# Vegetative anatomy and photosynthetic performance of the only known winter-green *Cypripedium* species: implications for divergent and convergent evolution of slipper orchids

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*Cypripedium subtropicum* is the only known winter-green species in the genus *Cypripedium*, whereas the other nearly 50 species keep their leaves for less than half the year. Life form has an important effect on carbon acquisition and adaptation of plants. However, the physiological mechanism behind it remains unclear. In this study, we investigated vegetative anatomy and photosynthetic performance of *C. subtropicum* across with its leaf ages. These anatomical and photosynthetic traits were also compared with typical *Cypripedium* spp. and other members of subfamily Cypripedioideae. The obtained results confirmed that this species exhibited many characters of shade plants, such as thin leaves, extremely low photosynthetic rate and light saturation point and high chlorophyll content. Unlike the strategy adopted by typical *Cypripedium* spp. that quickly achieve annual carbon gain with a high assimilation rate in a short growing season, *C. subtropicum* obtains its carbon through a low assimilation rate but a much longer leaf lifespan. The local climate and favourable light condition guaranteed the comparable carbon income in winter to compensate for its low photosynthetic capacity. The long-lived, thin leaves of *C. subtropicum*, differing from the long-lived, thick leathery leaves in conduplicate-leaved genera, represent a distinct adaptive strategy in subfamily Cypripedioideae. Our findings shed light on the divergent and convergent evolution in slipper orchids, and we hope these findings will contribute to the conservation of such an endangered orchid.

ADDITIONAL KEYWORDS: Cypripedioideae – *Cypripedium subtropicum* – endangered plant – habit shift – leaf trait – *Paphiopedilum* – physiological diversity – photosynthetic acclimation.

## INTRODUCTION

All the members of Orchidaceae subfamily Cypripedioideae are known as slipper orchids due to their conspicuous pouch-like flower labellum, which resembles a slipper. There are *c*. 200 species of slipper orchids, belonging to five accepted genera:

*Cypripedium* L., *Selenipedium* Rchb.f., *Mexipedium* V.A.Albert & M.W.Chase, *Paphiopedilum* Pfitzer and *Phragmipedium* Rolfe (Cox *et al*., 1997; Govaerts *et al*., 2020). According to growth patterns and leaf characters, two general types can be recognized: taxa with plicate leaves (*Cypripedium* and *Selenipedium*) and taxa with conduplicate leaves (*Mexipedium*, *Paphiopedilum* and *Phragmipedium*). Plants of the former type usually have short-lived shoots with conspicuous internodes and leaves folded into multiple pleats, whereas plants

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of the latter type are characterized by perennial shoots with condensed internodes and the leaves folded only along the midrib (Rosso, 1966; Atwood, 1984). The plicate-leaved *Cypripedium* spp. primarily occur in the north temperate zone, with a few species extending to subtropical areas of Asia and America. *Selenipedium* and the two conduplicate-leaved genera *Mexipedium* and *Phragmipedium* are restricted to the tropical regions of central and south America, and *Paphiopedilum* is confined to tropical and subtropical regions of Asia (Guo *et al*., 2012). Thus, the five genera of the subfamily Cypripedioideae may show divergent adaptation characteristics.

Slipper orchids exhibit remarkable diversity in flower morphology and have attracted much attention from pollination biologists and horticulturalists (Pemberton, 2013; Zhang, Huang & Zhang, 2016). Living in diverse climatic zones and habitats, members of the monophyletic subfamily also display various vegetative characters and different adaptive strategies to their habitats. *Paphiopedilum* spp. keep their leaves for years and lack a dormant period, whereas a dormant period, in which a plant shed its aboveground shoots, is critically for *Cypripedium* spp. surviving harsh winter conditions (Cribb, 1997, 1998). From an ecophysiological aspect, *Paphiopedilum* spp. usually have thick leathery leaves, low $CO_2$ assimilation rates and long leaf lifespans, whereas *Cypripedium* spp. are characterized by thin and delicate leaves, high $CO_2$ assimilation rates and short leaf lifespans (usually less than half a year) (Zhang, Hu & Li, 2008; Chang *et al*., 2011; Guan *et al*., 2011; Yang *et al*., 2018). As the leaves of slipper orchids are not shed from the stems like those of deciduous trees, the stems and leaves in this plant group have nearly the same longevity.

Detailed ecological observations are still lacking for most *Cypripedium* spp. (Cribb, 1997), and *C. subtropicum* S.C.Chen & K.Y.Lang is probably one of the most mysterious species in the genus. It was firstly described in south-eastern Tibet (Chen & Lang, 1986), and then similar plants were found in south-eastern Yunnan province and adjacent Vietnam, > 1000 km from the locality of the type specimen (Liu & Chen, 2009; Jiang & Liu, 2009; Averyanov *et al*., 2017). Although the distribution area of *C. subtropicum* could be relatively large, the species has been assessed as endangered (EN) on the Global Red List of IUCN, and is certainly at risk of full extinction due to over-collection in all known populations (Rankou & Averyanov, 2014; Averyanov *et al*., 2017). Nevertheless, little is known about *C. subtropicum* except the publication of its name because of its rareness and remote distribution. All previously known *Cypripedium* spp. have annual aerial shoots with a leaf lifespan usually not more than 6 months, but those of *C. subtropicum* have recently been

discovered to last for two or three years (Jiang & Liu, 2009). Based on similar habit, inflorescence and column structures, *C. subtropicum* was regarded as a missing link between *Cypripedium* and the Central and South American genus *Selenipedium* (Chen & Lang, 1986), although this notion was not supported by subsequent molecular phylogenetic analyses of *Cypripedium* (e.g. Li *et al*., 2011). *Cypripedium irapeanum* Lex., from Central America, resembles *C. subtropicum* in the large plant size and subtropical distribution (Chen & Lang, 1986), but they are not phylogenetically closely related (Li *et al*., 2011) and they do not share the long-lived leaf; *C. irapeanum*, like other members of the genus, has a noticeable five-month dormant stage (Hernandez *et al*., 2012). This makes *C. subtropicum* the only known species with perennial aerial shoots in *Cypripedium*. Furthermore, out of the plicate-leaved slipper orchids, *C. subtropicum* is more like a *Selenipedium* sp. in its habitat and life history, preferring tropical jungle, and it retains its leaves for more than one growing season (Atwood, 1984).

Previous studies have indicated divergent and convergent evolution in leaf anatomical structures and physiological functions between *Paphiopedilum*, with perennial aboveground shoots, and *Cypripedium*, with annual aboveground shoots (Guan *et al*., 2011; Chang *et al*., 2011). Evergreen leaves usually have a higher construction cost and lower photosynthetic capacity than deciduous leaves, but a longer period of photosynthetic activity, even in the unfavourable season. According to the cost–benefit theory, the adaptive significance of evergreen leaves over deciduous leaves is to maximize whole-plant carbon gain under various environmental conditions (Chabot & Hicks, 1982; Givnish, 2002). The plicate leaves of *C. subtropicum* are superficially similar to those typical leaves in the genus but have a longer lifespan, like *Selenipedium* spp., despite their isolated distribution and distant relation (Li *et al*., 2011; Guo *et al*., 2012). This probably represents a new adaptive strategy that reflects more complicated evolution history than previously expected in slipper orchids. However, the physiological mechanism for *C. subtropicum* for maintaining perennial aboveground shoots remains unclear, leaving *C. subtropicum* as an enigma in *Cypripedium*.

In this study, we investigated the anatomy of different vegetative organs, and photosynthetic performance of *C. subtropicum* across a range of leaf ages. These anatomical and physiological features were also compared with typical *Cypripedium* spp. and other slipper orchids. We aimed to answer the following questions. (1) How do the anatomical traits and photosynthetic performance of *C. subtropicum* differ from those of typical *Cypripedium* spp. and

other slipper orchids? (2) How do the leaf traits and photosynthetic ability of *C. subtropicum* change with leaf age, and what is the adaptive significance for a plicate-leaved slipper orchid?

