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Ecophysiological significance of leaf traits in *Cypripedium* and *Paphiopedilum*

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There is a close phylogenetic relationship between *Paphiopedilum* and *Cypripedium*, but these two genera diverge considerably in terms of their leaf traits. To understand the evolution and the ecophysiological significance of leaf traits, we investigated the leaf traits of three *Paphiopedilum* species and three *Cypripedium* species in southwestern China. *Cypripedium tibeticum* and *C. flavum* showed a significantly higher light-saturated photosynthetic rate (P_{max}), stomatal conductance (g_s), photosynthetic nitrogen utilization efficiency (PNUE) and specific leaf area (SLA), but lower ratio of leaf carbon to nitrogen content (C/N) and leaf construction cost (CC) than *Paphiopedilum*. These leaf traits of *Cypripedium* suggest its high resource use efficiency and high growth rate reflecting adaptation to a short growing period and abundant soil nutrients and water in alpine habitats. Conversely, the low P_{max}, g_s, PNUE, SLA and the ratio of chlorophyll *a* to chlorophyll *b* (Chl *a*/*b*), but high leaf nitrogen investment in light-harvesting component (P_L), CC and C/N in *Paphiopedilum* indicate its adaptation to a low light, nutrient-poor and limited soil water habitats in karst areas. As a sympatric species of *Paphiopedilum*, although *C. lentiginosum* retained the phylogenetic leaf traits of *Cypripedium*, such as high mass-based light-saturated photosynthetic rate ($P_{max\text{-}M}$), g_s and PNUE, it had some similar leaf traits to those of *Paphiopedilum*, such as low mesophyll conductance (g_m) and Chl *a*/*b*, and high P_L, which reflected an adaptation to the same habitat. Our results show that the evolution of the leaf traits of *Paphiopedilum* and *Cypripedium* are shaped by both phylogeny and environment.

Introduction

In the evolution and adaptation of plants, the leaf is more sensitive and plastic to environmental change than the other organs. Leaf traits are associated with the key aspects of leaf functions, and can provide a lot of information about the plant growth and resource-use strategies (Luo et al. 2005, Poorter and Bongers 2006, Vendramini et al. 2002). Many leaf traits, including photosynthesis, leaf life span (LLS), leaf nitrogen content

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Abbreviations – CC, leaf construction cost; C_i, intercellular CO_2 concentration; C/N, ratio of leaf carbon to nitrogen content; g_m, mesophyll conductance; g_s, stomatal conductance; J_{max}, maximum electron transport rate; LLS, leaf life span; N_a, total leaf nitrogen content; P_B, fraction of leaf nitrogen allocated to bioenergetics; P_C, fraction of leaf nitrogen allocated to Rubisco; P_L, fraction of leaf nitrogen allocated to light-harvesting components; P_{max}, light-saturated photosynthetic rate; $P_{max\text{-}M}$, mass-based light-saturated photosynthetic rate; P_n, photosynthetic rate; PNUE, photosynthetic nitrogen utilization efficiency; RSL, relative stomatal limitation; SLA, specific leaf area; V_{cmax}, maximum carboxylation rate; V_{cr}, maximum rate of RuBP carboxylation per Rubisco protein.

and specific leaf area (SLA), vary between species (Chapin 1980, Reich et al. 1992, Schulze et al. 1994). The variations in the leaf traits are associated with the ecological strategies and biogeographical ecology of plants. In fact, when some functional leaf traits appear in related taxa with similar or different habitats, it would suggest that the plants have undergone convergent and/or divergent evolution under natural selection (Ackerly and Reich 1999, Reich et al. 1992, 1997, 1998, Wright et al. 2001, 2002).

The LLS, SLA, nitrogen concentration and carbon assimilation rate are interpreted as an evidence of coordinated leaf physiological strategies in different environments (Körner 1991, Wright et al. 2002). These leaf traits are generally correlated among and within species (Körner 1989, Reich et al. 1992). In general, species with long LLS are considered to be adapted to low resource environments and tend to have a low photosynthetic rate (P_n), low photosynthetic nitrogen utilization, low mass-based leaf nitrogen content and low SLA (Mediavilla and Escudero 2003, Poorter and Bongers 2006, Reich et al. 1997, 1998, Wright et al. 2002). Conversely, species with short LLS are considered to be adapted to resource-rich habitats and short growing period and tend to have a high P_n, high mass-based leaf nitrogen concentration and high SLA (Reich et al. 1992, 1997, Villar and Merino 2001). Therefore the measurement of functional leaf traits allows us to address questions relating to leaf carbon balance, resource-use strategies and adaptive strategies to the environment.

Paphiopedilum and *Cypripedium* are very well-known slipper orchids in horticulture, and belong to the subfamily Cypripedioideae of the Orchidaceae (Cameron et al. 1999, Cox et al. 1997). It is reported that *Paphiopedilum* is an evolutionary branch of the genus *Cypripedium*, extending into tropical areas, and that *P. malipoense* is a 'link' between them based on morphology (Chen and Tsi 1984). Although they are closely related phylogenetically, there are significant differences in the leaf traits and geographical distributions of the two genera. Most species of *Cypripedium* are perennial geophytes and dormant in winter, occurring in alpine grasslands or under scrubs and forests, at altitudes of 2700–3700 m. The soil in areas where *Cypripedium* grows contains lots of nutrients and can store abundant water. However, *Paphiopedilum* are evergreen and usually occur in the understorey in karst limestone areas below at altitudes 2000 m (Cribb 1997, 1998). In karst areas, the mantle soil layer is very shallow, with a scarcity of soil, nutrients and water. With respect to leaf traits, *Cypripedium* has broad, plicate and deciduous leaves, whereas *Paphiopedilum* are sclerophylls and has coriaceous, green or tessellated and evergreen leaves (Atwood 1979, Cox et al. 1997, Cribb 1997, 1998). The genus *Paphiopedilum* has attracted considerable attention from physiologists because of the lack of chloroplasts in its guard cells. Williams et al. (1983) suggested that the comparative studies of *Paphiopedilum* and the related genera would be useful for further investigation of functional leaf traits in Orchidaceae. However, little has so far been done in this regard, especially in terms of the ecophysiological significances of the leaf traits in *Cypripedium* and *Paphiopedilum* and their convergent and divergent evolution.

