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REGULAR PAPER

The tiny-leaved orchid *Cephalanthera subaphylla* obtains most of its carbon via mycoheterotrophy

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Abstract The evolution of mycoheterotrophy has been accompanied by extreme reductions in plant leaf size and photosynthetic capacity. Partially mycoheterotrophic plants, which obtain carbon from both photosynthesis and their mycorrhizal fungi, include species with leaves of normal size and others that are tiny-leaved. Thus, plant species may lose their leaves in a gradual process of size reduction rather than through a single step mutation. Little is known about how the degree of mycoheterotrophy changes during reductions in leaf size. We compared the degree of mycoheterotrophy among five Japanese *Cephalanthera* species, four with leaves of normal size (*Cephalanthera falcata*, *Cephalanthera erecta*, *Cephalanthera longibracteata* and *Cephalanthera longifolia*), one with tiny leaves (*Cephalanthera subaphylla*), and one albino form of *C. falcata* (as reference specimens for fully mycoheterotrophic plants). The levels of mycoheterotrophy were determined by stable isotope natural abundance analysis. All *Cephalanthera* species were relatively enriched in ^{13}C and ^{15}N in comparison with surrounding autotrophic plants. *Cephalanthera subaphylla* was strongly enriched in ^{13}C and ^{15}N to levels similar to the albinos. Species with leaves of normal size were significantly less enriched in ^{13}C than *C. subaphylla* and the albinos. Thus, *C. subaphylla* was strongly mycoheterotrophic, obtaining most of its carbon from mycorrhizal fungi even though it has tiny leaves; species with leaves of normal size were partially mycoheterotrophic. Hence, during the evolutionary pathway to full mycoheterotrophy, some plant species appear to have gained strong mycoheterotrophic abilities before completely losing foliage leaves.

Keywords *Cephalanthera* · Leaf size · Mycoheterotrophy · Orchidaceae · Stable isotope

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Introduction

Approximately 880 species of vascular and non-vascular plants are mycoheterotrophic; they obtain carbon (C) from their associated mycorrhizal fungi (Merckx et al. 2013a). The evolution of mycoheterotrophy, which is a transition phase away from photoautotrophic nutrition, is accompanied by dramatic changes in plant morphology, especially an extreme reduction in leaf size (Merckx et al. 2013b). Leaves of mycoheterotrophs are generally reduced to very small achlorophyllous scales on the inflorescence axes

(Merckx et al. 2013b). Such leafless and achlorophyllous species are referred to as fully mycoheterotrophic plants, while plant species that obtain C from both photosynthesis and mycorrhizal fungi are partially mycoheterotrophic plants (Gebauer and Meyer 2003; Merckx 2013). Partial mycoheterotroph lineages include species with well-developed foliage leaves and others with strongly reduced leaves, such as *Epipactis microphylla* (Selosse et al. 2004) and *Pyrola aphylla*, which are typically leafless but often produce small leaves (Hynson et al. 2009). The existence of mycoheterotrophs with small leaves suggests that leaf loss during the evolutionary process did not result from a single step mutation, but rather a gradual process of size reduction. Little is known regarding the extent to which the mycoheterotrophic mechanism has been affected by reductions in leaf size.

The genus *Cephalanthera* includes both fully and partially mycoheterotrophic species and an ideal model to trace the character evolution in accordance with the evolution of mycoheterotrophy (Roy et al. 2009; Taylor and Bruns 1997). Five *Cephalanthera* species are distributed in Japan. *Cephalanthera falcata* (Thunb.) Blume, *C. erecta* (Thunb.) Blume, *C. longibracteata* Blume and *C. longifolia* (L.) Fritsch have foliage leaves with normal dimensions, while *C. subaphylla* Miyabe et Kudô has extremely reduced leaves (Fig. 1). *Cephalanthera subaphylla* is sister to the normal-leaved *C. erecta* by molecular phylogenetic analysis (Y. Sakamoto, unpublished data). In our study, we compared the extent of mycoheterotrophy among four species with normal leaves and the small-leaved species *C. subaphylla* using stable isotope natural abundance analyses.