## MATERIAL AND METHODS

### STUDY SITE AND PLANT MATERIAL

The study was performed near a protected area of *Cypripedium subtropicum* in Malipo County, Yunnan, China (elevation 1500 m a.s.l.). Plants were grown at a west-facing slope (*c*. 40°) under a semi-deciduous forest (mainly the deciduous *Alnus nepalensis* D.Don) for ten years before our experiment began. Voucher information for *C. subtropicum* and other species is provided in the Appendix. In most *Cypripedium* spp.,

populations are increased by clonal growth of ramets and plants finally grow in cluster (e.g. Kull, 1995). However, rather than a proliferation of the existing plant, the newly produced shoot of *C. subtropicum* just acts as a replacement of the old one. Two or rarely three shoots of different ages remain on a plant (Fig. 1A). The emergence of the new shoot occurs in May and it takes about two months for the leaves to reach their full size. Therefore, the ages of leaves on each stem were determined as two months and 14 months, respectively, in July, and eight months and 20 months, respectively, in January of the next year (Fig. 1A). All samplings and measurements were conducted in July 2018 and January 2019.

Climate data, including monthly precipitation, monthly mean temperature and monthly mean relative humidity of a nearby station, were also
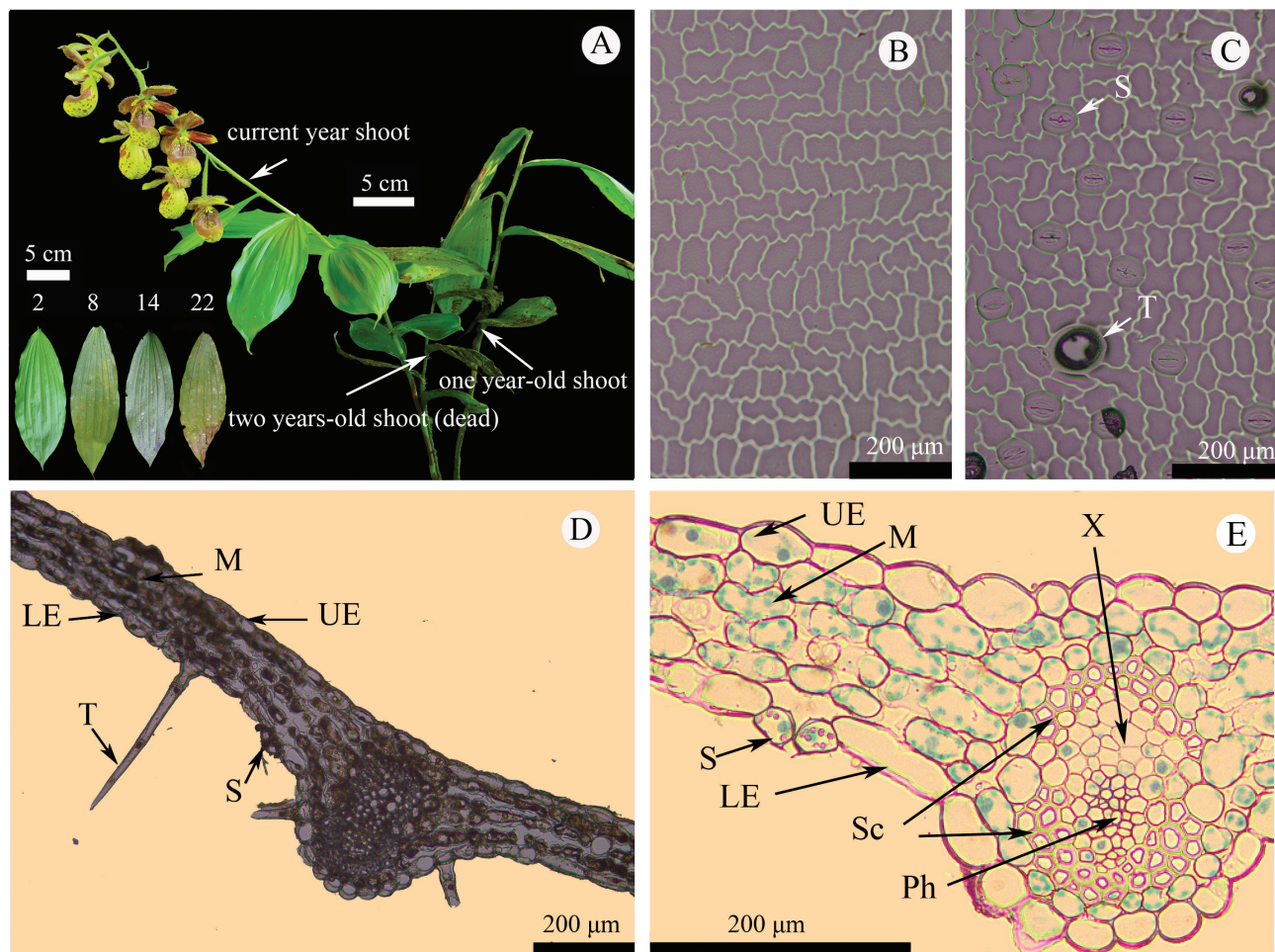


**Figure 1.** Plant habit and leaf anatomy of *Cypripedium subtropicum*. A, Plant habit and leaves of different ages (two to 22 months), showing flowers and two-month-old leaves on current year shoot, and 14-month-old leaves on one-year-old shoot; B, nail polish impression of adaxial surface of leaf; C, nail polish impression of abaxial surface of leaf; D, general view of the leaf cross section and E, details of the leaf cross section. S, stoma; T, trichome or its fracture surface impression; M, mesophyll cells; UE, upper epidermis; LE, lower epidermis; X, xylem; Ph, phloem; Sc, sclerenchyma.

compared with those of Shangri-La (elevation 3260 m) in north-western Yunnan, a typical habitat for *Cypripedium* where at least ten species have been recorded, including the closely related *C. wardii* Rolfe.

### FIELD MEASUREMENTS OF LEAF PHOTOSYNTHESIS

Leaf gas exchange was measured with a portable photosynthesis system (LI-6400; Li-Cor, Nebraska, USA). Recordings of $CO_2$ assimilation rate in response to incident photosynthetic photon flux density (PPFD) were made from 2000 to 0 µmol photons m$^{-2}$ s$^{-1}$, and a 3-minute interval was used for the reach of steady state of each point. Before taking measurements, each leaf was induced at a saturate light intensity (600 µmol photons m$^{-2}$ s$^{-1}$) for at least 30 min to ensure a steady state of photosynthesis. The atmospheric $CO_2$ concentration was maintained at 400 µmol mol$^{-1}$ by a $CO_2$ injector system (LI-6400–01; Li-Cor, Nebraska, USA), and relative air humidity and temperature of the leaf in the leaf chamber were maintained at 70% and 25 °C, respectively. The light compensation point (LCP) and light saturation point (LSP) were calculated from the light response curve, as described by Walker (1989). The light-saturated photosynthetic rate ($P_{max}$) was recorded at a light intensity of 600 µmol photons m$^{-2}$ s$^{-1}$ under the $CO_2$ concentration of 400 µmol mol$^{-1}$ during the period of 9:00–12:00 am. At least six leaves from different individuals were measured in the field.

