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Mycoheterotrophy evolved from mixotrophic ancestors: evidence in *Cymbidium* (Orchidaceae)

Hiroyuki Motomura[1], Marc-André Selosse[2], Florent Martos[2,3], Akira Kagawa[4] and Tomohisa Yukawa[1,*]

[1]*Tsukuba Botanical Garden, National Museum of Nature and Science, Tsukuba, Ibaraki 305-0005, Japan,* [2]*Centre d'Ecologie Fonctionnelle et Evolutive (CNRS, UMR 5175), Equipe Interactions Biotiques, 19129 Route de Mende, Montpellier cedex 5, France,* [3]*UMR C53 Peuplements Végétaux et Bioagresseurs en Milieu Tropical, Université de La Réunion, 15 Avenue René Cassin, BP 7151, 97715 Saint-Denis cedex 9, France and* [4]*Forestry and Forest Products Research Institute, Tsukuba Norin PO Box 16, Ibaraki 305-8687, Japan*

** For correspondence. E-mail yukawa@kahaku.go.jp*

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• *Background and Aims* Nutritional changes associated with the evolution of achlorophyllous, mycoheterotrophic plants have not previously been inferred with robust phylogenetic hypotheses. Variations in heterotrophy in accordance with the evolution of leaflessness were examined using a chlorophyllous–achlorophyllous species pair in *Cymbidium* (Orchidaceae), within a well studied phylogenetic background.
• *Methods* To estimate the level of mycoheterotrophy in chlorophyllous and achlorophyllous *Cymbidium*, natural ^{13}C and ^{15}N contents (a proxy for the level of heterotrophy) were measured in four *Cymbidium* species and co-existing autotrophic and mycoheterotrophic plants and ectomycorrhizal fungi from two Japanese sites.
• *Key Results* $\delta^{13}C$ and $\delta^{15}N$ values of the achlorophyllous *C. macrorhizon* and *C. aberrans* indicated that they are full mycoheterotrophs. $\delta^{13}C$ and $\delta^{15}N$ values of the chlorophyllous *C. lancifolium* and *C. goeringii* were intermediate between those of reference autotrophic and mycoheterotrophic plants; thus, they probably gain 30–50 % of their carbon resources from fungi. These data suggest that some chlorophyllous *Cymbidium* exhibit partial mycoheterotrophy (= mixotrophy).
• *Conclusions* It is demonstrated for the first time that mycoheterotrophy evolved after the establishment of mixotrophy rather than through direct shifts from autotrophy to mycoheterotrophy. This may be one of the principal patterns in the evolution of mycoheterotrophy. The results also suggest that the establishment of symbiosis with ectomycorrhizal fungi in the lineage leading to mixotrophic *Cymbidium* served as pre-adaptation to the evolution of the mycoheterotrophic species. Similar processes of nutritional innovations probably occurred in several independent orchid groups, allowing niche expansion and radiation in Orchidaceae, probably the largest plant family.

Key words: Mycoheterotrophy, nutritional mode, evolution, *Cymbidium*, Orchidaceae, symbiosis, mycorrhizal fungi, $\delta^{15}N$, $\delta^{13}C$.

INTRODUCTION

Unlike the autotrophic nutritional mode of chlorophyllous and leafy plants, which use atmospheric CO_2 as their sole carbon source, some achlorophyllous and leafless plant species obtain carbon from mycorrhizal fungi (Björkman, 1960; McKendrick *et al.*, 2000; Smith and Read, 2008). This fully heterotrophic nutrition, so-called mycoheterotrophy (MH), occurs in >400 species belonging to 87 genera in 11 families (Leake, 1994, 2005).

