *D. gibsonii*, *D. hancockii*, *D. linawianum*, *D. moschatum* and *D. crystallinum*. The photosynthetic carbon assimilation pathways of these species have not been previously examined. The innovation of this study lies in the application of a comprehensive analytical framework that integrates five categories of indicators: net CO_2 exchange, $\delta^{13}C$ values, titratable acid accumulation (ΔH^+), leaf anatomical traits and the mesophyll succulent index. This framework facilitates a systematic assessment of C_3, CAM and intermediate C_3/CAM photosynthetic types, revealing potential photosynthetic plasticity. Measurements of net CO_2 exchange enable the characterization of diel gas-exchange patterns (van Tongerlo et al. 2021). Changes in titratable acidity serve as an important indicator of carbon metabolism and reflect the magnitude of nocturnal CO_2 fixation and daytime decarboxylation (Cushman and Borland 2002). Carbon isotope ratios distinguish among C_3, C_4, and CAM pathways, providing long-term information about water-use efficiency (Farquhar et al. 1982). Leaf anatomical traits are closely linked to photosynthetic pathways; for instance, C_4 plants typically possess Kranz anatomy, while CAM plants often exhibit thick leaves with well-developed water-storage tissue (Orsenigo et al. 1997). The mesophyll succulent index offers a quantitative measure of xeromorphic structure, reflecting the degree of tissue water storage. Higher succulence is commonly

associated with typical CAM species and other plants adapted to arid habitats (Herrera 2009). By integrating physiological, biochemical and anatomical indicators, this study aims to accurately classify the photosynthetic pathways of the nine species and identify potential intermediate types between C_3 and CAM. The findings provide insights into the environmental adaptation mechanisms of *Dendrobium*, offering a scientific basis for taxonomy, conservation and cultivation management. This is particularly pertinent in the context of climate change and the sustainable utilization of germplasm resources.

Methods

Study site and plant materials

The experiment was conducted in 2023 at the Guangxi Institute of Botany, located in the Yanshan District of Guilin City, Guangxi, China (25°01' N, 110°17' E). The study site is situated at an elevation of approximately 180 m and is characterized by a subtropical monsoon climate. The region experiences a mean annual temperature of 18.8°C, with January being the coldest month, averaging 8.3°C, and July being the hottest month, averaging 28.3°C. Annual precipitation ranges from 1,900 to 2,000 mm, with over 70% occurring between April and August. The mean annual relative humidity is 78%, and the annual sunshine duration is approximately 1,500 h.

Nine *Dendrobium* species were introduced from Guangxi and Yunnan and cultivated in the germplasm nursery of the Guangxi Institute of Botany. The species included *D. parishii*, *D. aphyllum*, *D. anosmum*, *D. cariniferum*, *D. gibsonii*, *D. hancockii*, *D. linawianum*, *D. moschatum*, and *D. crystallinum*, as identified by Zhongchen Xiong of the Guangxi Institute of Botany. Detailed information regarding the plant materials is provided in Table 1. The plants were grown in plastic pots (height: 18 cm; inner diameter: 16 cm) filled with a substrate composed of tree bark, coconut fiber, and perlite in a ratio of 1:1:1. All test plants were three-year-old mature individuals maintained under mild drought stress. Shade nets with a transmittance of 30% were installed on the greenhouse roof, allowing the plants to grow under natural light conditions.Net CO_2 exchange measurements were conducted in early October 2023, and leaf samples were subsequently collected for analyses of carbon isotope ratios ($\delta^{13}C$), titratable acidity (ΔH^+), leaf anatomical structure, and mesophyll succulent index.

Table 1
Experimental materials

Species	Determiner	Determining Institution	Site of Introduction
D. parishii	Zhongchen Xiong	Guangxi Institute of Botany	Baoshan, Yunnan
D. aphyllum	Zhongchen Xiong	Guangxi Institute of Botany	Pu'er, Yunnan
D. anosmum	Zhongchen Xiong	Guangxi Institute of Botany	Longzhou, Guangxi
D. cariniferum	Zhongchen Xiong	Guangxi Institute of Botany	Tian'e, Guangxi
D. gibsonii	Zhongchen Xiong	Guangxi Institute of Botany	Baoshan, Yunnan
D. hancockii	Zhongchen Xiong	Guangxi Institute of Botany	Baise, Guangxi
D. linawianum	Zhongchen Xiong	Guangxi Institute of Botany	Jinxiu, Guangxi
D. moschatum	Zhongchen Xiong	Guangxi Institute of Botany	Jinghong, Yunnan
D. crystallinum	Zhongchen Xiong	Guangxi Institute of Botany	Tian'e, Guangxi

Measurement of diurnal patterns of net CO_2 exchange

Diurnal net CO_2 exchange was measured from 08:00 on October 7 to 06:00 on October 8, 2023. Healthy, fully expanded leaves located at the 3rd to 5th position from the shoot apex were selected for measurement. A portable photosynthesis system (LI-6400XT; LI-COR Biosciences, USA) was utilized under ambient CO_2 concentrations and natural light conditions. Measurements were conducted at 2-h intervals over a 24-h cycle, with each leaf measured three times to obtain a mean value. For each species, three individual plants were assessed.

According to Beijing local time, sunrise and sunset on October 7 occurred at 06:33:15 and 18:20:17, respectively; on October 8, they were recorded at 06:33:41 and 18:19:17. Photosynthetically active radiation (PAR), air temperature (T_a), and relative humidity (RH) were recorded simultaneously (Fig. 1).

Measurement of carbon isotope ratio

Carbon isotope ratios ($\delta^{13}C$) were determined according to the methodology established by Winter and Holtum (2002). Leaf samples were collected under mild drought stress, then ground in liquid nitrogen, freeze-dried, and weighed to a precision of 3 mg. The ratios of ^{13}C to ^{12}C were measured using a stable isotope ratio mass spectrometer (Delta V Advantage IRMS; Thermo Fisher Scientific, USA), with a precision of ± 0.05‰. The $\delta^{13}C$ values were calculated based on the PDB standard:

$$\delta^{13}\mathrm{C} = \left(\frac{\left({}^{13}\mathrm{c} / {}^{12}\mathrm{c} \right)_{\mathrm{sample}}}{\left({}^{13}\mathrm{c} / {}^{12}\mathrm{c} \right)_{\mathrm{standard}}} - 1 \right) \times 1{,}000$$

Each sample was measured in triplicate, and the mean value was utilized for subsequent analysis.

Measurement of titratable acidity

Titratable acidity was measured following the methodology outlined by Eastmond and Ross (1997). Leaf samples were collected at dawn (06:00) and dusk (18:00) under mild drought stress conditions. Approximately 1 g of leaf tissue was frozen in liquid nitrogen and subsequently stored at − 80°C. The leaf tissues were ground in pre-chilled mortars, extracted with 5 mL of deionized water, boiled for 20 min, cooled to room temperature, and then centrifuged at 5,000 × g for 15 min. Two milliliters of the supernatant were diluted to a final volume of 25 mL. Using 0.2% bromothymol blue as an indicator, the samples were titrated with 0.01 M KOH until a color change from yellow to blue was observed (pH 7.2). Five replicates were measured for each sample, and the mean value was calculated.

Measurement leaf anatomical structure

For each species, three plants were selected, and three mature leaves per plant were sampled from the same orientation as those used for CO_2 exchange measurements. Paraffin sections were prepared according to the method described by Atkinson and Wells (2017). Anatomical observations and imaging were conducted using a light microscope. Leaf structural traits, including upper epidermal thickness (UET), lower epidermal thickness (LET), mesophyll thickness (MT), and total leaf thickness (LT), were measured using CaseViewer software. For each leaf, three sections were examined, with five randomly selected fields analyzed per section.