### VEGETATIVE ANATOMY

Leaf area was first measured with a LI-3100 Portable Area Meter (Li-Cor, Nebraska, USA) after excision. A leaf sample was oven-dried at 80 °C for 48 h to determine the dry mass. Leaf mass per unit area (LMA) was expressed as the leaf dry mass divided by leaf area. The remained leaf, leafy stem and root (mature zone) samples were then fixed in FAA solution for further anatomical observations. They were dehydrated in an ethanol series and embedded in paraffin for transverse sectioning (Guan *et al*., 2011). The slices were then photographed under a light microscope (DM2500; Leica, Hesse-Darmstadt, Germany). Leaf epidermis and stomata were observed using nail polish to make impressions of adaxial and abaxial surface. Dried nail polish patches were then gently peeled from the leaf and photographed under the light microscope. Leaf characters, including the thickness (LT), upper epidermal thickness (UET), lower epidermal thickness (LET), mesophyll thickness (MT) and stomatal density, were then obtained from those digital photographs with Image J software (National Institutes of Health, USA). At least six leaves, stems or roots from different individuals were used in vegetative anatomy.

### LEAF BIOCHEMISTRY

Leaf chlorophyll was extracted and determined by following the method of Inskeep and Bloom (1985) with a spectrophotometer (UV-2550; Shimadzu, Kyoto, Japan). Leaf nitrogen content (N) was determined using a carbon–nitrogen analyser (Vario MAX CN; Elementar, Langenselbold, Germany). Photosynthetic nitrogen utilization efficiency (PNUE) was expressed as $P_{max}$ divided by area-based nitrogen content. Total phosphorus (P) and potassium (K) were determined with an inductively coupled plasma atomic-emission spectrometer (iCAP7400; Thermo Fisher Scientific, Massachusetts, USA).

### CLIMATIC RANGE ANALYSIS OF SPECIES

Distribution information of species was obtained from the online database Global Biodiversity Information Facility (GBIF, www.gbif.org/), and we discarded non-georeferenced records, records with equal longitude and latitude and points exceeding the elevational range of a given species. Climatic parameters were downloaded from WorldClim database (www.worldclim.org) using the raster package in R (Hijmans *et al*., 2020).

### DATA ANALYSIS

One-way analysis of variance (ANOVA) was used to examine significant differences among leaf ages, with means discriminated by LSD multiple comparison tests. A one-sample *t*-test was used to examine the significant differences of leaf traits between *C. subtropicum* and other slipper orchids. Data analyses were conducted in SPSS v.16.0 (SPSS Inc., Illinois, USA).

## RESULTS

### LEAF, STEM AND ROOT ANATOMY

Impressions of the adaxial leaf surface revealed sinuous or undulating walls of the epidermis cells (Fig. 1B). The pavement cells of abaxial (lower) epidermis are similar to those of adaxial (upper) epidermis, and stomata and trichomes only occur on the abaxial leaf surface (Fig. 1C, D). Cross sections showed the relatively simple structure of the leaf lamina. Both the adaxial and abaxial epidermises were made up by a single layer of cells. The mesophyll part was composed of three layers of loosely arranged cells, and no distinct differentiation of palisade and spongy layers was observed. Like other *Cypripedium* spp., leaves of *C. subtropicum* have several veins of similar size and lack a prominent midvein. Sclerenchyma associated with leaf vascular bundles is well-developed, made of a biseriate adaxial

sheath and a bi- to triseriate abaxial sheath, which are not attached to each other. The phloem is abaxial and the xylem adaxial. Guard cells of stomatal apparatus are extruded from the leaf surface (Fig. 1E).

The leafy stem consisted of the following tissues: epidermis, basic tissue/cortex and vascular bundles. Epidermis was made up by a single layer of cells, which was usually smaller in size when compared with inner basic tissue/cortex cells, and trichomes were observed associated with the epidermis. Vascular bundles scattered among the basic tissue and well-developed sclerenchyma associated with vascular bundles was also observed in the stem (Fig. 2). The epidermis of mature root was a single layer of living cells, and multiseriate velamen and root hairs were not observed at mature root zones. Exodermis consisted of uniseriate cells. The cells of the cortex located between the exodermis and the endodermis are usually larger than those of the exodermis and the endodermis and here were filled with an abundance of starch grains. The endodermis consisted of a single layer of cells and Casparian strips were not observed. The vascular cylinder comprised the central core of the root, and no continuous pericycle was observed (Fig. 3).

## LEAF PHOTOSYNTHETIC CAPACITY

The photosynthetic capacity of *C. subtropicum* derived from the photosynthetic response curves to PPFD was surprisingly low. At low light levels, the net photosynthetic rate increased with the increasing PPFDs and saturated at 60–80 µmol photons $m^{-2}$ $s^{-1}$, with a light-saturated photosynthetic rate of around 1.5 µmol $m^{-2}$ $s^{-1}$ (Fig. 4A). Photosynthetic rate was not significantly differed between leaf age of two and eight months. Then photosynthetic rate declined with leaf age (Fig. 4B).

## VARIATION OF LEAF TRAITS WITH LEAF AGE

All leaf structural traits (leaf thickness, upper epidermis thickness, lower epidermis thickness, MT and stomatal density) did not change significantly with leaf age (data not shown). Apart from photosynthetic capacity, leaf chemical and physiological traits such as content of nitrogen (N), phosphorus (P) and potassium (K), leaf chlorophyll and LMA varied with leaf age. Total phosphorus and potassium content decreased with leaf age. However, nitrogen content was the lowest in newly expanded leaves and then peaked at a leaf age of eight months, then decreased to a lower level at leaf ages of 14 and 20 months (Fig. 5A). The area-based chlorophyll content continued to increase before a leaf age of 14 months and decreased in the last stage (Fig. 5B). The ratio of chlorophyll *a* and chlorophyll *b* increased from two- to eight-month-old leaves, and declined at a leaf age of 14 months, making it the highest at the first winter of the leaf (Fig. 5C).
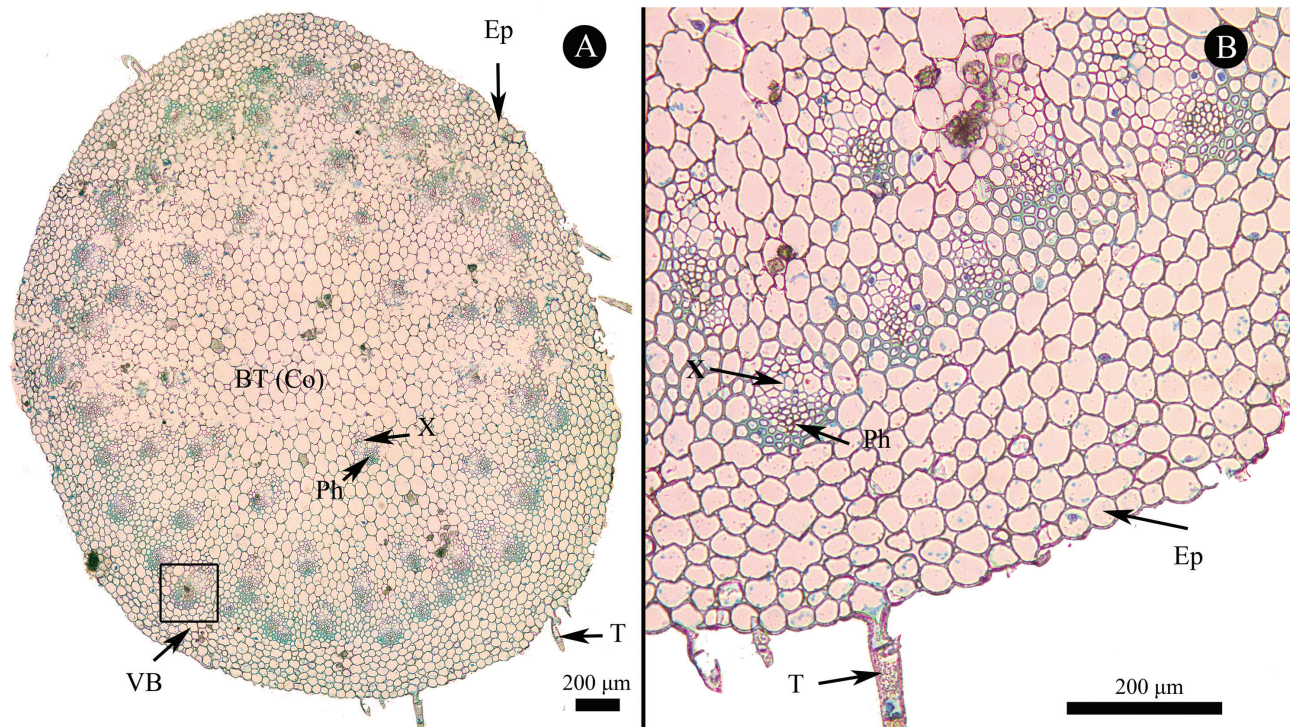