In this study, we investigate the photosynthesis, leaf nitrogen content and allocation, SLA, chlorophyll content and leaf construction cost (CC) of three *Cypripedium* species (*C. lentiginosum*, *C. tibeticum* and *C. flavum*) and three *Paphiopedilum* species (*P. micranthum*, *P. dianthum* and *P. malipoense*) to understand the ecophysiological significances of leaf traits in *Cypripedium* and *Paphiopedilum* and their convergent and divergent evolution. Among the three *Cypripedium* species, *C. lentiginosum* is a sympatric species of *Paphiopedilum* (Cribb and Chen 1999). We hypothesize that the variation in the leaf traits between *Paphiopedilum* and *Cypripedium* is shaped by both phylogeny and environment.

Materials and methods

Study sites and plant species

This study was performed at Shangri-la (altitude 3200 m, 27°46′N, 99°38′E) and Xichou (altitude 1526 m, 23°38′N, 104°78′E) in Yunnan province, southwestern China in July 2008. In Shangri-la, the annual mean temperature and precipitation are 5.4°C and 624.8 mm, respectively, and the relative humidity is about 60%. Xichou has a mean annual temperature of 15.9°C, a mean annual rainfall of 1294 mm and the relative humidity is about 70%.

Three species of *Cypripedium* (*C. lentiginosum*, *C. tibeticum* and *C. flavum*) and three species of *Paphiopedilum* (*P. micranthum*, *P. dianthum* and *P. malipoense*) were chosen for investigation. The ecological characteristics of the species are shown in Table 1 (Cribb 1997, 1998). Shangri-la is the natural distribution area of *C. tibeticum* and *C. flavum* in China; Xichou is the natural distribution area of *C. lentiginosum*, *P. micranthum*, *P. dianthum* and *P. malipoense* in China. The seedlings and plants were cultivated on the sites nearby their natural distribution areas to obtain natural growth performance respectively. Forty to fifty uniform seedlings (dormant) of *C. flavum* and *C. tibeticum* were collected from Napahai (altitude 3360 m, 27°55′N,

Table 1. Ecological characteristics and biological traits of six considered species.

	Cypripedium flavum	*Cypripedium tibeticum*	*Cypripedium lentiginosum*	*Paphiopedilum malipoense*	*Paphiopedilum micranthum*	*Paphiopedilum dianthum*
Distribution	Southwest China	Sikkim, Bhutan, China	Yunnan in China, Vietnam	Southwest China, Indo-China	Southwest China	Southwest China
Altitude (m)	2700–3700	2300–4200	1700–2200	760–1300	1000–1500	800–2250
Leaves	Ovate elliptic to lanceolate green leaves	Elliptic-ovate, obtuse to acute green leaves	Glossy, dark green leaves spotted with black or purplish margin	Blunt and chequered leaves, spotted purple beneath	Blunt and chequered leaves, spotted purple beneath	Dark green leaves
LLS	About 5 months	About 5 months	About 5 months	Over 3 years	Over 3 years	Over 3 years
Habitat	Grassland, forest or shrub	Grassland, forest or shrub	Rocky, karst limestone hills	Karst limestone hills	Karst limestone hills	Lithophytically in cracks of rocks
Natural habitat in China	Shangri-la in Yunnan province	Shangri-la in Yunnan province	Xichou in Yunnan province	Xichou in Yunnan province	Xichou in Yunnan province	Xichou in Yunnan province

99°33′E) in Shangri-la, and 100–120 uniform plants of *C. lentiginosum*, *P. micranthum*, *P. dianthum* and *P. malipoense* were collected from Xichou (altitude 1560 m, 23°27′N, 104°40′E) in 2007. The seedlings of *C. flavum* and *C. tibeticum* were transplanted into plastic cases containing alpine soil from their natural habitats and cultivated in a nursery at Shangri-la. The seedlings (dormant) of *C. lentiginosum* and the plants of *P. micranthum*, *P. dianthum* and *P. malipoense* were transplanted into plastic cases containing mixed substrates (brick, bark and muck) from natural habitats and were cultivated in a nursery at Xichou. The seedlings and plants were shaded with nylon netting to give about 40% full sunlight and watered regularly during dry periods.

Measurements of leaf photosynthesis

All measurements of leaf gas exchange were made on fully matured and healthy leaves on a clear day with a portable photosynthesis analysis system with a 6400-40 fluorescence chamber (LI-6400-40, Li-Cor, Lincoln, NE) in July 2008. Before the measurements were made, the plants were dark-adapted overnight for more than 10 h. After the minimal fluorescence (F_o) was determined with a weak modulated light, a 0.8 s saturating light of 6000 μmol m^{-2} s^{-1} was applied to the dark-adapted leaf to determine the maximal fluorescence (F_m). To ensure that the results could be compared, the two genera were analyzed under their optimal growth conditions. Therefore, the conditions of measurement of leaf photosynthesis were different between the two genera. The leaves of *C. tibeticum* and *C. flavum* were then illuminated with an actinic light of 800 μmol m^{-2} s^{-1} for about 20 min, and the leaves of *C. lentiginosum*, *P. micranthum*, *P. dianthum* and *P. malipoense* were illuminated with 300 μmol m^{-2} s^{-1} light for about 30 min at ambient CO_2 concentration. The ratio of blue light to red light was 1/9. Preliminary measurements showed that these settings for light intensity were above the photosynthetic light saturation point, but below the light intensity at which photoinhibition occurred. The measurements of *C. tibeticum* and *C. flavum* were made with the leaf temperature maintained at 20°C and the relative humidity maintained at about 50%; the measurements of *C. lentiginosum*, *P. micranthum*, *P. dianthum* and *P. malipoense* were made with the leaf temperature maintained at 25°C and the relative humidity maintained at about 65%.