Measurement of mesophyll succulent index

The succulence index (Sm) was determined following Males (2017), based on the formula established by Kluge and Ting (1978):

$$\mathrm{sm} = \frac{\text{Leaf water content } (\mathrm{g})}{\text{Total chlorophyll content } (\mathrm{mg})}$$

Six to ten mature leaves were weighed for fresh weight (FW) and subsequently dried at 75°C until a constant weight was achieved (DW). Leaf water content (LWC) was calculated as follows:

$$\mathrm{LWC} = \frac{\mathrm{FW} - \mathrm{DW}}{\mathrm{FW}}$$

A 0.2 g leaf sample was cut into pieces and extracted in 95% ethanol for 24 h in darkness. Absorbance at 665 nm and 649 nm was measured using a UV-Vis spectrophotometer (PerkinElmer, USA). Chlorophyll a and b concentrations were calculated using the following equations: Chl a = 13.95 × A_{665} − 6.88 × A_{649}; Chl b = 24.96 × A_{649} − 7.32 × A_{665}, where A_{665} and A_{649} denote absorbance readings at their respective wavelengths. Total chlorophyll content ($mg \cdot dm^{-2}$) was calculated using the formula: Total Chl = (C × V × N) / LA, where C represents the pigment concentration, V is the extraction volume, N is the dilution factor, and LA denotes total leaf area.Three replicates were performed for each species.

Data analyses

Data on net CO_2 exchange, $\delta^{13}C$, ΔH^+, leaf anatomical traits, and mesophyll succulent index were organized using Microsoft Excel 2019. Pearson correlation analyses (two-tailed) were conducted using SPSS Statistics 27.0 to evaluate linear relationships among variables. Graphing and curve fitting were performed using Origin 2021.

Results

Diurnal patterns of net CO_2 exchange

The nine *Dendrobium* species exhibited distinct diurnal patterns of net CO_2 exchange (Fig. 2). *D. cariniferum*, *D. gibsonii* and *D. hancockii* maintained a negative net CO_2 exchange throughout the entire night, which aligns with a typical C_3 photosynthetic pattern. In contrast, the remaining six species, including *D. parishii*, *D. aphyllum*, *D. anosmum*, *D. linawianum*, *D. moschatum* and *D. crystallinum*, displayed positive net CO_2 exchange during part or all of the nighttime, indicating varying degrees of CAM-like characteristics. Among these species, *D. parishii*, *D. aphyllum* and *D. anosmum* exhibited high nighttime CO_2 exchange, each reaching its peak during the late-night or early-morning hours. *D. linawianum* and *D. crystallinum* demonstrated weak but detectable nighttime exchange primarily between 18:00 and 22:00. *D. moschatum* maintained a positive net CO_2 exchange throughout the entire 24-hour cycle, although the magnitude of exchange was low during the night. These results highlight substantial variation in diel carbon acquisition strategies among the studied species.

Carbon isotope ratio and titratable acidity contents

Carbon isotope ratios ($\delta^{13}C$) exhibited variability among the species studied (Fig. 3). *D. parishii*, *D. aphyllum* and *D. anosmum* presented $\delta^{13}C$ values exceeding − 20‰, aligning with the characteristic range of CAM species. Conversely, the remaining species displayed $\delta^{13}C$ values below − 20‰, a range typically associated with C_3 plants. While $\delta^{13}C$ alone cannot conclusively differentiate C_3 species from potential C_3/CAM intermediates, the observed patterns provides significant long-term evidence of variations in carbon assimilation strategies.

The titratable acidity difference (ΔH^+) was positive across all species, indicating an increase in titratable acidity during the night (Fig. 3). *D. parishii*, *D. aphyllum*, *D. anosmum* and *D. crystallinum* recorded the highest ΔH^+ values, each above 45 μmol(H^+)·g^{-1} FW, with *D. aphyllum* reaching a maximum value of 143.544 μmol(H^+)·g^{-1} FW. In contrast, the other species exhibited lower ΔH^+ values, all below 19 μmol(H^+)·g^{-1} FW, with *D. hancockii* showing the lowest ΔH^+ at 1.088 μmol(H^+)·g^{-1} FW.

Leaf anatomical structure

All *Dendrobium* species exhibited fundamental anatomical characteristics, including a single-layered upper and lower epidermis, a compact mesophyll region and well-developed vascular tissues (Fig. 4). Notably, mesophyll cells lacked distinct palisade and spongy differentiation (Fig. 5). Variations in leaf thickness were observed among species, with *D. parishii* exhibiting the greatest total leaf thickness at 766.90 μm, including a mesophyll thickness of 714.07 μm. *D. anosmum* and *D. aphyllum* also displayed relatively thick leaves, both exceeding 500 μm. In contrast, *D. cariniferum* demonstrated moderate leaf thickness, while *D. linawianum* had the thinnest leaves, measuring 221.33 μm. These findings indicate that the species exhibited significant differences in their leaf anatomical parameters.

Mesophyll succulent index

The mesophyll succulent index (Sm) varied among the nine species (Fig. 6). *D. parishii*, *D. anosmum*, *D. gibsonii* and *D. moschatum* exhibited Sm values greater than 1.0. *D. aphyllum*, *D. linawianum* and *D. crystallinum* showed intermediate values of 0.98, 0.95 and 0.78 respectively. *D. cariniferum* and *D. hancockii* had lower values of 0.13 and 0.28. These results indicate that the species did not share the same degree of mesophyll succulence, with Sm values distributed across a broad range.

Correlation analysis

Nighttime net CO_2 exchange (NNEE) exhibited a strong positive correlation with $\delta^{13}C$ ($r = 0.894$). Additionally, $\delta^{13}C$ was positively correlated with ΔH^+ ($r = 0.805$), while NNEE showed a moderate positive relationship with ΔH^+ ($r = 0.676$). These three physiological indicators demonstrated coordinated changes across the species. Leaf thickness (LT) was positively related to these physiological parameters, with strong correlations observed NNEE ($r = 0.877$) and $\delta^{13}C$ ($r = 0.864$), and a moderate correlation with ΔH^+ ($r = 0.613$). In contrast, the mesophyll succulent index (Sm) displayed weaker relationships with physiological traits, evidenced by correlation coefficients of 0.649 with $\delta^{13}C$ and 0.521 with NNEE. These findings indicate that physiological traits are more closely interrelated than structural traits (Fig. 7).

Discussion

Judgment of photosynthetic carbon assimilation pathways

Continuous monitoring of diurnal net CO_2 exchange provides a reliable means of determining whether plants exhibit nocturnal CO_2 exchange, a the key criterion for identifying CAM activity (van Tongerlo et al. 2021). In this study, *D. parishii*, *D. aphyllum* and *D. anosmum* maintained positive net CO_2 exchange throughout the night, demonstrating the characteristic sequence of CAM phases II, III and IV during the night-to-morning transition. This pattern reflects coordinated nocturnal CO_2 fixation and daytime decarboxylation, corroborating observations in typical CAM epiphytic orchid reported previously (Ceusters et al. 2008; Hogewoning et al. 2021). These species appear capable of fixing CO_2 at night through phosphoenolpyruvate carboxylase (PEPC), which supplies carbon for daytime metabolism while

minimizing water loss, a strategy particularly advantageous in seasonally dry and warm environments (Zhang et al. 2014). In contrast, *D. cariniferum*, *D. gibsonii* and *D. hancockii* exhibited negative net CO_2 exchange from night to early morning, consistent with the C_3 pathway. Their diel rhythms align with those of typical C_3 plants, where CO_2 is assimilated through Rubisco during the day and released via respiration at night. These species are commonly found in shaded and humid habitats, where sufficient water availability supports continuous daytime CO_2 exchange and growth (Winter et al. 1983). *D. linawianum*, *D. moschatum* and *D. crystallinum* exhibited diel patterns intermediate between C_3 and CAM species. They relied primarily on C_3 photosynthesis during the day, although weak nocturnal CO_2 exchange was observed during certain nighttime periods. This suggests a limited capacity for nocturnal carboxylation that may be activated under specific environmental conditions to enhance carbon gain and water-use efficiency. Similar patterns have been documented in other facultative or intermediate C_3/CAM species, such as *Clusia minor* and *Mesembryanthemum crystallinum*, which modify their carbon assimilation pathways in response to environmental fluctuations (Borland et al. 1993; Winter and Holtum 2007). This flexibility is regarded as a crucial adaptive trait in environments characterized by intermittent drought or periodic variations in water availability.