**Figure 2.** Stem cross section of *Cypripedium subtropicum*. A, General view and B, detailed view. BT, basic tissue; Co, cortex; Ep, epidermis; Ph, phloem; T, trichome; VB, vascular bundle; X, xylem.
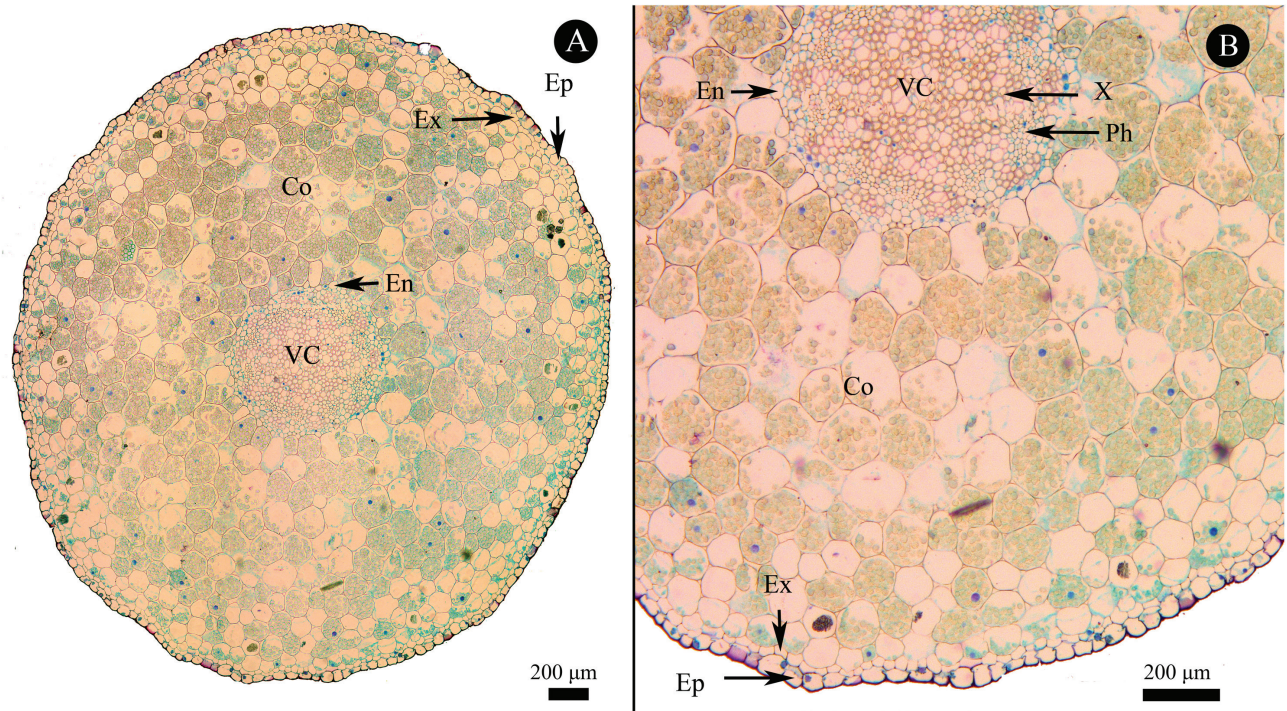
**Figure 3.** Root cross section of *Cypripedium subtropicum*. A, general view and B, detailed view. Co, cortex; En, endodermis; Ep, epidermis; Ex, exodermis; Ph, phloem; VC, vascular cylinder; X, xylem.
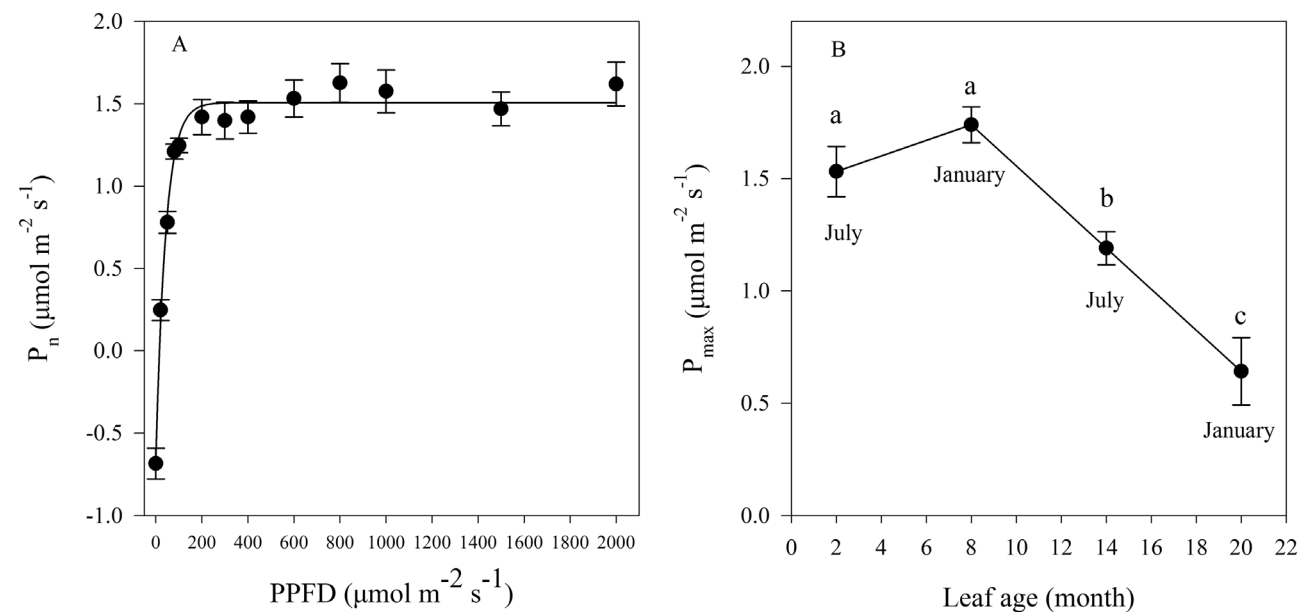


**Figure 4.** Photosynthetic light response curve for A, a two-month-old leaf and B, light-saturated photosynthetic rate at different leaf ages. Data are mean ± SE (*n* = 6–8), different lowercase letters indicate significant differences at *P* < 0.05 among leaf ages. July or January under each point indicates the time of the data collection.