When the photosynthetic light response curves were measured, the CO_2 concentration within the leaf chamber was maintained at 370 μmol mol^{-1} and the light intensity was gradually reduced from 2000 to 0 μmol m^{-2} s^{-1}, producing 10 sequential light levels. During these measurements, the waiting time was 2–4 min and the P_n and chlorophyll fluorescence were recorded when a steady state was reached.

The photosynthetic CO_2 response curves were determined with a range of CO_2 concentrations of 0–2000 μmol mol^{-1} at a light intensity of 800 μmol m^{-2} s^{-1} for *C. tibeticum* and *C. flavum* and a light intensity of 300 μmol m^{-2} s^{-1} for *C. lentiginosum*, *P. micranthum*, *P. dianthum* and *P. malipoense*. After the initial measurements were made at 2000 μmol mol^{-1} CO_2, the CO_2 concentration was reduced in steps and photosynthesis was recorded after a 2–4 min acclimation period at each concentration.

Calculation of photosynthetic parameters

The light response curves were fitted to a non-rectangular hyperbola. The light-saturated photosynthetic rate (P_{max}) was determined using Photosyn Assistant v.1.1 (Dundee Scientific, Dundee, Scotland, UK), following the method of Prioul and Chartier (1977).

The biochemical capacity for photosynthesis can be estimated using photosynthesis plotted against the intercellular CO_2 concentration (C_i). Using the P_n-C_i response curves, the light-saturated rate of maximum electron transport (J_{max}) and the maximum rate of carboxylation by ribulose-1,5-bisphosphate carboxylase/oxygenase (V_{cmax}) were calculated with the Photosyn Assistant software based on the photosynthetic model of von Caemmerer and Farquhar (1981).

The mesophyll conductance (g_m) was calculated using the method described by Harley et al. (1992)

$$g_m = \frac{P_n}{C_i - \left(\Gamma^*\left[J + 8(P_n + R_d)\right]/J - 4(P_n + R_d)\right)} \quad (1)$$

where R_d (daily respiration rate) was calculated from the CO_2 response curves, J was estimated from the chlorophyll fluorescence on the same leaf and Γ^* is 33.06 μmol mol^{-1} at 20°C and 37.43 μmol mol^{-1} at 25°C, according to Bernacchi et al. (2002). The values for g_m were calculated from the measurements of the net assimilation rate at $C_i = 100–300$ μmol mol^{-1} and the average values for g_m were determined for each leaf.

The relative stomatal limitation (RSL) of photosynthesis, an estimate of the proportion of the reduction in photosynthesis attributable to CO_2 diffusion between the atmosphere and the site of carboxylation, was calculated from the CO_2 response curves by the method of Farquhar and Sharkey (1982) as $RSL = (1 - P_n/P_{no}) \times 100$, where P_n is the photosynthetic rate at the ambient CO_2 concentration (370 μmol mol^{-1}) and P_{no} the photosynthetic rate at $C_i = 370$ μmol mol^{-1}.

Analysis of leaf structure and biochemistry

Leaf samples were taken from the same leaves used for the gas exchange measurements. The leaf areas were measured with a leaf area meter (LI-3000A, Li-Cor). The leaf dry mass was determined after the leaves had been dried for 72 h at 70°C, and SLA (m^2 g^{-1}) was calculated as the leaf area per unit mass. The total leaf nitrogen (N_a) and carbon contents were then measured using an N and C analyzer (Elementar Analysensysteme GmbH, Vario EL III, Hanau, Germany). Chlorophylls were extracted by the direct immersion of 10 intact discs (0.33 cm^2 disc^{-1}) into N, N-dimethylformamide. The absorbance of the extracts was measured at 664.5 and 647 nm with a UV-2550 spectrophotometer (Shimadzu, Tokyo, Japan). Leaf chlorophyll *a* and *b* contents were determined using the formula of Inskeep and Bloom (1985).

The proportion of apparent nitrogen investment in Rubisco (P_C), bioenergetics (P_B) and thylakoid light harvesting (P_L) were calculated from the estimated values of V_{cmax} and J_{max}, and the measured leaf N and chlorophyll contents were calculated according to Niinemets and Tenhunen (1997) as follows:

$$P_C = \frac{V_{cmax}}{(6.25 \times V_{cr} \times N_a)} \quad (2)$$

$$P_B = \frac{J_{max}}{(8.06 \times J_{mc} \times N_a)} \quad (3)$$

$$P_L = \frac{CC}{(N_m \times C_B)} \quad (4)$$

where V_{cr} [μmol CO_2 (g Rubisco)$^{-1}$ s^{-1}] is the maximum rate of RuBP carboxylation per Rubisco protein, J_{mc} [mol electron (mol cyt *f*)$^{-1}$ s^{-1}] is the capacity for photosynthetic electron transport per cytochrome *f*, C_B the ratio of chlorophyll binding to N invested in light harvesting and is constant (Niinemets et al. 1998); the coefficient 6.25 [g Rubisco (g nitrogen in Rubisco)$^{-1}$] converts nitrogen content to protein content and 8.06 [μmol cyt *f* (g nitrogen in bioenergetics)$^{-1}$] is used based on the assumption of constant 1:1:1.2 molar ratio for cyt *f*:ferredoxin NADP reductase:coupling factor. From the temperature dependency of V_{cr} and J_{mc} described by Niinemets and Tenhunen (1997), the values of V_{cr} and J_{mc} at 20°C were equal to 12.6 [μmol CO_2 (g Rubisco)$^{-1}$ s^{-1}] and 131.9 [mol electron (mol cyt *f*)$^{-1}$ s^{-1}], respectively, and at 25°C were equal to 20.78 [μmol CO_2 (g Rubisco)$^{-1}$ s^{-1}] and 155.65 [mol electron (mol cyt *f*)$^{-1}$ s^{-1}], respectively. N_a and N_m are leaf nitrogen content per area (g m^{-2}) and per dry mass (%), respectively. In addition, photosynthetic nitrogen utilization efficiency (PNUE, μmol g^{-1} s^{-1}) was calculated as the ratio of P_{max} to N_a.

The CC of the leaf tissue (grams of glucose necessary to synthesize 1 g of leaf tissue) was calculated according to the equation developed by Vertregt and Penning de Vries (1987).

Statistical analysis

Statistical analysis was conducted with SPSS 13.0 for Windows (SPSS Inc., Chicago, IL) and all graphs were produced in the software SIGMAPLOT 10.0 for Windows (Systat Software Inc., Richmond, CA). Differences among the physiological variables were determined using ANOVA and LSD tests for multiple comparisons. The relationships between the photosynthetic parameters and the leaf traits were assessed using Pearson's regression analysis.

Results

There were significant differences in all the physiological variables measured in this study among the six species examined, based on the results of a one-way ANOVA (Table 2).

For *C. lentiginosum*, the values of P_{max}, g_m, V_{cmax} and J_{max} were significantly lower and the values of RSL were significantly higher than the values for *C. tibeticum* and *C. flavum* (Tables 2 and 3). The P_{max}, g_m and RSL values obtained from *C. lentiginosum* were very similar to those of *Paphiopedilum* (Tables 2 and 3). The values of mass-based light-saturated photosynthetic rate (P_{max-M}) and g_S were similar in the three *Cypripedium* species, being significantly higher than those in *Paphiopedilum* (Tables 2 and 3).

The P_B and P_C values of *Cypripedium* were significantly higher than did *Paphiopedilum* (Tables 2 and 3). *Cypripedium lentiginosum* and *Paphiopedilum* had significantly higher P_L values than did *C. tibeticum* and *C. flavum* (Table 3). *Cypripedium* had significantly higher PNUE values than did *Paphiopedilum*, which contributed to its higher $P_{max\text{-}M}$ values (Fig. 4, Table 3).

Compared with *C. tibeticum* and *C. flavum*, *C. lentiginosum* and *Paphiopedilum* had significantly lower Chl *a/b* (Table 3). *Cypripedium lentiginosum* had significantly higher SLA values and significantly lower CC values than did the others. *Paphiopedilum* had significantly lower SLA but higher CC and higher leaf C/N values than did *Cypripedium* (Table 3). In addition, *P. malipoense* had similar SLA and CC values to those obtained from *Cypripedium* (Table 2). N_a was the lowest in *C. lentiginosum* (Table 3).

P_{max} increased significantly with V_{cmax}, J_{max}, N_a (Fig. 1) and g_m (Fig. 2), but decreased significantly with RSL (Fig. 2). The higher P_C and P_B of *Cypripedium* led to higher V_{cmax}, J_{max} and PNUE than those observed in *Paphiopedilum*, which in turn contributed to higher P_{max} and $P_{max\text{-}M}$ (Figs 1, 3 and 4). As SLA increased, P_{max}, V_{cmax}, J_{max}, N_a and CC decreased significantly, whereas P_L increased sharply (Fig. 5).

Table 3. Differences among *Cypripedium* (*C. flavum* and *C. tibeticum*), *C. lentiginosum* and *Paphiopedilum* (*P. malipoense, P. micranthum* and *P. dianthum*) according to a one-way ANOVA analysis. Mean species values ± SD are given (*Cypripedium*, n = 10; *C. lentiginosum*, n = 5; *Paphiopedilum*, n = 15). Different letters in the same row indicate significant differences among the species (LSD test).

	Cypripedium	*C. lentiginosum*	*Paphiopedilum*
P_{max}	10.36 ± 0.57 a	3.63 ± 0.36 b	2.79 ± 0.24 b
$P_{max\text{-}M}$	0.19 ± 0.00 a	0.13 ± 0.02 b	0.03 ± 0.00 c
V_{cmax}	25.0 ± 1.7 a	11.4 ± 1.1 b	14.1 ± 1.3 b
J_{max}	97.5 ± 3.0 a	39.0 ± 3.5 b	40.2 ± 4.1 b
g_s	0.17 ± 0.02 a	0.18 ± 0.01 a	0.04 ± 0.00 b
g_m	0.13 ± 0.03 a	0.01 ± 0.00 b	0.02 ± 0.00 b
RSL	21.4 ± 1.2 b	40.8 ± 1.7 a	42.2 ± 1.0 a
N_a	1.42 ± 0.08 a	0.58 ± 0.03 c	1.00 ± 0.10 b
P_B	0.07 ± 0.00 a	0.06 ± 0.00 a	0.03 ± 0.00 b
P_C	0.23 ± 0.02 a	0.16 ± 0.01 a	0.12 ± 0.01 b
P_L	0.15 ± 0.01 a	0.32 ± 0.01 b	0.42 ± 0.04 b
PNUE	7.47 ± 0.55 a	6.51 ± 1.02 a	2.93 ± 0.22 b
SLA	0.020 ± 0.000 b	0.0035 ± 0.000 a	0.010 ± 0.000 c
CC	61.6 ± 1.9 b	31.3 ± 2.4 c	115.7 ± 13.1 a
C/N	16.7 ± 0.6 b	21.9 ± 0.7 b	43.2 ± 2.2 a
Chl *a/b*	3.08 ± 0.07 a	2.38 ± 0.04 b	2.27 ± 0.02 b