Carbon isotope ratios provide additional long-term physiological evidence for distinguishing photosynthetic pathways. Typical C_3 plants generally exhibit $\delta^{13}C$ values ranging from − 33 to − 22.1‰, while strong CAM species typically fall between − 22 and − 12‰ (Elheringer and Osmond 1989; Pearcy et al. 2012). Silvera et al. (2009) demonstrated a bimodal distribution of δ13C values in orchid, with C_3 species centered around − 28‰ and CAM species near − 16‰. Intermediate values reflect C_3/CAM species that respond to environmental stress. Studies on *Dendrobium* and related *orchid* have also shown a clear bimodal pattern with an inflection point near − 20‰, and many recent works use $\delta^{13}C$ values greater than − 20‰ as indicators of CAM activity (Messerschmid et al. 2021). In the present study, *D. parishii*, *D. aphyllum* and *D. anosmum* exhibited $\delta^{13}C$ values of − 14.38‰, − 15.48‰ and − 15.95‰, respectively, which are higher than those typical of C_3 plants and suggest the presence of nocturnal CO_2 fixation. In contrast, *D. cariniferum*, *D. gibsonii* and *D. hancockii* displayed $\delta^{13}C$ values below − 26‰, consistent with the C_3 pathway and aligned with their negative nocturnal CO_2 exchange. *D. linawianum*, *D. moschatum* and *D. crystallinum* had $\delta^{13}C$ values ranging from − 27 to − 29‰, which fall within the C_3 range according to isotope-based classification. Although these values indicate a predominantly C_3 carbon source, $\delta^{13}C$ alone may not fully capture the potential for inducible CAM activity, as intermediate C_3/CAM species often exhibit C_3-like $\delta^{13}C$ signatures under non-stress conditions (Borland et al. 2009). Research on facultative CAM species has shown that δ13C values frequently resemble those of C_3 plants when CAM expression is weak and may only shift under conditions of severe drought or light stress (Ricalde et al. 2010). When considered alongside diel CO_2 exchange patterns, our results suggest that *D. linawianum*, *D. moschatum* and *D. crystallinum* primarily rely on C_3 photosynthesis under normal conditions but may activate CAM activity when exposed to environmental stress. Similar responses have been documented in *Agave deserti*, which functions as a

C_3 species in moist environments but exhibits weak CAM under drought or high irradiance (Hartsock and Nobel 1976). Therefore, while $\delta^{13}C$ values provide valuable insights into long-term carbon assimilation patterns, additional physiological indicators are necessary for accurately determining photosynthetic pathways.

As a central feature of the CAM pathway, nocturnal titratable acidity accumulation reflects the carboxylation activity of phosphoenolpyruvate carboxylase (PEPC) (Borland and Taybi 2004). In the present study, the analysis of ΔH^+ among nine *Dendrobium* species revealed differences among species, indicating diversity in their nocturnal organic acid metabolism. *D. parishii*, *D. aphyllum* and *D. anosmum* had ΔH^+ values of 143.54, 112.33 and 46.37 $\mu mol(H^+)\cdot g^{-1}$ FW respectively, indicating day–night variations in organic acid content and suggesting that these species synthesize organic acids during the night. In contrast, *D. cariniferum*, *D. gibsonii* and *D. hancockii* variations low ΔH^+ values, with *D. hancockii* recording a mere 1.088 $\mu mol(H^+)\cdot g^{-1}$ FW. These low values suggest that these species do not accumulate organic acids to a meaningful extent during the night, which aligns with the characteristics typically observed in C_3 plants. Although C_3 species generally do not build up large pools of organic acids overnight, small changes in acidity can occur due to of dark respiration and normal metabolic rhythms, potentially explaining their slightly positive ΔH^+ values rather than indicating CAM-related CO_2 fixation (Bräutigam et al. 2017). *D. linawianum*, *D. moschatum* and *D. crystallinum* had ΔH^+ values situated between those of the CAM-like and C_3 groups, indicating modest nocturnal organic acid accumulation and suggesting that these species may activate PEPC-mediated carboxylation specific particular environmental conditions. Silvera et al. (2005) examined 173 *orchid* species from Panama under drought conditions and reported broad and overlapping ranges of ΔH^+, with obligate CAM species displaying values from 10.7 to 275.7 $\mu mol(H^+)\cdot g^{-1}$ FW and facultative CAM species showing values from 1.7 to 36.1 $\mu mol(H^+)\cdot g^{-1}$ FW. This pattern is broadly consistent with the trends observed in the present study. Although the ΔH^+ values of the intermediate *Dendrobium* species in this study are lower than those of strong CAM plants, their ability to accumulate organic acids during the night indicates that they may activate components of the CAM pathway in response to water or light stress.

The integrated analysis of diurnal net CO_2 exchange, $\delta^{13}C$ values and titratable acid accumulation provides a systematic basis for elucidating the carbon assimilation pathways of the nine *Dendrobium* species. These indicators represent complementary aspects of carbon metabolism, reflecting short-term gas exchange, long-term carbon source patterns and aspects biochemical processes. Their combined application facilitates the identification of C_3, CAM and intermediate C_3/CAM types (Table 2). *D. parishii*, *D. aphyllum* and *D. anosmum* exhibited consistent CAM characteristics across all indicators. Conversely, *D. cariniferum*, *D. gibsonii* and *D. hancockii* demonstrated full alignment with the C_3 pathway. Meanwhile, *D. linawianum*, *D. moschatum* and *D. crystallinum* displayed intermediate characteristics across all indicators and may activate components of the CAM pathway under specific environmental conditions.

Table 2
Determination of photosynthetic carbon assimilation pathway types in nine *Dendrobium* species

Species	Net CO_2 exchange phenotype	$\delta^{13}C$ phenotype	ΔH^+ phenotype	Overall assessment
D. parishii	CAM	CAM	CAM	CAM
D. aphyllum	CAM	CAM	CAM	CAM
D. anosmum	CAM	CAM	CAM	CAM
D. cariniferum	C_3	C_3	C_3	C_3
D. gibsonii	C_3	C_3	C_3	C_3
D. hancockii	C_3	C_3	C_3	C_3
D. linawianum	CAM	C_3	CAM	C_3/CAM
D. moschatum	CAM	C_3	CAM	C_3/CAM
D. crystallinum	CAM	C_3	CAM	C_3/CAM

Discussion on leaf anatomical structure and the mesophyll succulent indexs

Leaf thickness is an important anatomical characteristic associated with CAM activity, as CAM species are often linked to succulent tissues. Succulence has been documented in numerous CAM lineages across various plant families, including *Geraniaceae*, *Orchidaceae* and *Clusiaceae* (Jones et al. 2003; Barrera Zambrano et al. 2014; Zhang et al. 2018). Increased tissue succulence in many drought-adapted CAM plants offers advantages by enhancing water-storage capacity compared to C_3 and C_4 species. A comparative analysis of tropical *orchid* species indicated that obligate CAM plants typically exhibit the greatest leaf thickness (Silvera et al. 2005). In *M. crystallinum*, leaf succulence also increases during the transition from C_3 to CAM under salt treatment (Guan et al. 2020). Recent studies further suggest that the evolution of C_3/CAM intermediate species may not necessitate major anatomical reconfiguration (Yang et al. 2019; Heyduk et al. 2021). Winter proposed that obligate CAM plants require substantial anatomical modification, whereas C_3/CAM intermediate species may operate CAM without such extensive structural changes, indicating that the transition from C_3 to CAM may proceed with minimal alteration to C_3 type anatomy (Winter and Holtum 2014). In this study, the nine *Dendrobium* species exhibited considerable variation in leaf thickness, which may correlate with their differing carbon assimilation pathways. *D. parishii*, *D. aphyllum* and *D. anosmum* had the highest leaf thickness values (764.14, 584.20 and 551.83 μm, respectively), consistent with their classification as CAM species based on $\delta^{13}C$ and net CO_2 exchange. Their well-developed leaf tissues likely provide the vacuolar space necessary for nocturnal malate storage and contribute to water conservation under fluctuating moisture