Isotopic analyses can be used to quantify fungal-derived organic C and nitrogen (N), thereby revealing the level of mycoheterotrophy in plant species (Gebauer and Meyer 2003). Previous studies have reported mycoheterotrophic levels in diverse *Cephalanthera* species (Bidartondo et al. 2004; Gebauer and Mayer 2003; Liebel et al. 2010). We evaluated the natural abundances of ^{13}C and ^{15}N stable isotopes in five Japanese *Cephalanthera* species. Since albino individuals of green *Cephalanthera* species are able to function as full mycoheterotrophs (Julou et al. 2005; Roy et al. 2013), we included the albinos of normal-leaved *C. falcata* as a reference with full mycoheterotrophy.

Materials and methods

Sample collection

Plant samples were collected from six sites in Japan (Table 1). The Mt. Aoba site was in an old forest dominated by *Abies firma* with a dense understory; the remaining sites were in an open, mixed stand dominated by *Quercus myrsinifolia*, *Q. serrata* and/or *Q. acutissima* accompanied by a species-rich understory vegetation. *Cephalanthera subaphylla* and *C. longifolia* were collected from the same sites (Mt. Aoba and Yokohama, respectively) during two different years. The albino and green individuals of *C. falcata* grew sympatrically at Machida B and Chigasaki. Leaves of *Cephalanthera* species and neighboring autotrophic reference plants were collected from five or six 1 m^2 plots per site following the procedures of Gebauer and Meyer (2003) (Table 2). Single 1 m^2 plots included *Cephalanthera* species and one to six reference plant species. Two green orchids, *Cymbidium goeringii* and *Calanthe discolor*, were also included in specimens taken from Machida A. Only fresh leaves from the current year were used for the analysis. *Cephalanthera subaphylla* is very small and sometimes provides inadequate leaf material for samples; in these cases, we included all fresh leaves and flowers in the analysis.

Isotopic analysis

Collected samples were dried at 105 °C for 48 h, ground to a fine powder and stored in silica gel until used. Relative C and N isotopic abundances were measured using an elemental analyzer (Thermo Fisher Scientific FLASH 2000) coupled to a continuous flow isotopic ratio mass spectrometer (IRMS; Finnigan MAT Delta PLUS, Thermo Electron, Bremen, Germany). Measured abundances are reported here as δ values calculated as follows: $\delta^{13}C$ or $\delta^{15}N = (R_{sample}/R_{standard} - 1) \times 1000$ ‰, where R is the ratio of heavy isotope to light isotope in the sample or the respective standard. The standards were Pee-Dee Belemnite (C) and atmospheric N_2 (N).

Calculation and statistics

δ values were normalized following the procedures of Preiss and Gebauer (2008) for our comparisons of plant C and N isotopic abundances among different sites and species. Enrichment factors ($\varepsilon^{13}C$ and $\varepsilon^{15}N$) were calculated for each site using δ values for *Cephalanthera* species and the reference plants as follows: $\varepsilon = \delta_s - \delta_{REF}$, where δ_s is a single $\delta^{13}C$ or $\delta^{15}N$ value for a *Cephalanthera* species, and δ_{REF} is the mean value of all reference plants from the same site.

To quantify proportional C and N gains from fungi in *Cephalanthera* species, we applied a linear two-source mixing model procedure to data collected at Machida B and Chigasaki, where fully mycoheterotrophic albinos grew sympatrically with pigmented individuals. The proportional C and N gains of mycoheterotrophic origin in autotrophic plants at the same site were set to 0 %, and