Discussion

This study suggests the significant difference in leaf functional traits between *Paphiopedilum* and *Cypripedium*. Consistent with our hypothesis, the differences in leaf

Table 2. Leaf traits of *Cypripedium* and *Paphiopedilum.* Mean species values ± SD are given (n = 5). Different letters in the same row indicate significant differences among the species (LSD test).

	Cypripedium flavum	*Cypripedium tibeticum*	*Cypripedium lentiginosum*	*Paphiopedilum malipoense*	*Paphiopedilum micranthum*	*Paphiopedilum dianthum*
P_{max}	11.30 ± 0.85 a	9.42 ± 0.52 b	3.63 ± 0.36 c	2.17 ± 0.16 c	3.34 ± 0.52 c	2.85 ± 0.39 c
$P_{max\text{-}M}$	0.21 ± 0.01 a	0.18 ± 0.01 a	0.13 ± 0.02 b	0.04 ± 0.01 c	0.02 ± 0.00 c	0.03 ± 0.00 c
V_{cmax}	28.4 ± 1.5 a	21.6 ± 0.5 b	11.4 ± 1.1 cd	9.3 ± 0.8 d	15.2 ± 2.0 c	17.9 ± 1.8 c
J_{max}	97.4 ± 5.9 a	97.6 ± 2.5 a	39.0 ± 3.5 c	23.3 ± 2.8 d	42.4 ± 4.5 c	54.8 ± 4.2 b
g_s	0.21 ± 0.03 a	0.13 ± 0.01 b	0.18 ± 0.01 a	0.03 ± 0.00 c	0.05 ± 0.00 c	0.04 ± 0.01 c
g_m	0.18 ± 0.06 a	0.08 ± 0.01 b	0.01 ± 0.00 c	0.02 ± 0.01 c	0.01 ± 0.00 c	0.01 ± 0.00 c
RSL	19.5 ± 1.4 b	23.3 ± 1.6 b	40.8 ± 1.7 a	43.4 ± 1.3 a	41.0 ± 1.3 a	42.3 ± 2.5 a
N_a	1.33 ± 0.11 a	1.51 ± 0.10 a	0.58 ± 0.03 bc	0.67 ± 0.05 c	1.40 ± 0.17 a	0.93 ± 0.07 b
P_B	0.07 ± 0.00 a	0.06 ± 0.00 ab	0.06 ± 0.00 ab	0.03 ± 0.00 c	0.02 ± 0.00 c	0.05 ± 0.01 b
P_C	0.27 ± 0.01 a	0.18 ± 0.01 b	0.16 ± 0.01 b	0.11 ± 0.01 c	0.08 ± 0.01 c	0.15 ± 0.02 b
P_L	0.13 ± 0.00 c	0.16 ± 0.01 c	0.32 ± 0.01 b	0.62 ± 0.05 a	0.32 ± 0.02 b	0.30 ± 0.03 b
PNUE	8.59 ± 0.61 a	6.35 ± 0.58 b	6.51 ± 1.02 b	3.32 ± 0.43 c	2.35 ± 0.12 c	2.99 ± 0.44 c
SLA	0.018 ± 0.001 b	0.019 ± 0.001 b	0.035 ± 0.003 a	0.016 ± 0.000 b	0.007 ± 0.001 d	0.011 ± 0.001 c
CC	62.0 ± 2.6 b	61.2 ± 3.1 b	31.3 ± 2.4 c	57.1 ± 4.4 b	162.9 ± 1.48 a	127.2 ± 11.3 a
C/N	18.0 ± 0.8 c	15.3 ± 0.6 c	21.9 ± 0.7 bc	30.5 ± 2.4 b	46.6 ± 1.5 a	49.2 ± 3.3 a
Chl *a/b*	2.90 ± 0.06 b	3.26 ± 0.03 a	2.38 ± 0.04 c	2.25 ± 0.03 c	2.25 ± 0.03 c	2.31 ± 0.07 c

