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Gas exchanges of three co-occurring species of *Cypripedium* in a scrubland in the Hengduan Mountains

S.B. ZHANG, H. HU*, K. XU, and Z.R. LI

Kunming Institute of Botany, the Chinese Academy of Sciences, Kunming 650204, China

Abstract

Gas exchanges and related leaf traits of three co-occurring species of genus *Cypripedium* (*C. yunnanense* Franch., *C. guttatum* SW., and *C. flavum* P.F. Hunt *et* Summerch.) were investigated in a scrubland at 3 460 m a.s.l. in the Hengduan Mountains. The considered species had similar photosynthetic responses to photosynthetic photon flux density (PPFD) and air temperature. The photosynthetic capacity (P_{max}), carboxylation efficiency (CE), apparent quantum efficiency (AQE), PPFD-saturated rate of electron transport (J_{max}), respiration rate (R_D), and leaf nitrogen content per unit area (LNC) of *C. guttatum* were higher than those of *C. yunnanense* and *C. flavum*. The highest P_{max} of *C. guttatum* was related to the highest LNC and the lowest ratio of intercellular CO_2 concentration to atmospheric CO_2 concentration (C_i/C_a). However, no significant differences in stomatal conductance (g_s) and relative stomatal limitations (RSL) were observed among the three species. Hence biochemical limitation had a dominant role in P_{max} differences among the considered species.

Additional key words: alpine environment; apparent quantum efficiency; carboxylation efficiency; CO_2 concentration; conservation; leaf nitrogen content; respiration rate; stomatal conductance.

Introduction

The genus *Cypripedium* includes 48 species having different sizes and colours. This genus has holarctic distribution, ranging from the Arctic Circle in Alaska and Scandinavia, to southern Honduras in Central America and the Himalayas (Cribb 1997). In the Hengduan Mountains of Southwestern China, 14 species of *Cypripedium* are found above 2 700 m a.s.l. (Lang 1990). In recent years, habitat destruction and illicit collection has determined a considerable decline of *Cypripedium* population (Cribb and Sandison 1998). Species of the genus *Cypripedium* are slowly growing plants that sometimes have limited reproductive capacities and often have very specific and limited conditions of flowering, seed production, germination, and offspring production (Kull 1998, Sugiura *et al.* 2001). Therefore, large-scale cultivation under artificial conditions is needed for the conservation of these species. Seedling micro-propagation has been proved successfully for several species of the genus *Cypripedium* (Shimura and Koda 2004), but artificial cultivation is still difficult (Cribb and Sandison 1998).

Successfully cultivating and conserving wild species requires information about growth condition (Cui *et al.* 2004, Aleric and Kirkman 2005). Unfortunately, little is known about the physiological ecology of *Cypripedium* in the native habitats (Kull 1999, Zhang *et al.* 2005). The capacity of plant species to grow in environments different from those of their original habitats depends on species acclimation capability (Pearcy 1977). Gas exchange measurements in the field can give information on capability of plant species acclimation to different environmental factors (Vats *et al.* 2002, Still *et al.* 2003).

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*Corresponding author; fax: +86-0871-522 3005, e-mail: huhong@mail.kib.ac.cn

Abbreviations: AQE = apparent quantum efficiency; Chl = chlorophyll; CE = carboxylation efficiency; CI = compensation irradiance for photosynthesis; C_i/C_a = the ratio of intercellular to the ambient CO_2 concentration; E = transpiration rate; g_s = stomatal conductance; J_{max} = PPFD saturated rate of electron transport; LNC = leaf nitrogen content per unit leaf area; P_{day} = daily mean P_N; P_{max} = light-saturated P_N; P_N = net photosynthetic rate; PPFD = photosynthetic photon flow density; R_D = respiration rate; RSL = relative stomatal limitation; SI = saturation irradiance for photosynthesis; T_l = leaf temperature; T_{opt} = optimum temperature for photosynthesis; TPU = rate of triose phosphate utilization; V_{cmax} = maximum RuBP saturated rate of carboxylation; WUE = water use efficiency.

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Three co-occurring species of the genus *Cypripedium* (*C. yunnanense, C. guttatum,* and *C. flavum*) with different distribution range were studied in a scrubland. Differences in gas exchange and physiological and biochemical leaf traits among the considered species to the alpine environment were analysed. The results may be used for cultivation and conservation management projects of these species.