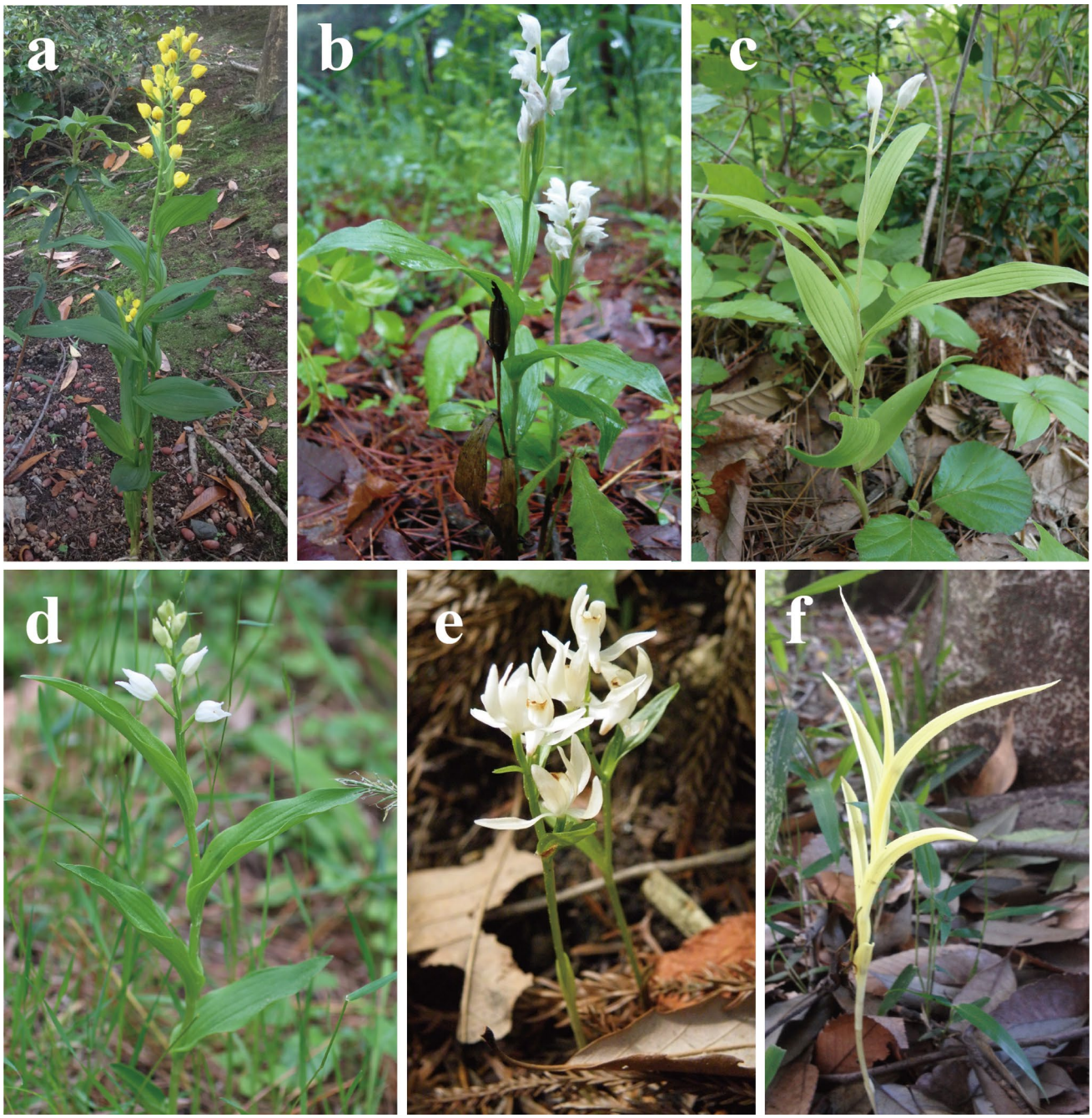

Fig. 1 *Cephalanthera* species used in this study. Four normal-leaves species, *C. falcata* (**a**), *C. erecta* (**b**), *C. longibracteata* (**c**) and *C. longifolia* (**d**), small-leaved *C. subaphylla* (**e**) and albino of *C. falcata* (**f**)

the gains of albino plants were set to 100 %. The proportional C and N gains from mycorrhizal fungi were calculated using the following expression (Gebauer and Meyer 2003): $\%x_{df} = (\delta x_{PMH} - \delta x_{REF})/\varepsilon_{MH} \times 100$, where $\%x_{df}$ is the percentage of fungal-derived C or N in a partially mycoheterotrophic plant, δx_{PMH} is the individual $\varepsilon^{13}C$ or $\varepsilon^{15}N$ value of a particular *Cephalanthera* species, δx_{REF} is the mean $\delta^{13}C$ or $\delta^{15}N$ value of autotrophic reference plants from the same site, and ε_{MH} is the mean enrichment factor for fully mycoheterotrophic plants (albinos of *C. falcata*) from the same site ($\varepsilon_{MH} = \delta x_{MH} - \delta x_{REF}$).