conditions. However, certain observations deviate from traditional expectations. For example, *D. cariniferum* exhibited a relatively high leaf thickness of 445.29 μm, yet both its $\delta^{13}C$ value (− 29.762‰) and net CO_2 exchange pattern clearly indicate a C_3 photosynthetic mode. This demonstrates that leaf thickness alone is not sufficient to determine the photosynthetic pathway in *Dendrobium*. Some C_3 species may develop thicker leaves as an adaptation to epiphytic microhabitats that experience high irradiance or nutrient limitation, without necessarily exhibiting CAM function. In contrast, species inferred to possess C_3/CAM intermediate characteristics, such as *D. crystallinum* and *D. linawianum*, showed relatively low leaf thickness values (296.94 and 221.33 μm, respectively). These findings align with Winter's hypothesis that intermediate species may operate inducible CAM within an essentially C_3 anatomical framework, relying on physiological and molecular regulation rather than major structural remodeling. Overall, leaf thickness in *Dendrobium* shows a general but not absolute association with CAM photosynthesis. Although obligate CAM species often possess thicker leaves, this trait does not reliably distinguish between C_3, C_3/CAM intermediate and CAM species on its own. The diversity of carbon assimilation pathways in the genus reflects long-term adaptation to heterogeneous microhabitats, shaped through coordinated evolution across anatomical, physiological and molecular levels.

The mesophyll succulent index (Sm), a structural indicator reflecting the balance between water storage and photosynthetic investment (Ripley et al. 2013), provides additional anatomical evidence supporting the inferred photosynthetic types in this study. Sm values exhibited patterned variation across species groups, complementing interpretations based on physiological parameters. Species inferred to be typical CAM types (*D. anosmum*, *D. aphyllum* and *D. parishii*) presented high Sm values (1.27, 0.98 and 1.06, respectively), consistent with their CAM physiology. A high Sm indicates substantial water-storage capacity relative to photosynthetic tissue, aligning with the requirements for nocturnal CO_2 fixation and malate accumulation, thereby supporting survival under periodic drought conditions. In contrast, C_3 species such as *D. gibsonii*, *D. hancockii* and D. *cariniferum* exhibited varied Sm levels. The elevated Sm of *D. gibsonii* (1.02) suggests that enhanced water storage is not exclusive to CAM species and may represent a drought-tolerance strategy in certain C_3 taxa. Meanwhile, the low Sm values of *D. hancockii* (0.28) and D. cariniferum (0.13) correspond with their C_3 CO_2 exchange and minimal nocturnal acid accumulation. C_3/CAM intermediate species (*D. linawianum*, *D. moschatum* and *D. crystallinum*) displayed intermediate Sm values (0.78, 1.10 and 0.95), spanning the ranges observed in both CAM and C_3 species. For *D. crystallinum* and *D. linawianum*, Sm values near 1 suggest sufficient but not extreme succulence to support inducible CAM under specific environmental conditions, aligning with their moderate nocturnal acid accumulation and $\delta^{13}C$ values remaining within the C_3 range. Notably, *D. moschatum* had an Sm value similar to that of CAM species (1.10), yet its $\delta^{13}C$ (− 28.453‰) and low ΔH^+ (6.435 μmol(H^+)·g^{-1} FW) indicate only weak or inducible CAM expression. This suggests that certain *Dendrobium* species may develop anatomical characteristics suited to CAM, while the physiological engagement of CAM remains conditional.

Correlation analysis between physiological and structural parameters

The correlation analysis revealed that the physiological parameters employed to assess photosynthetic carbon assimilation types exhibited a high degree of consistency with one another. Nighttime net CO_2 exchange (NNEE), $\delta^{13}C$ and the titratable acidity difference (ΔH^+) were positively correlated, indicating that nocturnal CO_2 exchange, long-term carbon isotope composition and overnight organic acid accumulation reflect similar trends in carbon assimilation across species. This coherence suggests that physiological indicators effectively capture the distinctions among C_3, CAM and C_3/CAM intermediate types, provideing a reliable basis for pathway determination. In contrast, the correlations between structural traits and physiological indicators were more variable. Leaf thickness (LT) demonstrated moderate correlations with certain physiological traits, while the mesophyll succulent index (Sm) exhibited weaker overall correlations, and consistency among structural traits was limited. These patterns indicate that structural characteristics can reflect aspects of CAM activity but are not determinative, as species may adopt different structural configurations to achieve similar photosynthetic outcomes. Consequently, structural parameters alone are insufficient for accurate discrimination among C_3, C_3/CAM intermediate and CAM pathways. Overall, the correlation results underscore the central role of physiological parameters in determining carbon assimilation type, while structural traits serve more appropriately as supplementary evidence. This distinction elucidate why some C_3/CAM intermediate species display transitional or inconspicuous anatomical features and supports the perspective that photosynthetic pathway differentiation involves multidimensional regulation and diverse adaptive strategies (Borland et al. 2009).

Ecological adaptability in photosynthetic carbon assimilation pathway variation

This study reveals that the photosynthetic carbon assimilation pathways of nine *Dendrobium* species exhibit a high degree of correlation with the characteristics of their native habitats. Based on descriptions from *Plants of the World Online* and *the Flora of China*, these species are predominantly epiphytic or lithophytic orchids found in tropical and subtropical montane forests, open woodlands or rocky valleys (Table 3). Such habitats typically experience fluctuating water availability, nutrient-poor substrates and variable light conditions, all of which are known to promote CAM evolution in orchids (Nelson and Sage 2008; Silvera et al. 2010). In this study, species identified as typical CAM types (*D. parishii*, *D. aphyllum* and *D. anosmum*) are commonly found in environments characterized by pronounced dry–wet seasonality, high irradiance and epiphytic or lithophytic growth forms, aligning with ecological conditions that favor CAM metabolism. Conversely, species characterized as C_3/CAM intermediates (*D. crystallinum*, *D. linawianum* and *D. moschatum*) often inhabit forest margins or areas with seasonally variable moisture, suggesting that their flexible photosynthetic strategies may reflect adaptive responses to these environments. In contrast, species classified as C_3 types (*D. cariniferum*, *D. gibsonii* and *D. hancockii*) are found in shaded, humid montane forests, ravines or high-elevation moist environments where water availability is relatively stable, consistent with C_3 photosynthesis. Collectively, these findings indicate that the diversity of carbon assimilation pathways among the nine *Dendrobium*

species corresponds to distinct habitat preferences, reflecting ecological differentiation. Although this study did not quantitatively analyze climatic variables, comparisons with habitat descriptions suggest that environmental factors such as water availability, growth form (epiphytic or lithophytic) and light conditions may serve as important selective pressures shaping carbon assimilation strategies in *Dendrobium*. Future research that integrates distribution data with environmental parameters may elucidate the mechanisms linking habitat characteristics to the evolution of photosynthetic pathways.