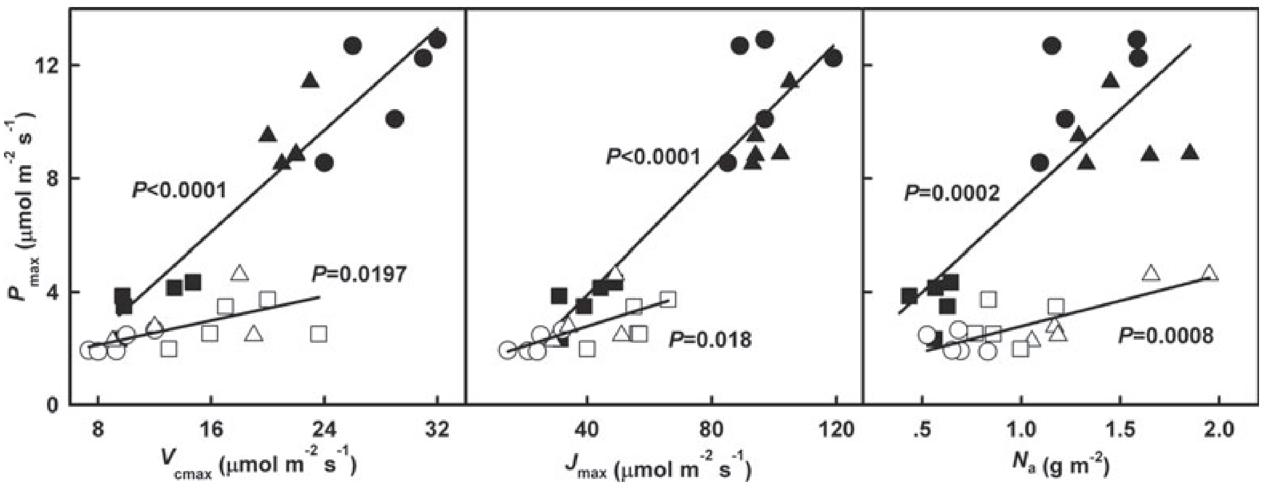

Fig. 1. The relationship between P_{max}, V_{cmax}, J_{max} and N_a of three *Paphiopedilum* and three *Cypripedium* species. ○, *P. malipoense*; △, *P. micranthum*; □, *P. dianthum*; ●, *C. flavum*; ▲, *C. tibeticum*; ■, *C. lentiginosum*; n = 5 for each species.

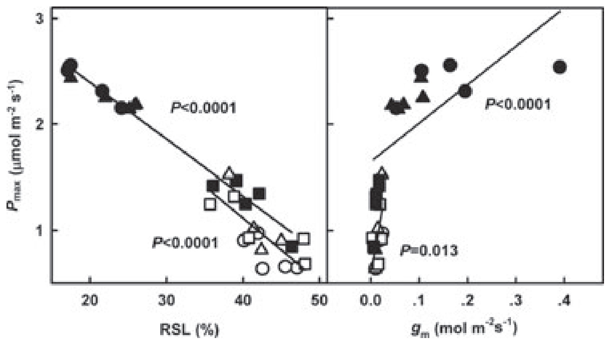

Fig. 2. The relationship between P_{max}, RSL and g_m of three *Paphiopedilum* and three *Cypripedium* species. The data of RSL and g_m have been transformed using the natural logarithm function. ○, *P. malipoense*; △, *P. micranthum*; □, *P. dianthum*; ●, *C. flavum*; ▲, *C. tibeticum*; ■, *C. lentiginosum*; n = 5 for each species.

traits between the two genera were regulated by phylogeny and environment and *C. lentiginosum* showed intermediate leaf traits between the two genera.

Compared with *Paphiopedilum*, *C. tibeticum* and *C. flavum* had a significantly greater photosynthetic capacity (P_{max}), whereas *C. lentiginosum* had a similar P_{max} to *Paphiopedilum*. The differences in P_{max} among species reflect differences in leaf physiology, anatomy and biochemistry (Durand and Goldstein 2001). The changes of P_{max} were linked to the changes of V_{cmax}, J_{max}, g_m and RSL (Figs 1 and 2). *Cypripedium lentiginosum* had the same g_s as *C. tibeticum* and *C. flavum*, whereas it had the same V_{cmax}, J_{max}, g_m and RSL as *Paphiopedilum*. The significantly lower V_{cmax}, J_{max}, g_s and g_m and the significantly higher RSL values of *C. lentiginosum* and *Paphiopedilum* than *C. flavum* and *C. tibeticum* resulted in their significantly lower P_{max} (Figs 1 and 2, Table 2). The remarkably low g_s and P_{max} of *Paphiopedilum* were also partly caused by the achlorophyllous stomata, which fail to respond to red light (Williams et al. 1983, Zeiger et al. 1985, 2002; Table 2). Guard-cell chloroplasts can contribute to stomatal opening and are conserved in all species except for the genus *Paphiopedilum* (Zeiger et al. 2002), so *C. lentiginosum* had higher g_s values than did the three species of *Paphiopedilum* (Table 3).

There is a strong positive correlation between P_{max} and leaf nitrogen content (Poorter and Evans 1998). We confirmed this result for the six species (Fig. 1). Furthermore, the efficient use of leaf nitrogen in the photosynthetic apparatus also affects plant photosynthesis (Niinemets and Tenhunen 1997). *Cypripedium* allocated more leaf nitrogen to bioenergetics and Rubisco of its photosynthetic machinery (P_B and P_C) (Tables 2 and 3) than did *Paphiopedilum*, which increased its P_{max} by increasing its biochemical capacity of photosynthesis (V_{cmax} and J_{max}; Figs 1 and 3, Table 1) and also contributed to the higher $P_{max\text{-}M}$ through a higher PNUE (Figs 3 and 4, Table 2). Therefore, the $P_{max\text{-}M}$ of *C. lentiginosum* was the same as that of the other *Cypripedium* species and significantly higher than that of *Paphiopedilum*. For *Paphiopedilum*, the long-lived leaves need to invest more nitrogen in their cell walls to increase leaf toughness and render the leaves more tolerant of physical hazards, at the expense of PNUE (Takashima et al. 2004, Wright et al. 2004). This may imply that the low PNUE of *Paphiopedilum* could be an adaptation to an unfertile habitat. However, *C. lentiginosum* retained the