The data were analyzed via a generalized linear model constructed using 'R' v. 3.0.1 software (R Development Core Team 2013). All post hoc tests were performed using the glht-function (Hothorn et al. 2008) in the R package 'multcomp', which performs multiple comparisons of the

Table 1 Study sites and *Cephalanthera* species used in this study

Population	Species	Collection date	Prefecture	Location		
				Latitude	Longitude	Altitude (m)
Tsukuba	*C. falcata, C. erecta C. longibracteata*	June 2012	Ibaraki	36°06′10″N	140°06′40″E	ca. 25
Machida A	*C. falcata C. erecta C. longibracteata*	May 2014	Tokyo	35°57′21″N	139°47′31″E	ca. 65
Machida B	*C. falcata C. erecta C. falcata* (albino)	May 2014	Tokyo	35°56′73″N	139°47′12″E	ca. 70
Chigasaki	*C. falcata C. falcata* (albino)	June 2013	Kanagawa	35°37′75″N	139°41′83″E	ca. 40
Yokohama	*C. longifolia*	May 2013 May 2014	Kanagawa	35°35′48″N	139°64′84″E	ca. 5
Mt. Aoba	*C. subaphylla*	June 2013 May 2014	Miyagi	38°24′7″N	140°83′9″E	ca. 115

means using Tukey contrasts; P values <0.01 were considered statistically significant.

Results

Intraspecific variation

To compare isotope abundances of the *Cephalanthera* species at different sites, we calculated enrichment factors (ε) for each location; these factors were used to quantify differences between the reference and target species (Fig. 2; Table S2). A very high ^{13}C enrichment was measured in the albinos and *C. subaphylla* populations. *Cephalanthera subaphylla* and *C. longifolia*, which were sampled from the same site during two different years, were significantly different in ^{13}C values between 2013 and 2014, but ^{15}N did not vary between years (Fig. 2). The mean ^{13}C enrichments in *C. falcata* were highly variable among populations (4.6 ± 1.8 to −0.3 ± 0.8; Table S2). At Machida A, *C. falcata* was significantly depleted in ^{13}C in comparison with other populations. The $\delta^{13}C$ values for this species at this site were not significantly different from any of the autotrophic references or two green orchids growing at the same site (Table S1); however, *C. erecta* and *C. longibracteata* were enriched in ^{13}C. The mean ^{15}N enrichment of *C. falcata* ranged from 4.8 ± 1.6 to 8.9 ± 1.2; the enrichment of this species at Machida B significantly exceeded those at Machida A and Chigasaki. ^{13}C and ^{15}N values of *C. erecta* and *C. longibracteata* were not significantly different among populations (Fig. 2; Table S2).

We calculated the proportion of C derived from mycorrhizal fungi using a mixing-model. The mean %Cdf ± SD values of *C. falcata* from Machida B and Chigasaki were 65 ± 26 and 19 ± 17 %; the value for *C. erecta* from Machida B was 42 ± 10 %. The proportional N gains from fungi in *C. falcata* were 85 ± 12 and 65 ± 6 % in Machida B and Chigasaki, respectively; the proportional gain of *C erecta* was 118 ± 8 % in Machida B.