Table 3
Natural habitat distribution of nine *Dendrobium* species

Species	Native distribution	Habitat type (as documented in the literature)	References
D. parishii	Northeast India, Myanmar, Thailand, Laos, Vietnam, China (Yunnan)	Epiphytic on trees in montane evergreen forests or on rocks; elevation 250–1200 m	Flora of China;POWO
D. aphyllum	Nepal, Bhutan, India, Myanmar, Thailand, Laos, Vietnam, China (Yunnan)	Epiphytic or lithophytic in tropical moist forests; elevation 200–1,500 m	Flora of China;POWO
D. anosmum	Philippines, Malaysia, Laos, Vietnam, Myanmar, China (Yunnan)	Epiphytic in lowland to montane forests, often near riverbanks or humid valleys	New Guinea Orchids;POWO
D. cariniferum	China (Yunnan), Myanmar, Northeast India	Epiphytic in humid montane forests; elevation 1,200–2,000 m	Flora of China;POWO
D. gibsonii	China (Yunnan, Guangxi), India, Myanmar	Epiphytic or lithophytic on cliffs, rocks or forest trunks; elevation 800–1,500 m	Flora of China;POWO
D. hancockii	China (Yunnan, Guizhou, Guangxi), Vietnam, Northern Thailand	Epiphytic or lithophytic in montane forests; elevation 700–1,500 m	Flora of China;Smithsonian
D. linawianum	Taiwan, China (Guangdong)	Epiphytic in montane evergreen forests; elevation 700–1,400 m	Flora of China;POWO
D. moschatum	Northeast India, Nepal, Bhutan, Myanmar, China (Yunnan)	Epiphytic in cliffs or montane moist forests; elevation 1,000–1,800 m	Useful Tropical Plants;POWO
D. crystallinum	Thailand, Myanmar, Laos, Vietnam, China (Yunnan, Guizhou, Hainan)	Epiphytic or lithophytic in montane forests with seasonal moisture variation; elevation 700–1,700 m	Flora of China;POWO

Conclusions

In summary, *D. parishii*, *D. aphyllum* and *D. anosmum* exhibited characteristics consistent with typical CAM plants, including nocturnal net CO_2 exchange, $\delta^{13}C$ values exceeding − 16‰ and substantial

nocturnal titratable acidity accumulation ($\Delta H^+ > 46$ μmol(H^+)·g^{-1} FW), and their anatomical traits, such as increased leaf thickness and elevated mesophyll succulent indices, further substantiated their classification within the CAM category. Conversely, *D. cariniferum*, *D. gibsonii* and *D. hancockii* displayed characteristics indicative of C_3 plants, including the absence of nocturnal CO_2 exchange, $\delta^{13}C$ values below − 26‰, and minimal overnight acid accumulation. *D. linawianum*, *D. moschatum* and *D. crystallinum* demonstrated intermediate C_3/CAM characteristics; moreover, leaf thickness and mesophyll succulent inices were generally higher in most CAM species, although some overlap occurred with values from C_3/CAM intermediates and C_3 species. Correlation analysis revealed that physiological indicators NNEE, $\delta^{13}C$ and ΔH^+ were highly consistent with one another, reflecting a continuum of carbon assimilation strategies within the genus *Dendrobium*. In contrast, the associations between structural traits and physiological parameters were generally weaker. Leaf thickness exhibited moderate correspondence with specific physiological variables, while the mesophyll succulent index showed weak associations with physiological parameters and lacked a clear correlation with photosynthetic types. These findings indicate that structural traits alone are insufficient for reliably distinguishing between C_3, C_3/CAM intermediate and CAM species, and they should analyzed used in conjunction with physiological parameters for more accurate classification. The various photosynthetic types correspond to distinct habitat preferences: typical CAM species are commonly found in seasonally dry, high-light epiphytic or lithophytic environments; C_3 species are typically associated with humid and shaded conditions; while C_3/CAM intermediate species thrive in habitats that experience alternating wet and dry conditions. This pattern reflects the relationship links between photosynthetic strategy and the availability of environmental moisture and light. Overall, the diversity of carbon assimilation pathways in *Dendrobium* illustrates both physiological plasticity and long-term ecological adaptation. This study offers a valuable foundation for ecological assessment, germplasm conservation and cultivation management of this orchid genus.

Declarations

Authors contributions

Conceptualization, Z.Y., S.C. and H.P.; data curation, Z.Y. and N.C.; formal analysis, L.P.; investigation, N.C.; methodology, L.P. and Z.Y.; software, N.C. and Z.Y.; supervision, L.P., J.W., Q.J. and N.C.; writing—original draft, D.T.; writing—review and editing, Z.Y. and S.C. All authors have read and agreed to the published version of the manuscript.

Funding

This study was supported by the National Key Research and Development Program of China (2022YFF1300700), Guangxi Forestry Bureau Project (2024LYKJ01), and Guangxi Key Laboratory of Plant Functional Substances and Sustainable Utilization (ZRJJ2024-12).

Data availability

All data generated or analyzed during this study are included in this published article.

The authors have no relevant financial or non-financial interests to disclose.

Ethics, Consent to to participate

Not applicable.

Consent for publication

All authors have approved the manuscript for submission to BMC Plant Biology.

Clinical trial number

Not applicable.

Conflicts of Interest

The authors declare no conflicts of interest.