**Figure 5.** A–C, Changes of leaf chemistry and D, leaf dry mass per area with leaf ages. Data are mean ± SE ($n$ = 6), different lowercase letters indicate significant differences at $P < 0.05$ among leaf ages. July or January under each point indicates the time of the data collection.
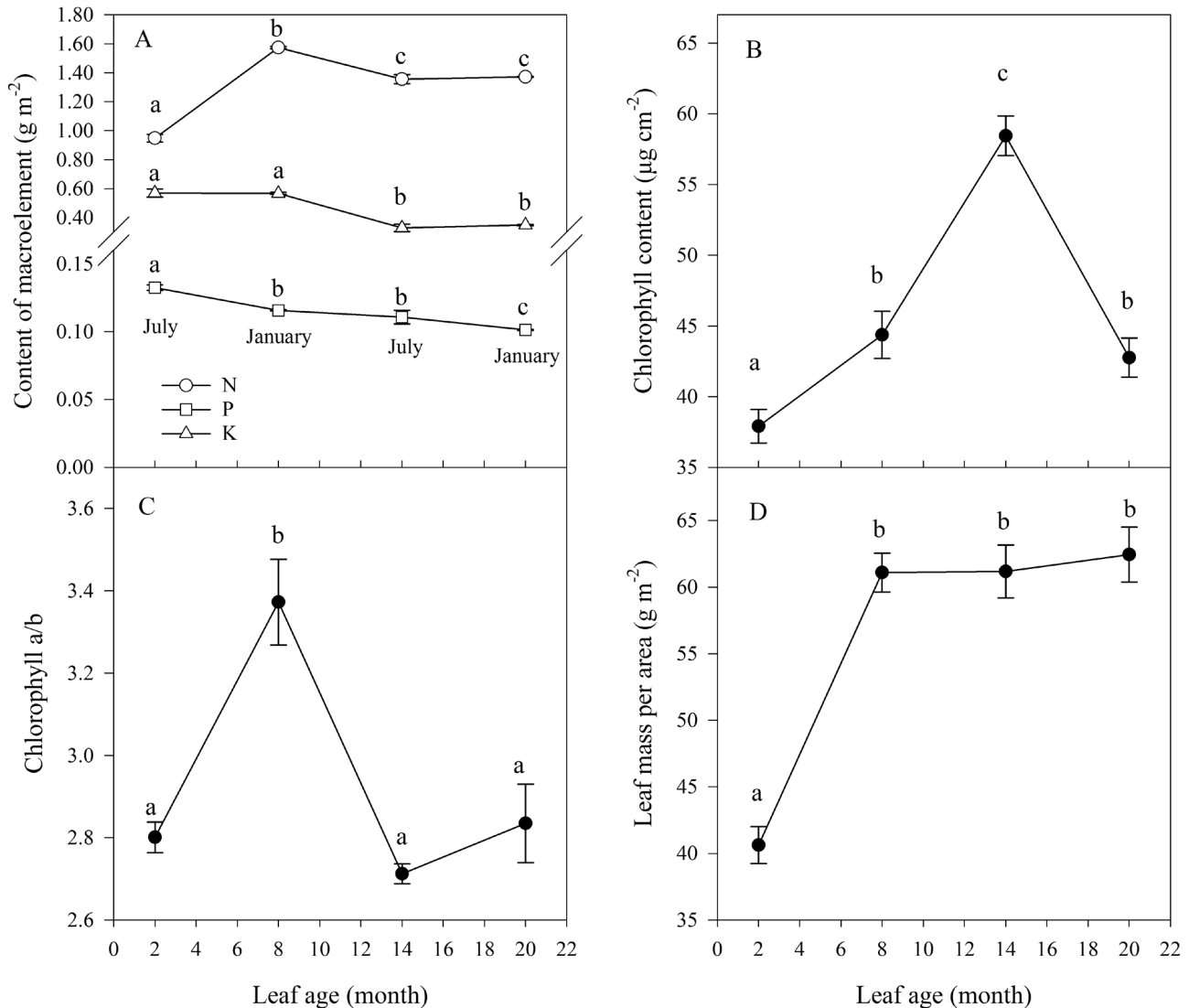
LMA was relatively low at a leaf age of two months, and increased to a significantly higher level at leaf ages from eight to 20 months (Fig. 5D).

### COMPARISON OF LEAF TRAITS WITH OTHER SLIPPER ORCHIDS

Leaf anatomical and physiological traits of two-month-old leaves in *C. subtropicum* were used since most available data of *Cypripedium* spp. from previous studies were obtained from leaves of a similar age. When compared with previous studied *Cypripedium* spp., *C. subtropicum* had the lowest values of leaf thickness (LT) and thickness of epidermis (UET and LET) and mesophyll (MT) (Table 1). All *Cypripedium*

spp. had thinner leaves than those of the conduplicate-leaved genus *Paphiopedilum*. Although data for *Phragmipedium* and *Mexipedium* are limited, we can infer from qualitative studies (Rosso, 1966; Sandoval *et al*., 2003) that leaf structures of these two conduplicate-leaved genera are more like those of *Paphiopedilum*. Limited data also showed similar leaf epidermis thickness of *C. subtropicum* and the plicate-leaved genus *Selenipedium*, which was *c*. 20 µm and equivalent to approximately half the value of other *Cypripedium* spp. (Table 1). The values of stomatal density and LMA of *C. subtropicum* fall within the range of other *Cypripedium* spp., even with the consideration of the increase of LMA in older leaves (Fig. 5D, Table 1).

**Table 1.** Comparison of leaf traits of *Cypripedium subtropicum* and other slipper orchids

| | LT | UET | LET | MT | SD | LMA |
|---|---|---|---|---|---|---|
| *Cypripedium subtropicum* | 137.59 ± 1.52* | 22.95 ± 0.84 | 28.32 ± 0.78* | 83.50 ± 1.56* | 28.79 ± 1.03 | 40.62 ± 1.38 |
| *C. flavum* | 259.64–312.60 | 46.70–49.90 | 42.70–50.29 | 157.56–224.20 | 33.30–34.06 | 46.70–66.70 |
| *C. tibeticum* | 275.20 | 51.00 | 43.00 | 182.10 | 28.40 | 28.43–63.20 |
| *C. yunnanense* | 290.10–300.62 | 48.90–50.63 | 43.28–44.60 | 191.40–210.72 | 36.90–44.52 | 45.40–61.33 |
| *C. guttatum* | - | - | - | - | - | 49.99–59.83 |
| *C. lichiangense* | 412.32 | 75.98 | 47.98 | 284.60 | 20.15 | 56.33–69.88 |
| *C. lentiginosum* | - | - | - | - | - | 28.57 |
| *C. japonicum* | - | - | - | - | - | 62.00 |
| *Selenipedium* | - | 17.00 | 23.00 | - | - | - |
| *Paphiopedilum* | 751.40–1536.64 | 104.70–606.18 | 43.81–91.54 | 348.20–927.40 | 17.03–66.23 | 97.00–237.39 |
| *Phragmipedium* | | 159.00–420.00 | 41.00–140.00 | - | - | - |

LT (µm), leaf thickness; UET (µm), upper epidermis thickness; LET (µm), lower epidermis thickness; MT (µm), mesophyll thickness; SD (mm$^{-2}$), stomatal density; LMA (g m$^{-2}$), leaf dry mass per area; Chla+b (µg cm$^{-2}$), chlorophyll *a* and *b* content per unit area; Chla:b, ratio of chlorophyll *a* and *b*; N$_{area}$ (g m$^{-2}$), area-based nitrogen content; $P_{max}$ (µmol m$^{-2}$ s$^{-2}$), light-saturated photosynthetic rate; PNUE, photosynthetic nitrogen utilization efficiency; LCP (µmol m$^{-2}$ s$^{-2}$), light compensation point; LSP (µmol m$^{-2}$ s$^{-2}$), light saturation point. "*", significantly difference when compared with other slipper orchids using one-sample *t*-test at $P < 0.05$. "-", data not available.
References: [1] Guan *et al*. 2011; [2] Zhang *et al*. 2005; [3] Li *et al*. 2008; [4] Zhang *et al*. 2006a; [5] Zhang *et al*. 2008; [6] Yang *et al*. 2018; [7] Zhang *et al*. 2006b; [8] Chang *et al*. 2011; [9] Zheng *et al*. 2017; [10] Zhang *et al*. 2007; [11] Wang *et al*. 2014; [12] Cho *et al*. 2019; [13] Rosso 1966; [14] Zhang *et al*. 2011; [15] Zhang *et al*. 2012; [16] Chang 2010; [17] Assmann *et al*. 1985.