Interspecific variation

The mean ^{13}C and ^{15}N enrichments of the species are depicted in Fig. 3. All *Cephalanthera* species were relatively enriched in C ($\varepsilon^{13}C > 1.5$) and N ($\varepsilon^{15}N > 5.9$). The albinos and small-leaved *C. subaphylla* specimens were not significantly different, but both were significantly enriched in ^{13}C in comparison with other *Cephalanthera* species. *Cephalanthera subaphylla* and the albinos were similarly highly enriched in ^{15}N. *Cephalanthera erecta* had the highest level of ^{15}N enrichment.

Discussion

All of the *Cephalanthera* species that we evaluated were relatively enriched in ^{13}C and ^{15}N (Fig. 3), indicating that they obtained C and N from their mycorrhizal fungi. Full mycoheterotrophy in the albinos was associated with strong enrichment in ^{13}C and ^{15}N (Figs. 2, 3). The small-leaved species *C. subaphylla* was also strongly enriched in ^{13}C and ^{15}N, the levels of which were not significantly different from those in the albinos, indicating that *C. subaphylla* was strongly mycoheterotrophic and obtained most of its carbon from mycorrhizal fungi and not from the small leaves borne by the plants. Single albino individual of *C. subaphylla* found on Mt. Aoba in 2014 was even more enriched than were green specimens of *C. subaphylla* collected at the same site ($\varepsilon^{13}C = 9.0$, $\varepsilon^{15}N = 13.4$). It is likely that green *C. subaphylla* plants obtain some C from their

Table 2 Number of samples for isotope analysis

Species	Tsukuba	Machida A	Machida B	Chigasaki	Yokohama 2013	Yokohama 2014	Mt. Aoba 2013	Mt. Aoba 2014
Cephalanthera								
C. falcata	9	5	5	5				
C. erecta	7	5	5					
C. longibracteata	5	4						
C. longifolia					16	7		
C. subaphylla							12	6
C. subaphylla (albino)								1
C. falcata (albino)			3	3				
Other orchids								
Calanthe discolor		5						
Cymbidium goeringii		5						
Reference								
Ophiopogon japonicus	6							
Pleioblastus chino	5	5	5					
Ilex crenata	4							
Akebia trifoliata	4	5						
Pittosporum tobira	3							
Castanopsis sieboldii	3							
Disporum smilacinum		5					5	4
Sasa sp.				5			3	5
Quercus myrsinifolia			5	1				
Paederia scandens				1				
Oplismenus undulatifolius				1				
Houttuynia cordata			4	2				
Eurya japonica				2				
Acer pycnanthum			4					
Aucuba japonica							5	5
Coptis japonica							4	5
Poa trivialis					5	5		
Trigonotis peduncularis					4			
Ixeris stolonifera					3	5		
Artemisia indica var. *maximowiczii*					3	5		
Quercus serrata		5		6				
Youngia japonica					2			
Galium gracilens					1			

photosynthesis, but further study with larger samples is needed to confirm this fact.

The mycorrhizal fungi associated with the *Cephalanthera* species included in this study were identified through molecular genetic analyses by Sakamoto et al. (2015, 2016). *Cephalanthera subaphylla* specimens from Mt. Aoba sites dominated by ectomycorrhizal *Abies firma* trees were specifically associated with ectomycorrhizal basidiomycetes in the family Thelephoraceae. Mycoheterotrophic plants associated with ectomycorrhizal fungi obtain carbon from surrounding autotrophic plants via shared mycorrhizal mycelia. This association has been described as a 'tripartite symbiosis' (Merckx 2013). *Cephalanthera subaphylla* plants probably obtain C and N from thelephoracean mycorrhizal fungi that are also associated with surrounding autotrophic trees; Yagame and Yamato (2013) demonstrated such transfers in *C. falcata*. We showed that *C. subaphylla* has strong mycoheterotrophic abilities comparable to those of fully mycoheterotrophic plants. However, a more detailed analysis of samples from geographically separated populations growing in different forest types is required to fully evaluate mycoheterotrophy in *C. subaphylla*.