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Figures

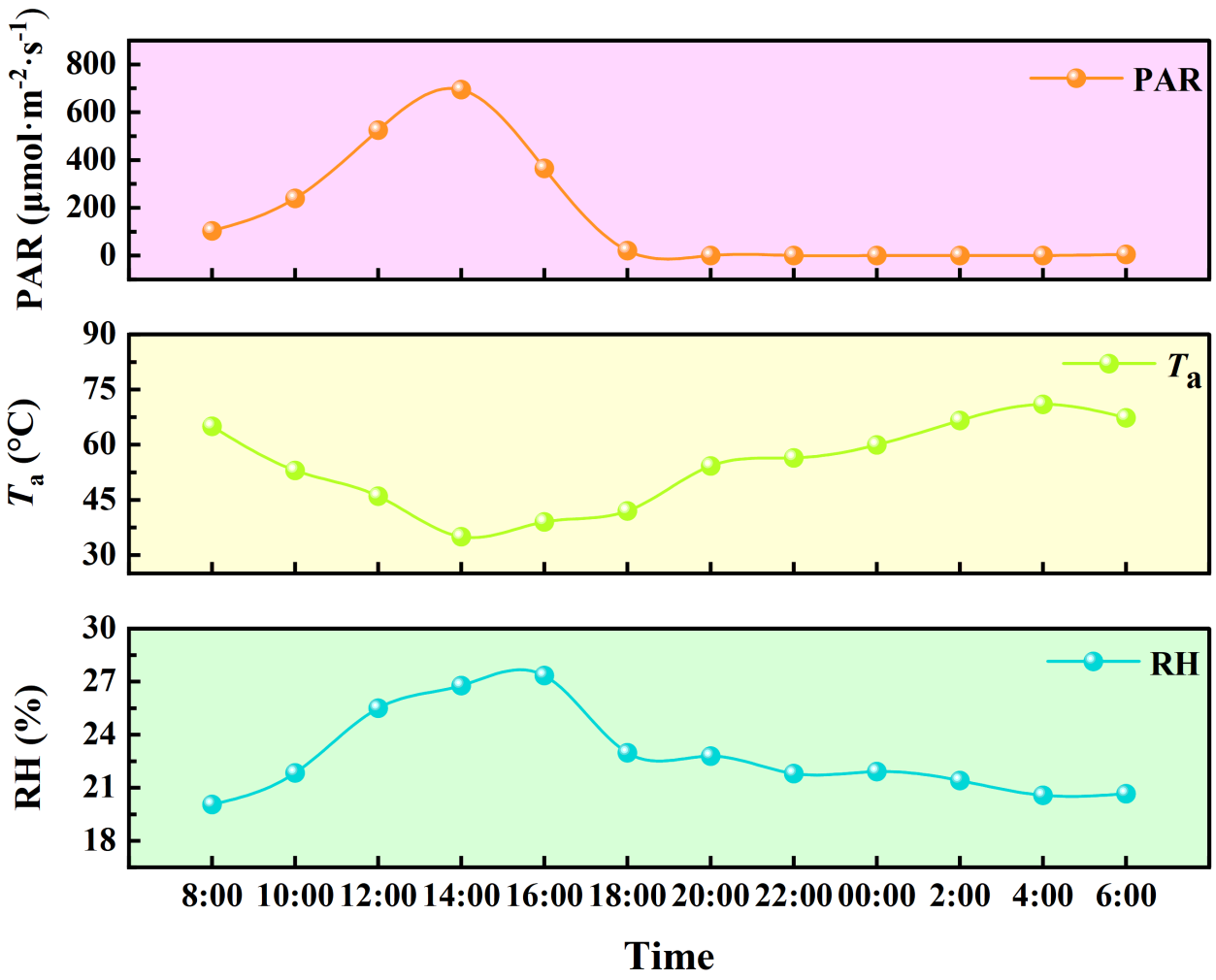

Figure 1

Diurnal variation curves of environmental factors

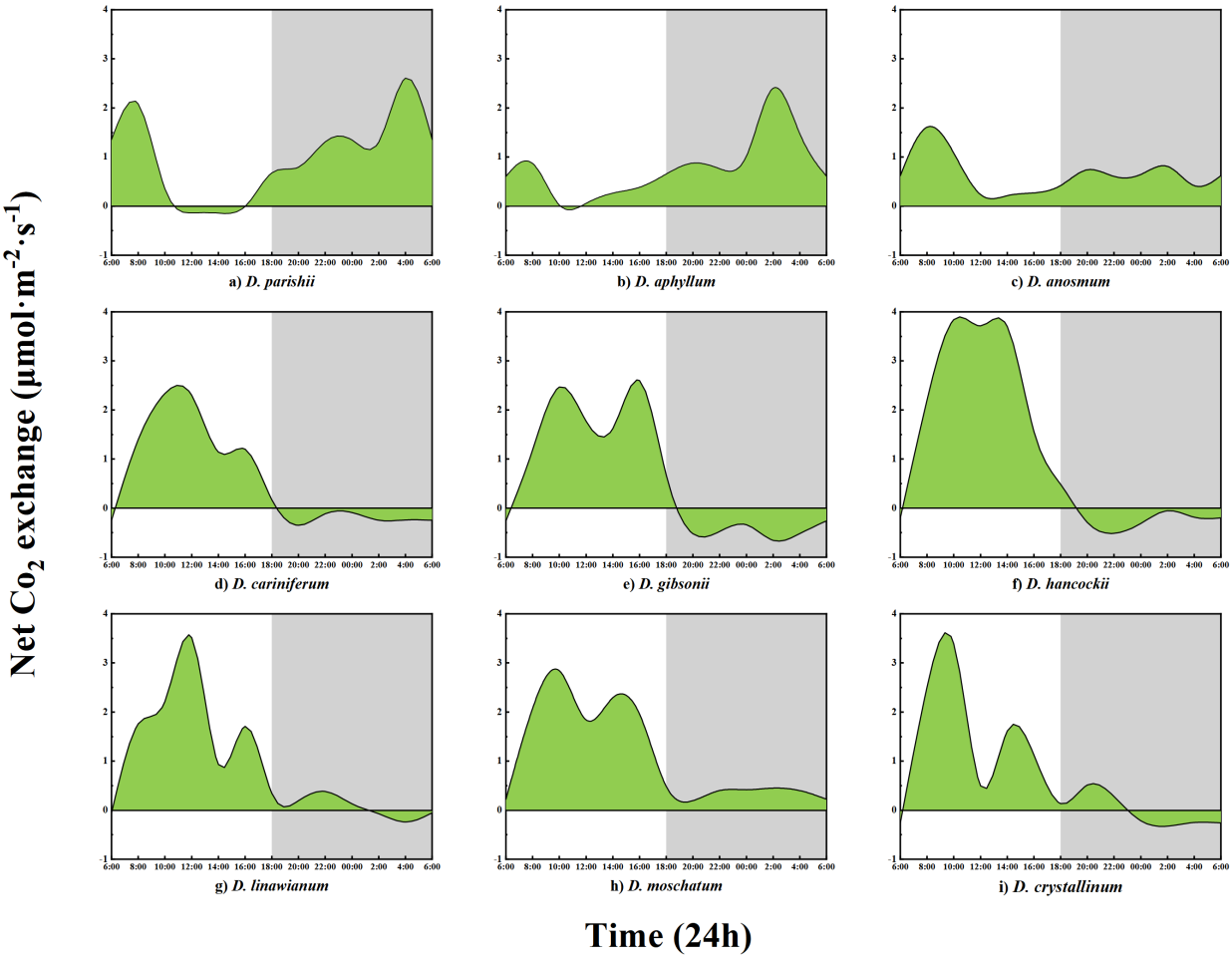

Figure 2

The diurnal variation in net CO2 exchange among nine *Dendrobium* species. Note: The white background denotes the daytime period, while the gray shaded area indicates the nighttime period. Each subfigure illustrates the diurnal and nocturnal dynamics of net CO_2

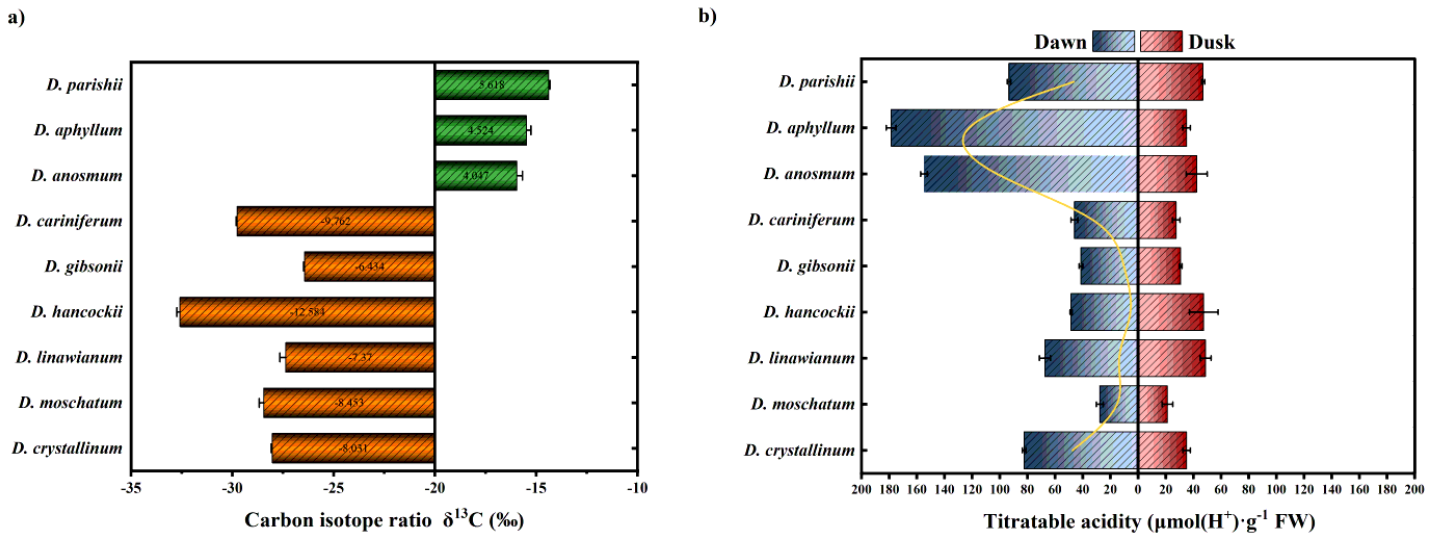

Figure 3

Carbon isotope ratios and titratable acidity contents of the nine *Dendrobium* species. Note: that panel a) illustrates the carbon stable isotope ratios $\delta^{13}C$ (‰). Different bar colors indicating interspecific variation in $\delta^{13}C$ values. Green bars represent values associated with predominantly CAM-type carbon assimilation, while yellow bars indicate values linked to predominantly C_3-type carbon assimilation. Panel b) displays leaf titratable acidity measured at dawn and dusk for each species, with blue bars representing acidity at dawn and red bars representing acidity at dusk. The yellow curve illustrates the difference in acidity between dawn and dusk.