Materials and methods

Experimental site and plant species: Field surveys were conducted in the Shangrila County of the Hengduan Mountains, southwestern China. The climate is characterized by a mean annual air temperature of 5.4 °C and a total annual rainfall of 625 mm, much of which occurring from May to October; dry season lasts from November to April (data by the Meteorological Station of Shangrila County in 1958−2002).

Seedlings (10 seedlings per species) of three cooccurring species (*C. yunnanense*, *C. guttatum*, and *C. flavum*) of the genus *Cypripedium* were selected for investigation in a scrubland at 3 460 m a.s.l. (99˚50.11´E, 27˚47.76´N). *Salix taiwanalpina, Betula utilis*, and *Rhododendron vernicosum* were the dominant shrubs of the study site, and *Nomocharis forrestii* Balf., *Stellera chamaejasme* L., *Deyeuxia scabrescens* Munro*,* and *Polygonum macrophylum* co-occurred.

The ecological characteristics of the considered species are shown in Table 1. The brown soil of the site was characterized by 7.67 % organic matter, 0.32 % total nitrogen, 0.17 % total phosphorus, 1.76 % potassium; pH was 7.25. The growth irradiance of study site was 40–50 % of full sunlight.

Table 1. Ecological characteristics of three considered species.

	C. yunnanense	*C. guttatum*	*C. flavum*
Distribution	Western China	Europe, North America, and Asia	Yunnan, Sichuan, Hubei, Gansu, Xizang of China
Altitude [m]	2 700–3 800	1 000–4 100	1 800–3 700
Habitat	Sparse wood or shrub	Grassland, forest, or shrub	Grassland, forest, or shrub
Plant height [cm]	20–40	15–25	30–60
Number of leaves	3−4	2	6−7
Flowering period	June–July	May–July	June–July
Fruiting period	July–October	June–October	July–October

Gas exchange was measured on the clear days on 16–22 June, 2003 (flowering period). Diurnal variations in gas exchange of three species were measured at the same day (on June 16), the measurements of photosynthetic response curves (including P_N-PPFD, P_N-C_i, and P_N-T curves) on 17, 21, and 22 June. Diurnal variations of gas exchange were measured on five fully expanded leaves per species from 08:00 to 18:00 at one-hour intervals. After steady-state of gas exchange had been achieved, net photosynthetic rate (P_N), intercellular CO_2 concentration (C_i), leaf temperature (T_l), transpiration rate (E), and photosynthetic photon flow density (PPFD) were measured using a portable infrared gas exchange open-system with a *PLC-B* (*CIRAS-1*, *PP Systems*, Herfordshire, UK) leaf chamber. Water use efficiency (WUE) was calculated as the ratio between P_N and E.

Photosynthetic responses to PPFD were measured on fully expanded leaves using a *CIRAS-1* infrared gas analyzer under constant temperature (20 °C) and CO_2 concentration (350 μmol mol^{-1}). PPFD was increased from 0 μmol m^{-2} s^{-1} to 13 subsequent irradiances and P_N was recorded. Three plants per species were measured. Data were fit by a non-rectangular hyperbola (Prioul and Chartier 1977). Using P_N-PPFD curves, light-saturated photosynthesis (P_{max}), respiration rate (R_D), apparent quantum efficiency (AQE), compensation irradiance (CI), and saturation irradiance (SI) were estimated by *Photosyn Assistant* software (version *1.1*, *Dundee Scientific*, Scotland, UK).

Following P_N-PPFD curves, P_N-C_i response curves (photosynthesis plotted against intercellular CO_2 concentration) were generated to investigate photosynthetic characteristics of three species. For each species, three recently matured leaves were placed in the leaf chamber at a CO_2 concentration of 350 μmol mol^{-1}, 20 °C, and 500 μmol m^{-2}s^{-1} PPFD. The relative air humidity within the chamber was maintained at 70 % to minimize stomatal heterogeneity. Following a 15 min acclimation, photosynthetic rate was recorded, then CO_2 concentration was reduced to 50 μmol mol^{-1} and another reading was taken. A gradual increase in ambient CO_2 to a final concentration of 2 000 μmol mol^{-1} in 13 increments occurred with a reading taken at each increment following a 3-min acclimation period. Using P_N-C_i curves, maximum carboxylation rate by ribulose-1,5-bisphosphate carboxylase/oxygenase, RuBPCO (V_{cmax}), PPFD-saturated electron transport (J_{max}), rate of triose phosphate utilization (TPU), and carboxylation efficiency (CE) were calculated by *Photosyn Assistant* software that applied the biochemical models described by Caemmerer and Farquhar (1981), Sharkey (1985), Harley and Sharkey (1991), and Harley *et al.* (1992). 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 P_N-C_i curves by the method of Farquhar and Sharkey (1982) as RSL = $(1 - P_N/P_{No})\times100$, where P_N is the photosynthetic rate at the environmental CO_2 concentration (350 µmol mol^{-1}) and P_{No} is the photosynthetic rate at C_i = 350 µmol mol^{-1}.