For leaf chemistry, *C. subtropicum* had significantly higher chlorophyll content than other slipper orchids, but was without a high value of nitrogen content. Most *Cypripedium* spp. had high photosynthetic capacity according to light-saturated photosynthetic rate ($P_{max}$) and are followed by *Phragmipedium* and *Paphiopedilum*. In addition, a typical *Cypripedium* sp. needs a light intensity of > 400 µmol m$^{-2}$ s$^{-1}$ to achieve its saturated photosynthetic rate, whereas the values for *Paphiopedilum* spp. are 156–280 µmol m$^{-2}$ s$^{-1}$. However, the LSP is 67.85 µmol m$^{-2}$ s$^{-1}$ for *C. subtropicum*. Due to the low $P_{max}$, C. *subtropicum* also had the lowest value for PNUE (Table 1).

## CLIMATE DIFFERENCE BETWEEN TYPICAL *CYPRIPEDIUM SPECIES* AND *C. SUBTROPICUM*

Although available data for some of species are limited, the analysis of climatic ranges of *Cypripedium* spp. suggested that C. *subtropicum* grows at places with higher annual mean temperature than most species apart from *C. irapeanum*. Temperature seasonality and temperature annual range (maximum temperature of warmest month minus minimum temperature of coldest month) for *C. subtropicum* are lower than most of the species. *Cypripedium subtropicum* also received the highest annual precipitation of all species (Table 2).

Annual trends of precipitation and temperature are similar between Malipo and Shangri-La. The highest temperature and precipitation were recorded in July for these two sites, and the lowest temperature and precipitation in January and December, respectively. Nevertheless, the Malipo site always had higher monthly precipitation and monthly mean temperature (Fig. 6A, B). That site also showed higher but less strong seasonality of relative humidity (Fig. 6C).

The light environment for *C. subtropicum* was also different from those species growing in Shangri-La. The photosynthetically active radiation at noon of a sunny day in July was recorded at *c*. 100 µmol m$^{-2}$ s$^{-1}$ (5% of the full sunlight) for *C. subtropicum* under the *Alnus nepalensis* forest, which is far lower than that of *C. flavum* P.F.Hunt & Summerh. (700–1600 µmol m$^{-2}$ s$^{-1}$) (Zhang *et al*., 2005), *C. guttatum* Sw. (400–1500 µmol m$^{-2}$ s$^{-1}$) (Zhang *et al*., 2007) and *C. tibeticum* King ex Rolfe (180–1900 µmol m$^{-2}$ s$^{-1}$) (Zheng *et al*., 2017) recorded in natural habitats in Shangri-La.

## DISCUSSION

### DISTINCTIVENESS OF TRAITS OF *CYPRIPEDIUM SUBTROPICUM*

With much longer lifespan, the leaves of *C. subtropicum* had some anatomical or physiological similarities with and some differences from those of typical species in the genus. Previous studies have indicated that *Cypripedium* spp. have higher photosynthetic capacity than *Paphiopedilum* spp. due to differences in their leaf anatomical structures and physiological

| Chla + b | Chla:b | $N_{area}$ | $P_{max}$ | PNUE | LCP | LSP | Reference (see footnotes) |
|---|---|---|---|---|---|---|---|
| 37.89 ± 1.19* | 2.80 ± 0.03 | 0.95 ± 0.02 | 1.53 ± 0.11* | 1.61* | 15.14 ± 1.34 | 67.85 ± 5.68* | Present study |
| 17.60–34.20 | 2.90 | 1.24–1.33 | 5.40–11.30 | 7.02–8.78 | 8.30–32.60 | 594.00–700.00 | 1–6 |
| 14.70 | 3.26 | 0.56–1.51 | 3.81–9.42 | 6.35 | 4.61–12.98 | 655.00–987.00 | 6–9 |
| 15.10–17.80 | 3.35 | 0.59–0.63 | 6.87–9.32 | - | - | - | 1, 4, 6–7 |
| 17.90–21.50 | 3.25–3.91 | 0.70–0.85 | 8.13–9.69 | - | 16.77–21.57 | 528.00–608.00 | 4, 7, 10 |
| 19.30 | - | 0.66 | 6.03 | - | - | - | 1, 7 |
| - | 2.38 | 0.58 | 3.63 | 6.51 | - | - | 8 |
| - | 5.20 | - | 6.07–6.90 | | 6.00 | 416.50 | 11–12 |
| - | - | - | - | - | - | - | 13 |
| 9.22–12.90 | 2.31-2.25 | 0.67–1.40 | 1.94–3.43 | 2.35–3.32 | 8.00–28.00 | 156.10–280.10 | 1, 6, 8, 14–16 |
| - | - | - | -6 | - | - | 300.00–400.00 | 13, 17 |

characteristics (Chang *et al.*, 2011; Guan *et al.*, 2011; Yang *et al.*, 2018), but $P_{max}$ of *C. subtropicum* was even lower than those of *Paphiopedilum* spp. (Table 1). Light-saturated photosynthetic rate is strongly affected by leaf traits, including leaf or mesophyll thickness (McClendon, 1962; Oguchi, Hikosaka & Hirose, 2003), LMA(Wright *et al.*, 2004; Poorter *et al.*, 2009) and leaf nitrogen concentration (Evans, 1989; Wright *et al.*, 2004). *Cypripedium subtropicum* had lower leaf and mesophyll thickness but similar LMA and leaf nitrogen concentration compared with other *Cypripedium* spp. (Table 1). There are two possible explanations for the extremely low $P_{max}$ in *C. subtropicum*. The first is low MT of this species, because $P_{max}$ increases with the increase in MT within a certain range (Oguchi *et al.*, 2003). The other is the difference in photosynthetic nitrogen partition pattern between *C. subtropicum* and other *Cypripedium* spp. reflected by the ratio of chlorophyll content and total nitrogen. Although *C. subtropicum* had comparable leaf nitrogen concentration with other *Cypripedium* spp., it also had a significantly higher chlorophyll content (Table 1). Plants grow under low irradiance greatly increase the partitioning of nitrogen into chlorophyll-related light harvesting components, and decrease the investment of nitrogen into $CO_2$ fixation enzymes, resulting in a low $P_{max}$ and low PNUE (Evans, 1989; Hikosaka & Terashima, 1995). Thus, we speculate that the mechanisms for the low photosynthetic capacity of *C. subtropicum* and *Paphiopedilum* could be different. The low photosynthetic capacity for the former is probably caused by higher biochemical limitations, whereas in *Paphiopedilum* spp. it can largely be explained by diffusional limitations (Yang *et al.*, 2018).

Members of both *Cypripedium* and *Selenipedium* are characterized by thin, not fleshy leaves with a relatively large proportion of mesophyll cells (Rosso, 1966). As a member of *Cypripedium*, *C. subtropicum* shares the long leaf lifespan and thin epidermis with *Selenipedium* (Table 1). However, there are also some differences in leaf anatomy. For example, the epidermis anticlinal cell wall of *C. subtropicum* is sinuous or undulating (Fig.1B), whereas that of *Selenipedium* is straight (Rosso 1966). Little is known about the detailed leaf anatomical structures and photosynthetic performance of *Selenipedium*, but we can infer from limited descriptive information that *Selenipedium* spp. share most leaf anatomical and physiological traits with *C. subtropicum*, because they have similar leaf appearance and texture and comparable leaf lifespan and they live in similar habitats (Rosso, 1966; Atwood, 1984; Dressler, 1989; Cribb, 2009).

Most anatomical features of the stem of *C. subtropicum* fall within the range of previous studied *Cypripedium* spp. (Rosso, 1966), despite its longer lifespan. As for the root anatomy, all *Cypripedium* spp., including *C. subtropicum*, and *Selenipedium* possess a uniseriate epidermis rather than multiseriate velamen found in *Paphiopedilum* and *Phragmipedilum* (Rosso, 1966). This is probably because *Cypripedium* and *Selenipedium* are terrestrial, whereas *Paphiopedilum* and *Phragmipedilum* occur in a more-or-less epiphytic habitat, in which they need multiseriate velamen for efficient water and nutrient uptake and retention (Zotz & Winkler, 2013).