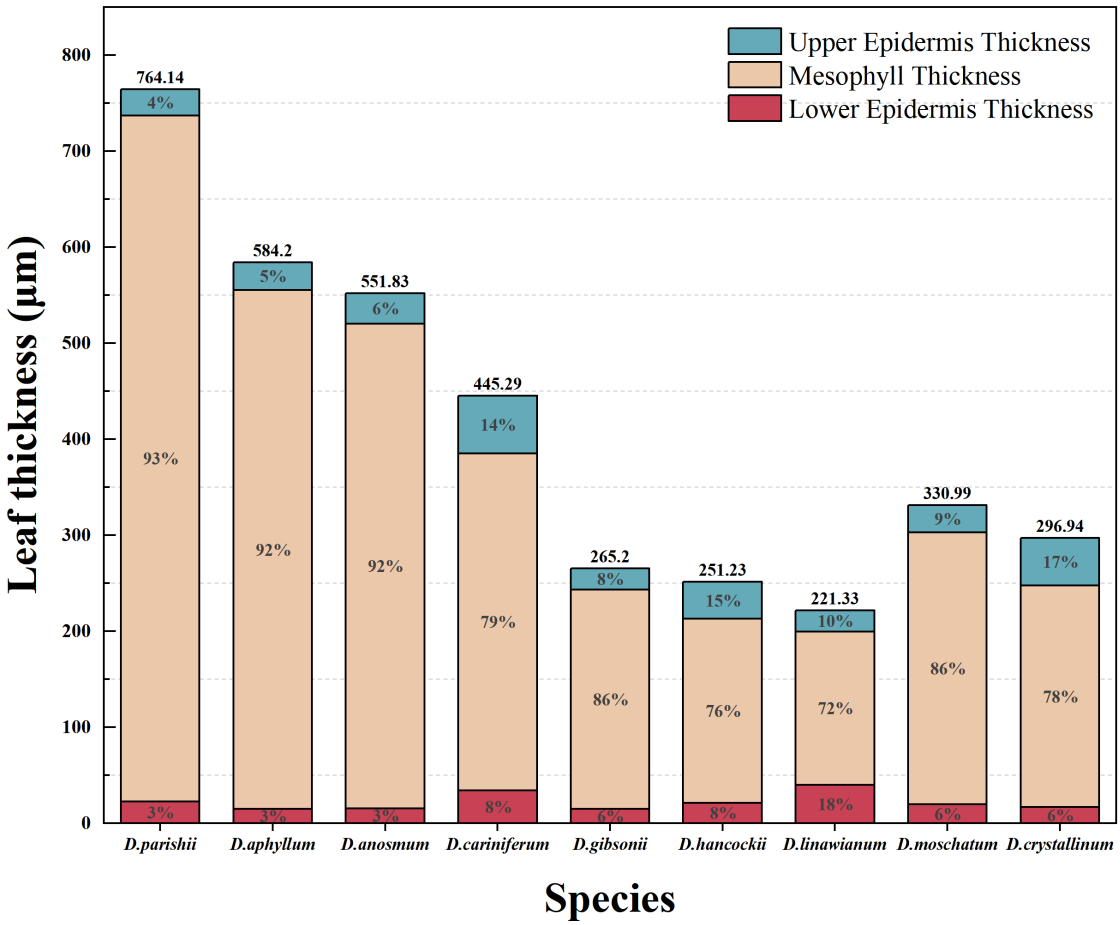

Figure 4

Leaf thickness and tissue composition in nine *Dendrobium* species. Note: The stacked bars represent the thickness (μm) of the upper epidermis (blue), mesophyll (yellow), and lower epidermis (red). The numerical values above each bar indicate total leaf thickness, and the percentages within each color segment denote the relative contribution of each tissue type.

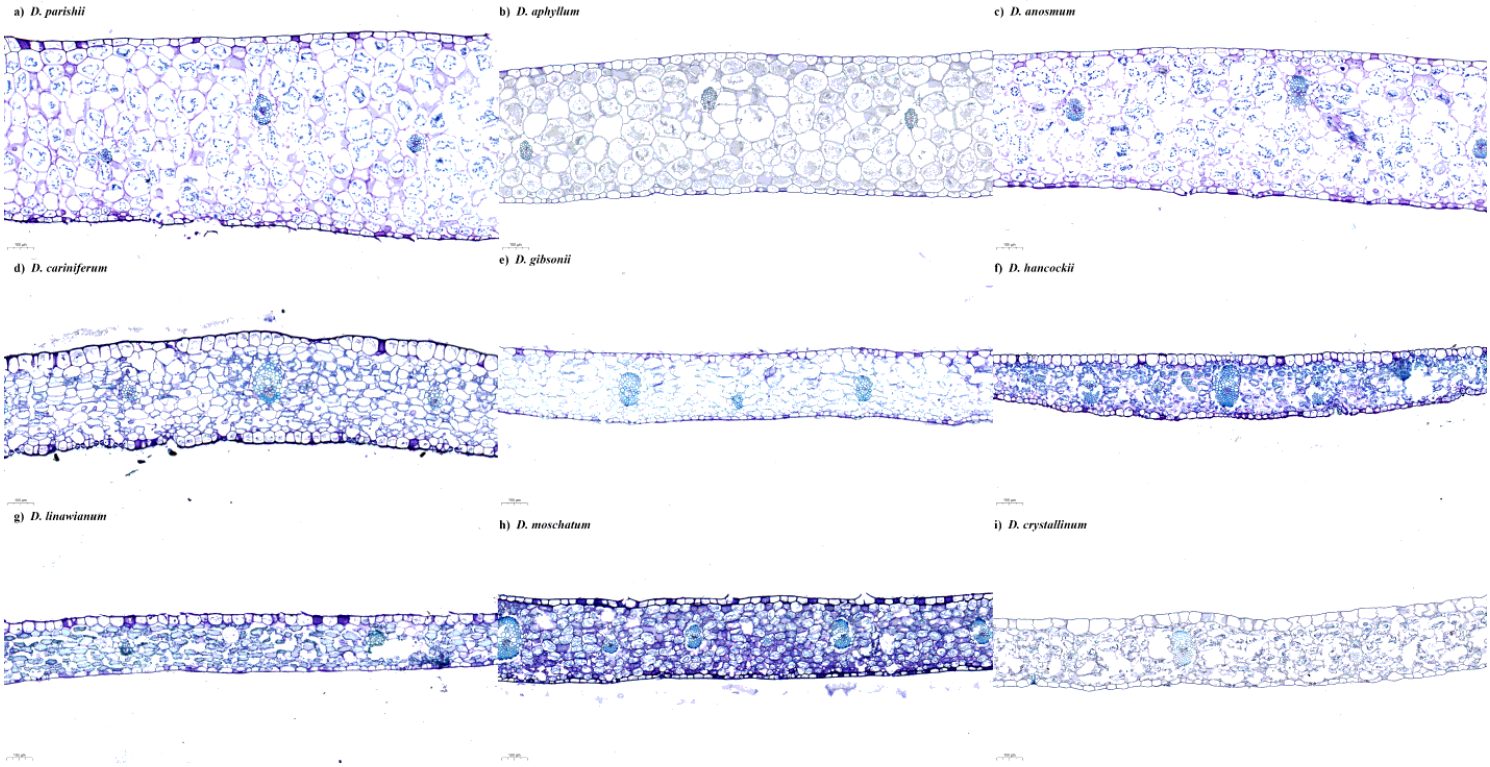

Figure 5

Leaf anatomical structure of nine *Dendrobium* species. Note: Images are presented in the correct anatomical orientation, with the upper (adaxial) epidermis positioned at the top and the lower (abaxial) epidermis at the bottom. Labels (a-i) correspond to the nine *Dendrobium* species examined in this study.