The dependences of P_N on temperature were measured on three fully expanded leaves for each species using an infrared gas analyzer between 08:00 and 11:00 to avoid the high temperature at midday. Measurements were made between 10 and 35 °C at 350 µmol mol^{-1} CO_2 concentration and 500 µmol m^{-2} s^{-1} PPFD. A second-order polynomial equation was used to fit the optimum temperature for photosynthesis.

Physiological and biochemical leaf traits: After gas exchange measurements, 26–30 leaves (5 seedlings) per species were harvested from the sample seedlings. N was analyzed using an N analyzer (*Leco FP-428*, Michigan, USA). Chl was extracted from 20 leaf disks (0.38 cm^2 per disk) from five seedlings per species with N,N-dimethylformamide (DMF) and Chl amounts were analyzed with a spectrophotometer (*UV-2550*, *Shimadzu*, Kyoto, Japan) and calculated using the method of Inskeep and Bloom (1985). Three repetitions were made for each species.

Statistical analysis was performed using *SPSS* version *10.0* (*SPSS*, Chicago, USA). Differences in physiological and biochemical leaf variables among the considered species were determined using the one-way ANOVA and LSD test for multiple comparisons. Correlations among the considered leaf traits were addressed using regression analysis.

Results

Diurnal patterns of gas exchange: PFPD and T_l of *C. yunnanense*, *C. guttatum*, and *C. flavum* increased rapidly after 08:00, reaching the maximum values between midday and 14:00 (Fig. 1). There were no significant differences in daily averaged PPFD (LSD = 0.356, *p*=0.701) and T_l (LSD = 0.243, *p*=0.785) among the three species.

The maximum g_s values occurred in the early morning, decreasing towards midday, and increased in the late afternoon (Fig. 1). The diurnal variation of g_s was opposite to that of T_l. There was no significant difference in g_s values among the three species (LSD = 0.915, *p*=0.402). C_i/C_a was significantly different among the three species (LSD = 140.280, *p*<0.001) (Table 2). The highest *E* was observed between midday and 14:00, but no significant difference was observed in *E* daily mean

Table 2. Photosynthetic parameters and physiological and biochemical leaf traits of *C. yunnanense, C. guttatum,* and *C. flavum.* Means ± SE. *Different letters within the same parameters* indicate means significantly different (*p*<0.05).

	Species				
	yunnanense	*guttatum*	*flavum*	LSD	*p*
P_{day} [µmol m^{-2} s^{-1}]	4.21±0.24a	5.70±0.28b	4.82±0.26a	8.054	0.000
E [mmol m^{-2} s^{-1}]	5.38±0.22a	5.59±0.24a	5.88±0.18a	1.327	0.000
WUE [µmol mmol^{-1}]	0.77±0.03a	1.03±0.04b	0.80±0.03a	17.433	0.000
P_{max} [µmol m^{-2} s^{-1}]	6.87±0.56a	8.58±0.35b	7.78±0.21ab	5.582	0.042
CE [µmol Pa^{-1}]	0.468±0.009a	0.631±0.037b	0.489±0.011a	14.656	0.005
AQE [mol(CO_2) mol^{-1}(photon)]	0.039±0.001a	0.066±0.005b	0.054±0.003c	16.188	0.004
R_D [µmol m^{-2} s^{-1}]	1.09±0.07ab	1.39±0.17a	0.84±0.07b	5.924	0.038
RSL [%]	22.27±0.14a	19.15±1.14a	19.71±2.58a	1.043	0.408
V_{cmax} [µmol m^{-2} s^{-1}]	20.30±1.56a	26.83±0.90b	24.43±1.33ab	6.541	0.031
J_{max} [µmol m^{-2} s^{-1}]	84.73±4.97a	114.40±4.85b	97.57±5.43ab	8.548	0.018
TPU [µmol m^{-2} s^{-1}]	5.53±0.17a	6.08±0.12b	5.42±0.17a	5.325	0.047
g_s [mmol m^{-2} s^{-1})	274.4±5.6a	270.3±6.21a	261.4±8.6a	0.915	0.402
C_i/C_a	0.74±0.01a	0.66±0.01b	0.71±0.01c	140.280	0.000
CI [µmol m^{-2} s^{-1}]	25.7±3.1a	20.5±2.3ab	16.0±0.9b	4.477	0.065
SI [µmol m^{-2} s^{-1}]	344.4±20.3a	340.5±22.4a	317.7±17.6a	0.511	0.624
T_{opt} [^{o}C]	18.4±0.5a	20.1±0.8a	19.2±0.4a	2.076	0.206
Chl *a* [g m^{-2}]	0.116±0.002a	0.136±0.002b	0.152±0.003c	4.716	0.000
Chl *b* [g m^{-2}]	0.035±0.002a	0.040±0.004a	0.044±0.003a	0.194	0.022
Chl *a*+*b* [g m^{-2}]	0.151±0.005a	0.176±0.002b	0.195±0.004c	3.533	0.000
Chl *a/b*	3.35±0.15a	3.48±0.40a	3.51±0.21a	0.100	0.907
LNC [g m^{-2}]	0.587±0.033a	0.791±0.026b	0.656±0.011a	17.054	0.003