ADAPTATION TO THE HABITAT IN DENSE JUNGLE

Leaves usually have a weak ability to change their structures once they are fully expanded and their adaptation to the changes in irradiance is mainly via physiological adjustments (Sims & Pearcy, 1992; Oguchi, Hikosaka & Hirose, 2005; Zhang, Huang & Zhang, 2017).

**Table 2.** Climatic ranges of *Cypripedium* species

| Species | Annual mean temperature (°C) | Temperature seasonality | Temperature annual range (°C) | Annual precipitation (mm) | Precipitation seasonality | Available records |
|---|---|---|---|---|---|---|
| *C. subtropicum* | 15.87 (15.53–16.33) | 510 (472–574) | 22.85 (20.17–27.50) | 1528 (1411–1602) | 89.54 (88.24–93.14) | 3 |
| *C. lentiginosum* | 13.98 (13.22–15.19) | 450 (419–484) | 19.85 (18.72–21.28) | 1509 (1217–1720) | 87.53 (83.57–92.70) | 3 |
| *C. calceolus* | 4.85 (–11.12–15.99) | 818 (405–2221) | 30.77 (16.65–70.28) | 873 (236–2102) | 30.87 (6.51–120.91) | 4366 |
| *C. flavum* | 5.09 (–1.41–9.99) | 630 (448–1003) | 28.54 (21.97–42.47) | 747 (281–1494) | 76.99 (18.61–94.56) | 44 |
| *C. guttatum* | –1.01 (–16.85–10.88) | 1327 (360–2340) | 46.99 (15.01–71.70) | 508 (218–1845) | 61.42 (18.71–129.61) | 943 |
| *C. irapeanum* | 19.78 (7.8–26.25) | 171 (84–370) | 19.86 (13.40–30.40) | 1199 (366–4561) | 94.09 (48.13–123.97) | 115 |
| *C. japonicum* | 12.60 (7.09–15.57) | 788 (736–1029) | 29.87 (27.72–37.26) | 1339 (652–2116) | 58.65 (26.07–101.23) | 26 |
| *C. lichiangense* | 9.60 (6.78–13.57) | 528 (478–594) | 25.63 (24.10–28.18) | 767 (708–799) | 65.55 (63.16–67.23) | 3 |
| *C. tibeticum* | 7.07 (–0.55–13.68) | 641 (474–1003) | 29.06 (24.10–46.30) | 717 (61–105) | 84.98 (60.86–104.62) | 46 |
| *C. yunnanense* | 7.40 (2.96–11.35) | 663 (536–1003) | 31.45 (21.17–41.47) | 647 (281–873) | 85.54 (67.24–94.86) | 9 |

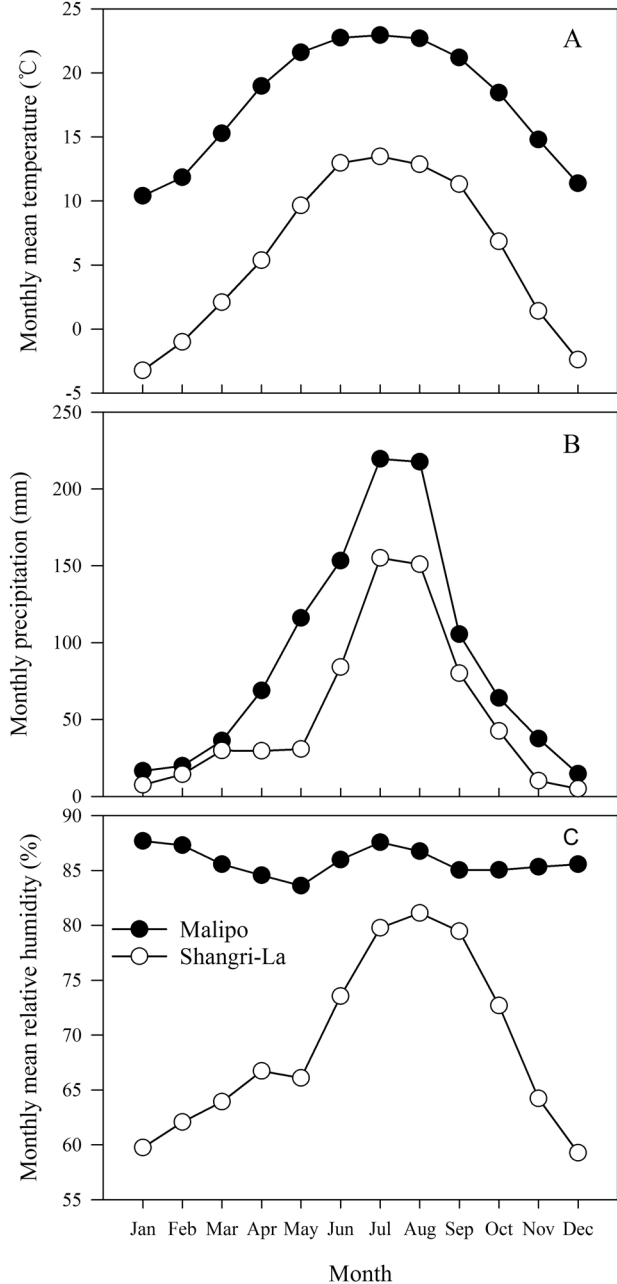Data are means of all records followed by the minimum and maximum values.



**Figure 6.** A, Monthly mean temperature, B, monthly mean precipitation and C, monthly mean relative air humidity in Malipo (natural habitat of *Cypripedium subtropicum*) and Shangri-La (natural habitat of several typical *Cypripedium* spp.), Yunnan province.

All leaf structural traits did not change significantly at different leaf ages. This is because leaves have already reached their full size at the age of two months. We observed similar changes of $P_{max}$ along with leaf ageing, but different patterns of changes in leaf nitrogen, LMA and chlorophyll content in *C. flavum* (Zhang *et al*., 2008). Area-based leaf nitrogen content, chlorophyll

content and LMA increased significantly after full leaf expansion in *C. subtropicum* (Fig. 5A, B, D). This is probably due to the differences in habitat and leaf lifespan of these two species. *Cypripedium subtropicum* usually grows under semi-deciduous forests (Averyanov *et al.*, 2017), whereas *C. flavum* and other *Cypripedium* spp. are usually found under sparse woods and at the margin of subalpine or boreal forests (Kull, 1998; Brzosko, 2002; Li *et al.*, 2008; Zheng *et al.*, 2017). Due to its retaining of leaves in winter, *C. subtropicum* has to face much stronger seasonality in light environment caused by the canopy closure and openness. Many traits such as the low LT, high concentration of chlorophyll, low LSP and $P_{max}$ reflect the adaptation of *C. subtropicum* to a shaded forest understory, whereas the seasonal changes in chlorophyll content, ratio of chlorophyll *a* to *b* and LMA reflect the physiological acclimation of the leaves to the changing light environment (Zhang *et al.*, 2007; Li *et al.*, 2008; Zheng *et al.*, 2017).

Similar intraspecific variations of traits were also observed in plants from different natural habitats in other *Cypripedium* spp. Plants grow at shady sites usually have lower $P_{max}$, LMA, LSP and higher chlorophyll content than those under a favourable light condition (Zhang *et al.*, 2007; Li *et al.*, 2008; Zheng *et al.*, 2017). Poor light availability (e.g. canopy closure) also has an adverse effect on ramet growth, flowering, fruiting and seedling establishment of *Cypripedium* plants (Kull, 1998; Brzosko, 2002; Hurskainen *et al.*, 2017). Moreover, deep shade not only leads to the decline in flower production of *Cypripedium* plants, but even a vegetative dormancy, a state in which a plant produces no aboveground shoots for one year or more to escape from environmental stress (Shefferson, Kull & Tali, 2005; Shefferson *et al.*, 2012). Therefore, selective tree removal, which increases light transmission, has been proposed as a management method to increase population size of the understory *Cypripedium* plants (Hurskainen *et al.*, 2017).