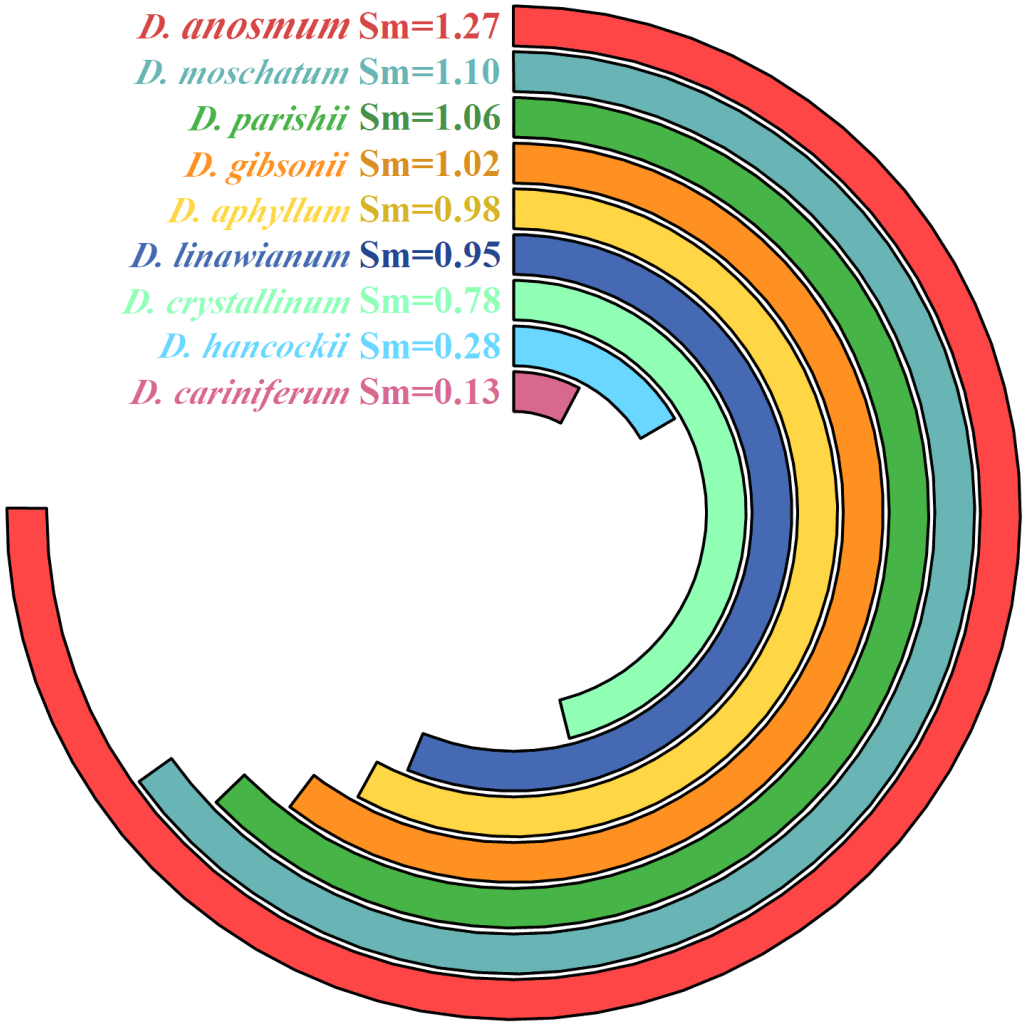

Figure 6

The mesophyll succulence of nine *Dendrobium* species. Note:Each species is represented by a colored arc, with arc length proportional to its Sm value; larger Sm values correspond to longer.

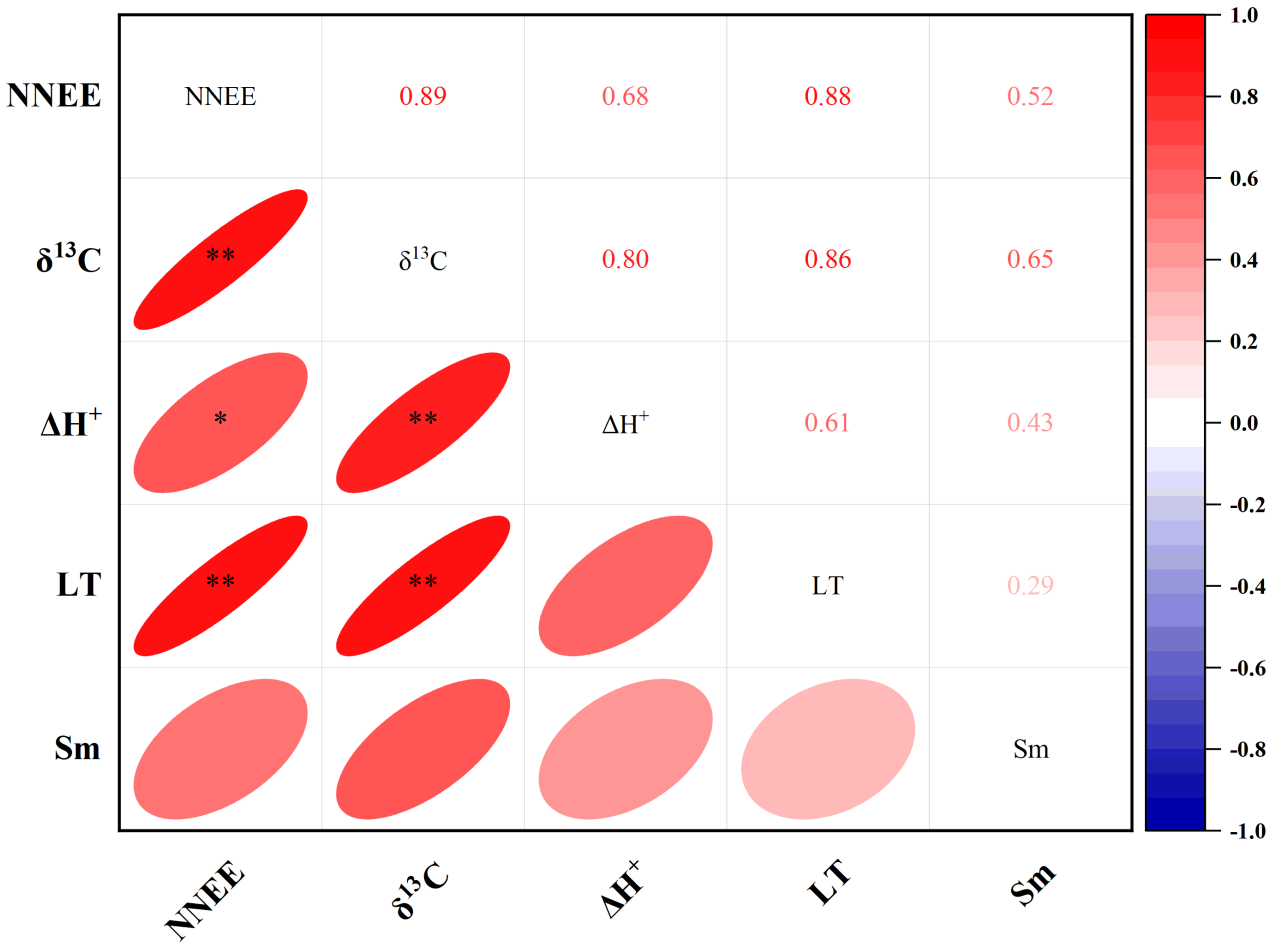

Figure 7

The heatmap illustrates the correlations between physiological parameters and structural traits of nine *Dendrobium* species. Note: This heatmap illustrates the correlations among key physiological indicators (NNEE, Nighttime Net CO_2 Exchange; $\delta^{13}C$, Carbon Isotope Ratio; ΔH^+, Titratable acidity difference) and leaf structural traits (LT, leaf thickness; Sm, mesophyll succulent index) . The color intensity reflects the strength of the correlations (−1 to +1), with red indicating positive correlations and blue indicating negative correlations. Greater color saturation corresponds to stronger correlation coefficients. Statistical significance is denoted as *p < 0.05, **p < 0.01, and ***p < 0.001.