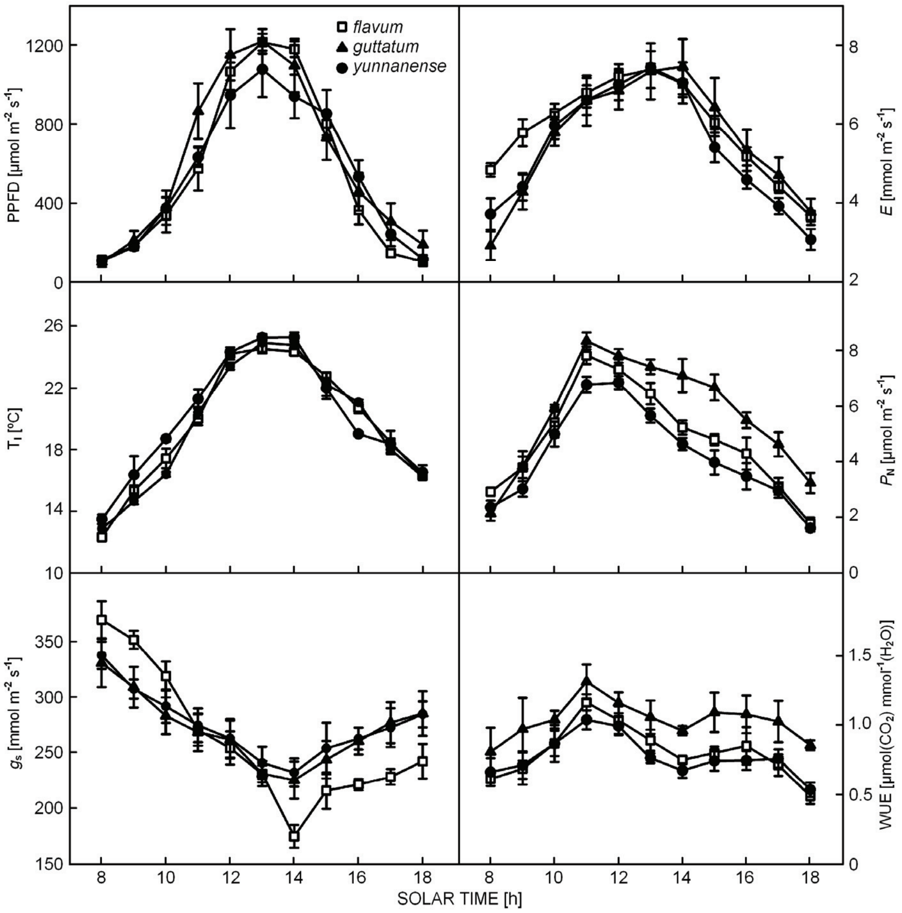

Fig.1. Diurnal courses of photosynthetic photon flow density (PPFD), leaf temperature (T$_l$), stomatal conductance (g_s), transpiration rate (E), net photosynthetic rate (P_N), and water use efficiency (WUE) of *Cypripedium flavum* (□), *C. guttatum* (▲), and *C. yunnanense* (●). Means of five measurements. *Error bars* represent ± SE.