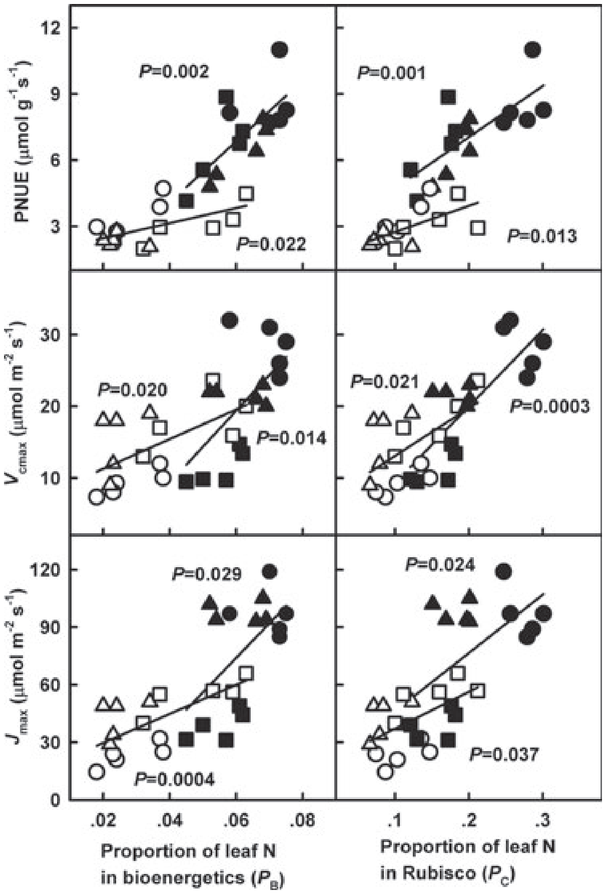

Fig. 3. PNUE, V_{cmax} and J_{max} as functions of the P_C and to P_B of three *Paphiopedilum* and three *Cypripedium* species. ○, *P. malipoense*; △, *P. micranthum*; □, *P. dianthum*; ●, *C. flavum*; ▲, *C. tibeticum*; ■, *C. lentiginosum*; n = 5 for each species.

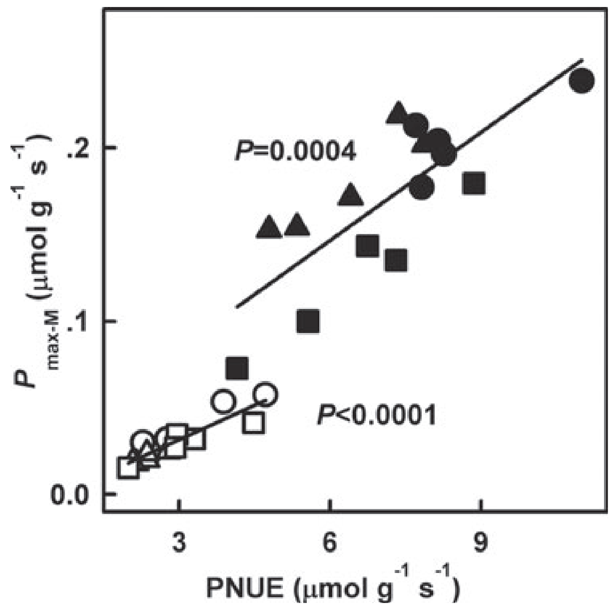

Fig. 4. The relationship between $P_{max\text{-}M}$ and PNUE of three *Paphiopedilum* and three *Cypripedium* species. ○, *P. malipoense*; △, *P. micranthum*; □, *P. dianthum*; ●, *C. flavum*; ▲, *C. tibeticum*; ■, *C. lentiginosum*; n = 5 for each species.

phylogenetic characteristics of *Cypripedium* in terms of efficient use of leaf nitrogen. Moreover, *C. lentiginosum* and *Paphiopedilum* had higher P_L values than did *C. tibeticum* and *C. flavum* (Tables 2 and 3), indicating the efficient use of a limited light resource of its habitat. Furthermore, a lower Chl *a*/*b* ratio of *C. lentiginosum* and *Paphiopedilum* means more light-harvesting complexes in the photosystem II (PSII) and fewer PSII reaction centers (Hikosaka and Terashima 1996), as required by plants adapted to low light habitats. The higher P_L and lower Chl *a*/*b* ratio of *Paphiopedilum* might also be adaptation to their achlorophyllous stomata, which affect the stomatal response to light quality (Lawson 2009, Williams et al. 1983).

As one of the key leaf traits, SLA is related to resource capture and utilization (Grime et al. 1997, Vendramini et al. 2002, Westoby 1998). *Paphiopedilum* had significantly lower SLA and higher CC values than did *Cypripedium* (Table 3), whereas similar SLA and CC were found in *P. malipoense* and *Cypripedium* (Table 2). The low SLA is associated with lower g_m and a lower P_n (Sims and Pearcy 1992) because of the long and narrow intercellular spaces in these leaves (Evans and von Caemmerer 1996, Parkhurst 1994). The thick leaves with low SLA allocate little leaf nitrogen to the photosynthetic machinery and have low PNUE and therefore lower P_{max} (Mediavilla et al. 2001). Our results for *Paphiopedilum* are consistent with these studies because *Paphiopedilum* have sclerophyllous leaves. The low g_m but high SLA of *C. lentiginosum* are linked to its typical mesomorphic leaves which also result in the high RSL and low N_a. Sclerophyllous leaves and mesomorphic leaves are able to store more water and prevent water transpiration, which are required for plants living in karst area with low soil water content. *Paphiopedilum* may also benefit from low SLA and high CC, as a leaf with low SLA and high CC has a longer longevity and extend the life-time carbon gain per unit leaf mass (Harrington et al. 1989, Lambers and Poorter 1992, Poorter and Villar 1997, Reich et al. 1997). However, the significantly higher leaf C/N of *Paphiopedilum* relative to that of *Cypripedium* indicates increased defense against damage for a longer LLS, reflected in smaller amounts of structural carbon compounds, such as cellulose and lignin (Herms and Mattson 1992), which have an energetic cost. These results indicate the long duration of the revenue stream from the high investment of CC (Cunningham et al. 1999,