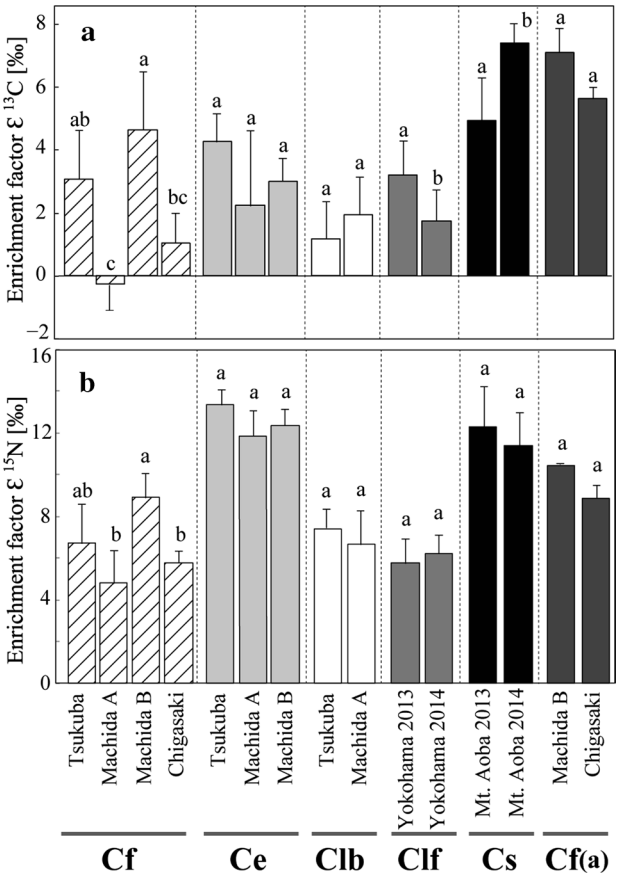

Fig. 2 Enrichment factors (ε) for ^{13}C (**a**) and ^{15}N (**b**) of *Cephalanthera* populations. The mean ± SD was calculated for each populations. *Different letters* indicate significant differences among populations of each species at $P < 0.01$ (a generalized linear model and Tukey post hoc test). *Cf C. falcata*, *Ce C. erecta*, *Clb C. longibracteata*, *Clf C. longifolia*, *Cs C. subaphylla*, *Cf(a)* albinos of *C. falcata*

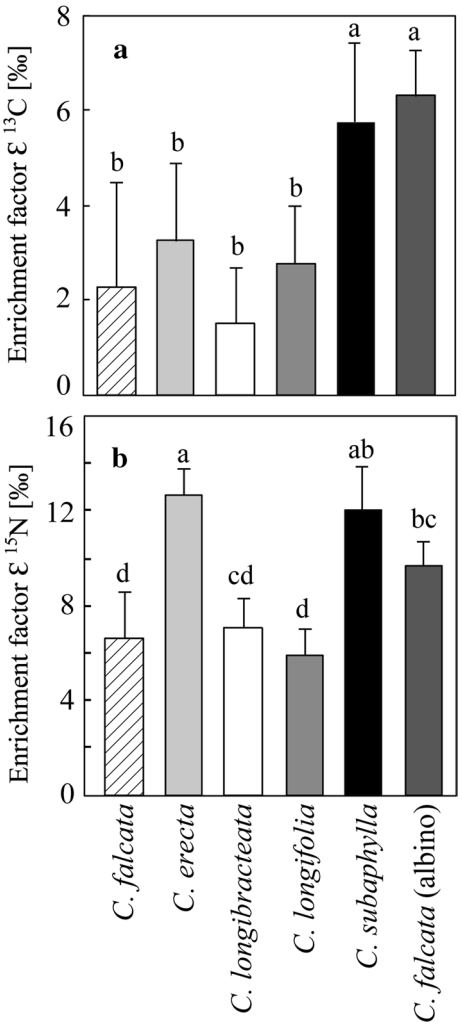

Fig. 3 Enrichment factors (ε) for ^{13}C (**a**) and ^{15}N (**b**) of *Cephalanthera* species. The mean ± SD was calculated for each *Cephalanthera* species using all individuals from the study site