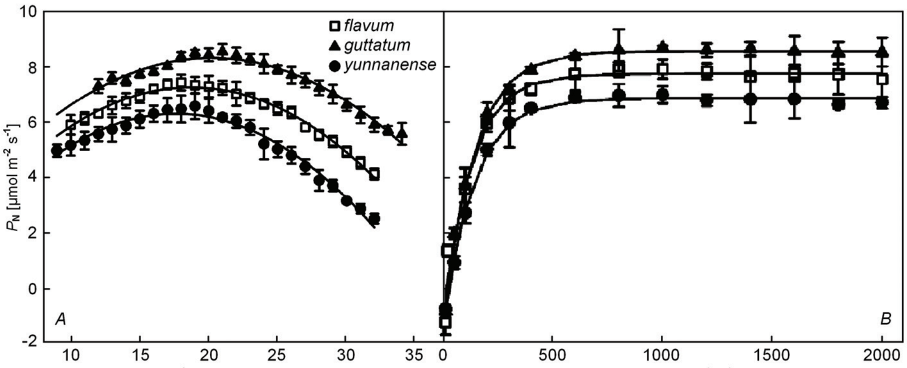

Fig. 2. Photosynthetic responses of *Cypripedium flavum* (□), *C. guttatum* (▲), and *C. yunnanense* (●) to (*A*) temperature and (*B*) PPFD. *Vertical bars* indicate standard errors of means for three measurements.

among the three species (LSD = 1.327, p=0.268). P_N of the three species increased rapidly with the increasing PPFD after 08:00, reaching the maximum values at 11:00, and no midday decrease was observed for the three species (Fig. 1). *C. guttatum* had the highest mean P_N (LSD = 8.054, p=0.000) and WUE (LSD = 17.433, p=0.000) among the three species, while *C. yunnanense* the lowest one (Table 2).

Effects of irradiance and temperature: The response of P_N to temperature was similar in the considered species (Fig. 2*A*). The highest P_N was obtained at T$_l$ between 18.4

and 20.1 ^{o}C for the three species, nevertheless, the difference in photosynthetic optimum temperature was not significantly different (LSD = 2.076, *p*=0.206) (Table 2). SI was attained between 317.7 and 344.5 μmol m^{-2}s^{-1} PPFD for three species (Fig. 2*B*, Table 2), however, there were no significant differences in saturation PPFD (LSD = 0.511, *p*=0.624). CI of *C. flavum* was lower than that of *C. yunnanense* (LSD = 4.477, *p*=0.065) (Table 2).

Differences in photosynthetic capacity: *C. guttatum* had the highest P_{max} among the three species (Table 2), while *C. yunnanense* had the lowest one (LSD = 4.582, *p*=0.062). R_D of *C. guttatum* was significantly higher than that of *C. flavum* (LSD = 5.924, *p*=0.038), but *C. yunnanense* did not differ significantly from *C. flavum* and *C. guttatum* in R_D (Table 2). AQE, CE, V_{cmax}, J_{max}, and TPU were also higher in *C. guttatum* than in *C. flavum* and *C. yunnanense* (Table 2). P_{max} was related to AQE, CE, J_{max} and V_{cmax}, respectively (Table 3). However, RSL of the three species were similar (LSD = 1.043, *p*=0.0408), they were not significantly related to P_{max} ($r^2 = 0.362$, *p*=0.087) and to C_i/C_a ($r^2 = 0.199$, *p*=0.229) (Table 3).

Table 3. Regression analysis of factorial correlations for three species *of* genus *Cypripedium* (*n* = 9).