Unlike the strategy adopted by typical *Cypripedium* spp. that quickly achieves annual carbon gain with a high assimilation rate in a short growing season, the shaded plant *C. subtropicum* obtains its carbon through a low assimilation rate but a much longer leaf lifespan. We found that photosynthetic rate in winter was comparable with that in summer (Fig. 4B), which addressed the importance of carbon income in winter. *Cypripedium subtropicum* is never exposed to frost or severe drought (Fig. 6), and this might be an important factor in its ability to retain the foliage all year. The suitable climate and the good light condition provided by leaf shedding of canopy trees also make it possible for *C. subtropicum* to maintain a relatively high assimilation rate in winter. Orchids growing under a shade understory like *C. subtropicum* may obtain carbon not only from their own photosynthesis, but also their fungal partners (Preiss, Adam & Gebauer, 2010; Gonneau *et al.*, 2014). Unfortunately, there is no study so far concerning to mycorrhizal fungi or isotopic abundance of *C. subtropicum* to the best of our knowledge, limiting the understanding of the fungus-derived carbon income. According to our field observation, this species showed weak vegetative propagation ability through the increase of ramet number, and it is also proved to be difficult to germinate seeds in flasks and transplant adult plants from the wild (unpublished data). Thus, we suggest *in situ* conservation of this endangered species.

## DIVERGENT AND CONVERGENT EVOLUTION OF LEAF TRAITS IN SLIPPER ORCHIDS

In the evolution of slipper orchids, the plicate-leaved genus *Cypripedium* diverged first, followed by *Selenipedium* and finally the conduplicate-leaved genera *Paphiopedilum*, *Mexipedium* and *Phragmipedium* (Guo *et al.*, 2012). The disjunction of three conduplicate-leaved genera could be explained by fragmentation of the boreotropical flora, i.e. the ancestor of the conduplicate slipper orchids had a continuous distribution in the boreotropics, and migrated southwards to both sides of the Pacific Ocean due to climate cooling in the late Cenozoic and then evolved into separate genera. As for the plicate-leaved genus *Cypripedium*, Guo *et al.* (2012) suggested an origin in the subtropics, and the Bering land bridge acted as a corridor for the dispersal of temperate *Cypripedium* between East Asia and North America from the mid to late Tertiary. Guo *et al.* (2012) also proposed vicariance is responsible for the disjunct distribution of conduplicate slipper orchids in Palaeotropical and Neotropical regions. The longer lifespan of leaves of *C. subtropicum* and *Selenipedium* is associated with shift from aboveground shoots that live for one season to shoots that live for more than one. Since *C. subtropicum* is proved not the earliest-diverging species of *Cypripedium* (Li *et al.*, 2011), the similar disjunction of *C. subtropicum* and *Selenipedium* clearly reflects the convergent adaptation of perennial areal shoots (long-lived plicate leaves) to tropical habitats on both sides of the Pacific Ocean.

Previous study has revealed the divergence in leaf anatomical structures and physiological functions between *Paphiopedilum* spp. and *Cypripedium* spp., reflecting adaptations to contrasting habitats of these two groups (Guan *et al.*, 2011). A study on another species that grows in sympatry with *Paphiopedilum*
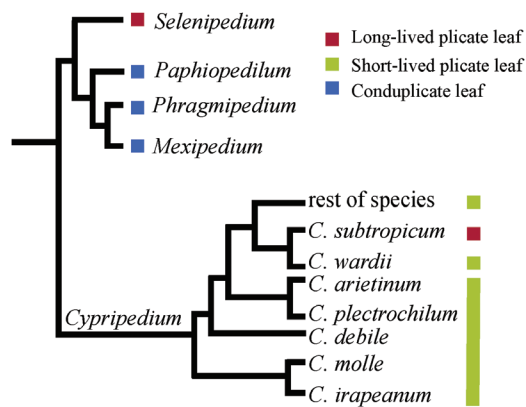
**Figure 7.** Three ecological types in subfamily Cypripedioideae. The tree topology was based on Guo *et al.* (2012) and Li *et al.* (2011).

spp., *C. lentiginosum* P.J.Cribb & S.C.Chen, suggested the convergent evolution of some leaf traits and physiology of *Paphiopedilum* spp. and *Cypripedium* spp. in similar habitats (Chang *et al.*, 2011). Including results for *C. subtropicum* from this study, all three ecological types of slipper orchids (conduplicate-leaved, long-lived plicate leaved and short-lived plicate leaved, see Fig. 7) can be found in a relative narrow area in south-eastern Yunnan, China, and northern Vietnam. *Paphiopedilum* spp. usually occur below 2000 m, and *C. subtropicum* is found at *c.* 1500 m, whereas *C. lentiginosum* grows between 2100 and 2300 m a.s.l. (Chen & Cribb, 2009; Averyanov *et al.*, 2017). Previous studies (Chang *et al.*, 2011; Guan *et al.*, 2011) and our results together reveal the rich physiological diversity of co-occurring orchids (Zhang, Hu & Zhang, 2016; Zhang *et al.*, 2018). The occurrence of all three ecological types also indicates the irreplaceable value of conservation for slipper orchids in this region.

## CONCLUSIONS

In this study, we investigated anatomy and photosynthetic performance of *C. subtropicum*, the only species with aboveground shoots in *Cypripedium* that survive for more than one season. The species exhibited many characters of shaded plants. Long leaf lifespan and local climate guarantee the carbon income of this species in winter to compensate for its low photosynthetic capacity. *Cypripedium subtropicum*, with long-lived plicate leaves, represents a new adaptive strategy in *Cypripedium*, and adds to our understanding of the evolution history of leaf traits and habit shift in slipper orchids.

## CONFLICT OF INTEREST

The authors declared that they have no conflicts of interest to this work.

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# APPENDIX

**Table S1.** Voucher information of species involved in the present study

| Taxon | Voucher information |
|---|---|
| *Cypripedium subtropicum* | *H. Jiang 08890*, YAF; *L. Averyanov et al. CPC 8243a*, LE |
| *C. flavum* | No specific information in original documents, but see *Z.D. Fang 0004*, SABG; *Y.J. Guo, J.D. Ya & Y. Su, 14CS8288*, KUN |
| *C. tibeticum* | No specific information in original documents, but see *Z.D. Fang et al. K-515*, SABG; *L.M. Gao & J. Liu, GLM-081028*, KUN |
| *C. yunnanense* | No specific information in original documents, but see *S. Jang 10103*, PE |
| *C. guttatum* | No specific information in original documents, but see *Z.D. Fang 0944*, SABG; *K.M. Feng 1394*, KUN |
| *C. lichiangense* | No specific information in original documents, but see *Z.D. Fang et al. K-1908*, SABG; *Z.X. Ren & H.D. Li, 2012-RZX-0016*, KUN |
| *C. lentiginosum* | No specific information in original documents, but see *Li in Luo s.n.*, K |
| *C. japonicum* | No specific information in original documents, but see *P.C. Tsoong 4070*, PE |
| *Selenipedium* | *Steyermark & Allen 17606*, MO (*Selenipedium chica*) |
| *Paphiopedilum* | No specific information in original documents, but see *F.Y. Liu 81-A*, PE (*Paphiopedilum armeniacum*); *C.W. Wang 86152*, KUN (*P. dianthum*); *W.Z. Zhang 47*, PE (*P. micranthum*) |
| *Phragmipedium* | Cultivated at Missouri Botanical Garden according to Rosso, 1966 (*Phragmipedium longifolium*) |