^{13}C was significantly less enriched in the *Cephalanthera* species with normal leaves (*C. falcata*, *C. erecta*, *C. longibracteata* and *C. longifolia*) than in *C. subaphylla* and the albinos (Fig. 3), indicating that these species with normal leaves are partially mycoheterotrophic orchids. Partial mycoheterotrophy of European *C. longifolia* specimens has been demonstrated by isotopic analyses (Abadie et al. 2006, Liebel et al. 2010, Johansson et al. 2015). The *Cephalanthera* species with normal leaves that we evaluated are associated with thelephoracean fungi that are not phylogenetically distinct from the fungi associated with *C. subaphylla*. Furthermore, *C. falcata* and *C. longibracteata* are additionally associated with basidiomycete ectomycorrhizal fungi in the Russulaceae and Sebacinales (Sakamoto et al. 2015, 2016). Thus, *Cephalanthera* species with normal leaves that grew in a habitat with abundant ectomycorrhizal *Quercus* trees likely obtained C and N via tripartite symbiosis via a mechanism similar to that in *C. subaphylla*.

Cephalanthera subaphylla and *C. longifolia* had significantly different ^{13}C enrichments between the years evaluated (Fig. 2). Furthermore, ^{13}C enrichment in *C. falcata* varied strongly among the populations (Fig. 2). These intraspecific variations in enrichment may be due to variations in environmental factors, particularly light flux, which is strongly correlated with the levels of mycoheterotrophy in the partial mycoheterotrophs *C. damasonium* and *C. rubura* (Preiss et al. 2010). Under low light conditions, *C. damasonium* and *C. rubura* plants increased their fungus-derived C contents to approximately 50 % of the level that occurred in fully mycoheterotrophic orchids. When irradiance was increased, the proportional contribution of heterotrophic nutrition declined markedly. Microsite differences in irradiance created by variation in the incidence of forest canopy gaps may have strong influenced the ^{13}C enrichment variation that we detected, but other factors, such as annual variability, genetic effects,

meteorological factors, soil nutrients and mycorrhizal associations should also be taken into consideration.

The highest enrichment in ^{15}N (significantly higher than that in albinos) occurred in *C. erecta*, which is a sister taxon of *C. subaphylla* (Fig. 3). Strong ^{15}N enrichment in *C. erecta* was also reported by Motomura et al. (2010). *Cephalanthera erecta* is mainly associated with the thelephoracean fungi closely related to members of the same family associated with *C. falcata* and *C. longifolia* (Matsuda et al. 2009; Sakamoto et al. 2016). Thus, the high ^{15}N enrichment in *C. erecta* cannot be attributed to unique mycorrhizal taxa. The high enrichment levels may be related to strong mycoheterotrophic N uptake, to particular physiological processes within the tissues of *C. erecta*, to soil nutrient levels, or to host tree identity. The mechanism of N enrichment in *C. erecta* has not been identified, but it may be part of a process that paved the way to the evolution of almost complete mycoheterotorophy in *C. subaphylla*; importantly, these two orchids are closely related.

In conclusion, we showed that *C. subaphylla* had strong mycoheterotrophic carbon and nitrogen uptake despite the presence of small leaves. During the evolutionary pathway from autotrophy to full mycoheterotrophy, plant leaves have generally become extremely reduced (to achlorophyllous scales on inflorescence axes) (Merckx et al. 2013b). Our findings indicate that during the evolutionary process from partial to full mycoheterotrophy, plant species gained strong mycoheterotrophic abilities before the complete loss of foliage leaves. Strong mycoheterotrophic ability can balance the loss of photosynthetic carbon gain resulting from reductions in leaf size, which may explain why partial mycoheterotrophy leads to a reduction in leaf size that in turn triggers the evolution of full mycoheterotrophy. We found significant interspecific variation in the ^{13}C enrichment of *Cephalanthera* species. Studies on *C. damasonium* and *C. rubura* (Preiss et al. 2010) indicate that the interspecific variation found in this study may to be related to the light environment in habitats occupied by the plants. *Cephalanthera* species may increase their dependency on fungal-derived C when growing under low light levels, which likely confers flexible adaptability to diverse environments.

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