Y-X	*a* (±SE)	*b* (±SE)	*F*	R^2	*P*
P_{max}–CE	2.795(1.256)	9.352(2.348)	15.869	0.694	0.005
P_{max}–AQE	4.703(1.026)	57.135 (18.818)	9.218	0.568	0.019
P_{max}–Chl *a*	3.280(2.515)	3.322(1.860)	3.190	0.313	0.117
P_{max}–Chl *b*	4.064(1.890)	9.324 (4.739)	3.871	0.356	0.090
P_{max}–Chl *a*+*b*	3.077(2.553)	2.682 (1.459)	3.381	0.326	0.109
P_{max}–LNC	1.587(0.904)	9.083 (1.321)	47.288	0.871	0.000
P_{max}–RSL	11.851(1.792)	–0.201(0.101)	3.966	0.362	0.087
P_{max}–V_{cmax}	1.466(0.717)	0.263(0.030)	78.180	0.918	0.000
P_{max}–J_{max}	1.731(0.679)	0.061 (0.007)	80.063	0.920	0.000
P_{max}–TPU	–3.594(3.062)	1.998 (0.538)	13.773	0.663	0.008
V_{cmax}–LNC	5.130(3.372)	27.622 (4.914)	31.598	0.819	0.001
J_{max}–LNC	24.160(7.615)	110.249 (40.364)	7.460	0.516	0.029
C_i/C_a–RSL	0.591(0.086)	0.006 (0.004)	1.740	0.199	0.229
CE–C_i/C_a	3.412(0.655)	–3.595 (0.931)	14.916	0.681	0.006

Physiological and biochemical leaf traits: Chl *a* and Chl *a*+*b* contents of *C. flavum* were higher than those of *C. guttatum* and *C. yunnanense*, but Chl *a* content and ratio of Chl *a* to Chl *b* were similar among the three species, and differences were not significant (Table 2). Chl contents were not correlated significantly to P_{max} (Table 3). *C. guttatum* had the highest LNC among the three species (LSD = 17.054, *p*=0.003) (Table 2). LNC was correlated with P_{max} ($r^2 = 0.871$, *p*=0.000) (Table 3).

Discussion

Sunlight is one of the major environmental factors influencing photosynthesis, growth, and distribution (Aleric and Kirkman 2005), especially for plants inhabiting places under the forest (Zhang *et al.* 2005). Irradiance at the ground surface of scrubland is affected by the canopy cover, which creates complex light dynamics for *Cypripedium*. The diurnal variations in T_l and gas exchange of the three species of *Cypripedium* are mainly controlled by the change of sunlight. Under natural condition, the three species did not show midday depression in P_N (Fig. 1). Although T_l, PPFD, and g_s of the three species were similar, *C. guttatum* had the highest P_{day} and WUE.

Among the three species, *C. guttatum* had also the highest P_{max}. Differences in photosynthetic capacity reflect differences in leaf physiology, anatomy, and biochemistry (Durand and Goldstein 2001). The interspecific differences in photosynthesis may be caused by the difference in Chl content (Friend and Woodward 1990). However, Chl contents per unit area of the three species were not significantly correlated with P_{max} in our study.

A strong positive correlation between the P_{max} and N has been widely recognized (Cordell *et al.* 1999), and it is justified by the large proportion of leaf N in chloroplasts, most of it being in the photosynthetic apparatus (Evans 1989). Our study confirms this result for three *Cypripedium* species, in which LNC significantly correlates to P_{max}, J_{max}, and V_{cmax}. Photosynthesis is primarily limited by the maximum V_{cmax} (Warren and Adams 2004). Species with higher LNC have higher RuBPCO content, therefore higher photosynthetic capacity according to the results of Kogami *et al.* (2001) and Warren and Adams (2004). Higher LNC may be a critical determinant of higher P_{max} of *C. guttatum.*

Plant photosynthesis may be either limited by stomatal diffusion or leaf biochemical capacity (Geber and Dawson 1997). If the ratio of C_i/C_a decreases, the CE of leaves might increase (Körner and Diemer 1987). We did not find any significant differences in g_s and RSL among the three species, but their C_i/C_a values were significantly different. The lower C_i/C_a of *C. guttatum* was not mainly caused by stomatal limitation, but by the biochemical process.

Variation in abundance of a plant species along an ecological gradient is the result of its physiological tolerance to adverse situation and its interaction with other species. The photosynthetic trait would be one of the factors accounting for the ability to compete with other species (Gulías *et al.* 2002, McDowell 2002). Several studies show that endemic species have lower photosynthetic capacity than their widespread congeners (Gulías *et al.* 2003, Cui *et al.* 2004). Our study supports these results. *C. guttatum* with widespread distribution has higher photosynthetic capacity than *C. flavum* and *C. yunnanense* with narrow distribution. It is likely that the differences in photosynthetic capacity among species reflect physiological adaptability to different climates. Physiological profile would be one of endemic causes for species (Richards *et al.* 2003).

In conclusion, the three species are characterized by similar T_{opt}, CI, and SI, but P_N and other photosynthetic parameters of *C. guttatum* are the highest. These results might be considered for conservation and cultivation strategies of the genus *Cypripedium*.

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