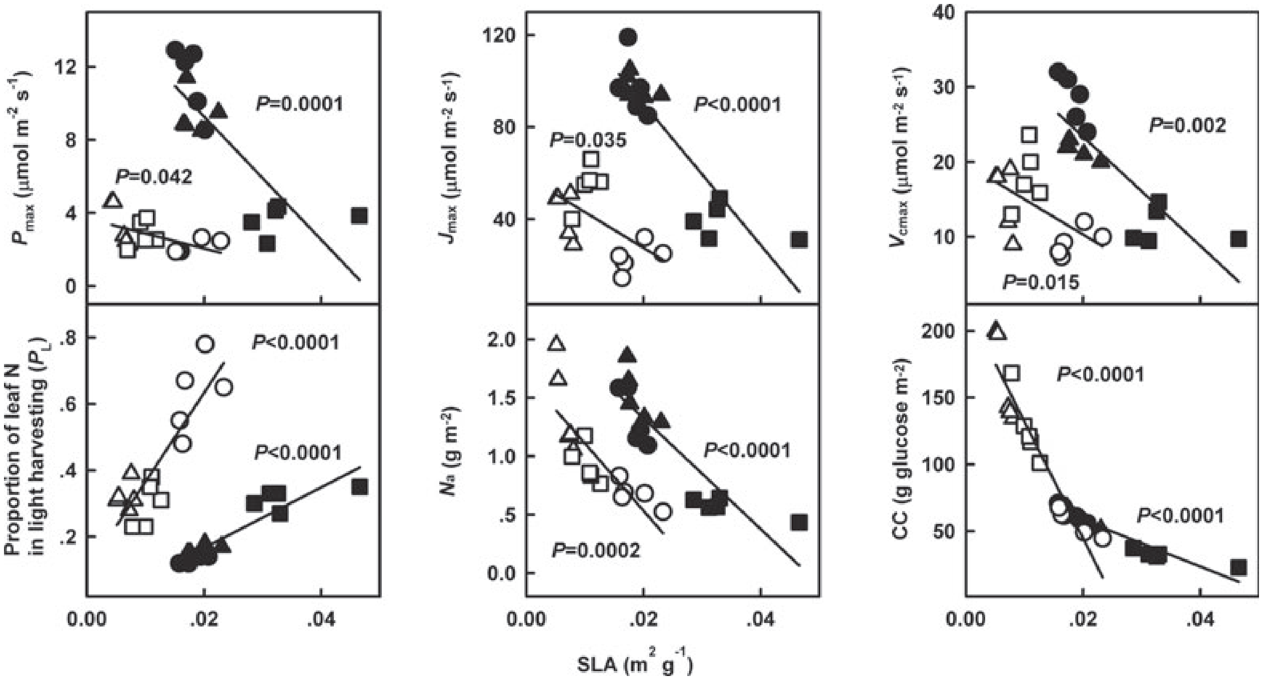

Fig. 5. The correlations of SLA, P_{max}, J_{max}, V_{cmax}, P_{L}, N_{a} and CC of three *Paphiopedilum* and three *Cypripedium* species. ○, *P. malipoense*; △, *P. micranthum*; □, *P. dianthum*; ●, *C. flavum*; ▲, *C. tibeticum*; ■, *C. lentiginosum*; n = 5 for each species.

Niinemets 2001, Wright et al. 2002), which reflects low resource requirements in a nutrient-poor environment. For *Cypripedium*, the high SLA, low CC and low leaf C/N reflect adaptations to a short growth season and abundant soil humus that is nutrient-rich and able to store water.

The common leaf traits in the *Paphiopedilum* species (long LLS, low P_{max}, g_{s}, g_{m}, SLA and Chl *a/b*; and high P_{L}, leaf C/N and CC; and achlorophyllous stomata) would be beneficial for resource acquisition in the nutrient-poor soils, low soil water content and low light environment in karst limestone shrub areas. Conversely, *Cypripedium* has a significantly higher P_{max}, $P_{\text{max-M}}$ and SLA and a lower C/N and leaf CC, which indicate highly efficient resource use and high growth rates (Nagel and Griffin 2001). Being a perennial geophyte with 'adult dormancy', the common leaf traits of *Cypripedium* not only indicate adaptation to the short growth season, abundant soil nutrients and water but also facilitate nutrient storage in rhizome, which would be beneficial for their survival and reproduction in alpine habitat.

The leaf traits measured in this study indicate convergent and divergent evolution between *Cypripedium* and *Paphiopedilum*. As a sympatric species of *Paphiopedilum*, *C. lentiginosum* has not only retained the phylogenetic characteristics of *Cypripedium* in $P_{\text{max-M}}$, P_{B}, P_{C}, PNUE and winter dormancy, but is also very close to *Paphiopedilum* in terms of P_{L}, RSL, g_{m}, Chl *a/b* and their similar leaf morphologies. The similar leaf traits observed in *C. lentiginosum* and *Cypripedium* reflect highly efficient resource use which is an adaptation to a short growth season, while the similar leaf traits in *C. lentiginosum* and *Paphiopedilum* reflect an adaptation to similar habitats with low soil water content and limited light resource. Contrary to the expectation, *P. malipoense* did not display significant transitional leaf traits between those of *Cypripedium* and *Paphiopedilum* in our study even though the SLA and CC of *P. malipoense* and *Cypripedium* are similar.

Overall, the results suggest that the leaf traits variations between *Cypripedium* and *Paphiopedilum* are related to contrasting habitats and that the difference in leaf traits between *Paphiopedilum* and *Cypripedium* are shaped by both phylogeny and environment. This comparative study should provide useful data for the further investigation of the adaptation and evolution of the subfamily Cypripedioideae which may reflect environmental or selective pressures on the Cypripedioideae.

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