MH nutrition has evolved repeatedly from autotrophy (AT) in various plant lineages, but detailed evolutionary processes that lead to MH remain unclear. Recent studies demonstrated that several chlorophyllous species in Orchidaceae and Ericaceae obtain carbon not only from their photosynthetic activity, but also from mycorrhizal fungi (Gebauer and Meyer, 2003; Bidartondo *et al.*, 2004; Selosse *et al.*, 2004; Julou *et al.*, 2005; Cameron *et al.*, 2006; Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007, 2008). This nutritional mode, called mixotrophy (MX), has been suggested to be a pre-adaptation in the evolution of MH nutrition (Bidartondo *et al.*, 2004; Selosse *et al.*, 2004, 2006; Abadie *et al.*, 2006). In orchids, both MX and MH species tend to associate with unusual mycorrhizal fungi: instead of the usual saprobic or parasitic *Rhizoctonia* fungi that are the almost exclusive associates of autotrophic orchids (Rasmussen, 1995), most MX and MH orchids recruit various ascomycetes and basidiomycetes, which are ectomycorrhizal fungi on surrounding tree roots (Taylor *et al.*, 2002). MX and MH species thus indirectly exploit tree photosynthates as a carbon source (McKendrick *et al.*, 2000).

Nutritional changes associated with the evolution of achlorophyllous plant species have to date been inferred from assumptions without using clades in which leaflessness appears (Bidartondo *et al.*, 2004; Tedersoo *et al.*, 2007; Roy *et al.*, 2009) or from comparisons between green plants and albino mutants that exist in some species (Julou *et al.*, 2005; Abadie *et al.*, 2006). For these reasons, it is necessary to

clarify fluctuations of heterotrophy in accordance with the evolution of leaflessness using a relationship between chlorophyllous and achlorophyllous species based on credible phylogenetic hypotheses.

Cymbidium, an orchid genus distributed from east and south-east Asia to Australia, comprises about 52 species (DuPuy and Cribb, 2007). This genus exhibits distinctive ecological diversification (Motomura *et al.*, 2008) and occurs in terrestrial, epiphytic and lithophytic life forms. Two species, *C. macrorhizon* and *C. aberrans*, lack foliage leaves and are thus assumed to be MH (Fig. 1).

Yukawa *et al.* (2002) analysed phylogenetic relationships among 36 *Cymbidium* spp. using nucleotide variation in the nuclear and plastid genomes and found that these achlorophyllous species are sister species, in a clade successively connecting with *Cymbidium lancifolium* and the clade including *Cymbidium goeringii* (Fig. 2). *Cymbidium lancifolium* and *C. goeringii* develop foliage leaves and appear to be capable of AT nutrition. On the other hand, the evolution of characters related to nutritional traits has been well analysed in *Cymbidium*: in an investigation of the vegetative anatomy of the genus, Yukawa and Stern (2002) found degeneration of stomata in *C. macrorhizon*, indicating a lack of CO_2 exchange in this species. Yokoyama *et al.* (2002) and Y. Ogura-Tsujita and T. Yukawa (unpubl. res.) found a shift of mycobionts between chlorophyllous and achlorophyllous *Cymbidium*. Chlorophyllous *C. lancifolium* and *C. goeringii* both harbour saprobic (Tulasnellaceae) and tree ectomycorrhizal (Sebacinales, Russulaceae, Thelephoraceae, etc.) fungi, whereas achlorophyllous *C. macrorhizon* and *C. aberrans* establish symbiosis exclusively with ectomycorrhizal fungi. Furthermore, Motomura *et al.* (2008) demonstrated a large diversification of photosynthetic modes in *Cymbidium*. Therefore, *Cymbidium* is an ideal model taxon to test contributions of carbon from mycorrhizal fungi, in accordance with the evolution of leaflessness and associated characters related to nutritional innovations.

MH plants are highly enriched in ^{13}C and ^{15}N relative to AT plants (Gebauer and Meyer, 2003; Trudell *et al.*, 2003). Their ^{13}C contents are similar to, or slightly more elevated than, those of associated mycorrhizal fungi, whereas their ^{15}N contents tend to be higher (Trudell *et al.*, 2003; Selosse and Roy, 2009). Fractionation against heavy isotopes occurs commonly in physical and metabolic processes, and thus analysis of the natural abundance of stable isotopes allows tracking of nutrient sources and fluxes in ecosystems (Dawson *et al.*, 2002; Post, 2002). Isotopic abundance in MH plants correlates with the use of resources derived from their mycorrhizal fungi (and ultimately from nearby AT plants). As expected, ^{13}C and ^{15}N abundances in MX orchid species range between those of AT and MH species (Gebauer and Meyer, 2003; Bidartondo *et al.*, 2004; Julou *et al.*, 2005; Roy *et al.* 2009), and are indicative of variable levels of heterotrophy from one species or one site to another.

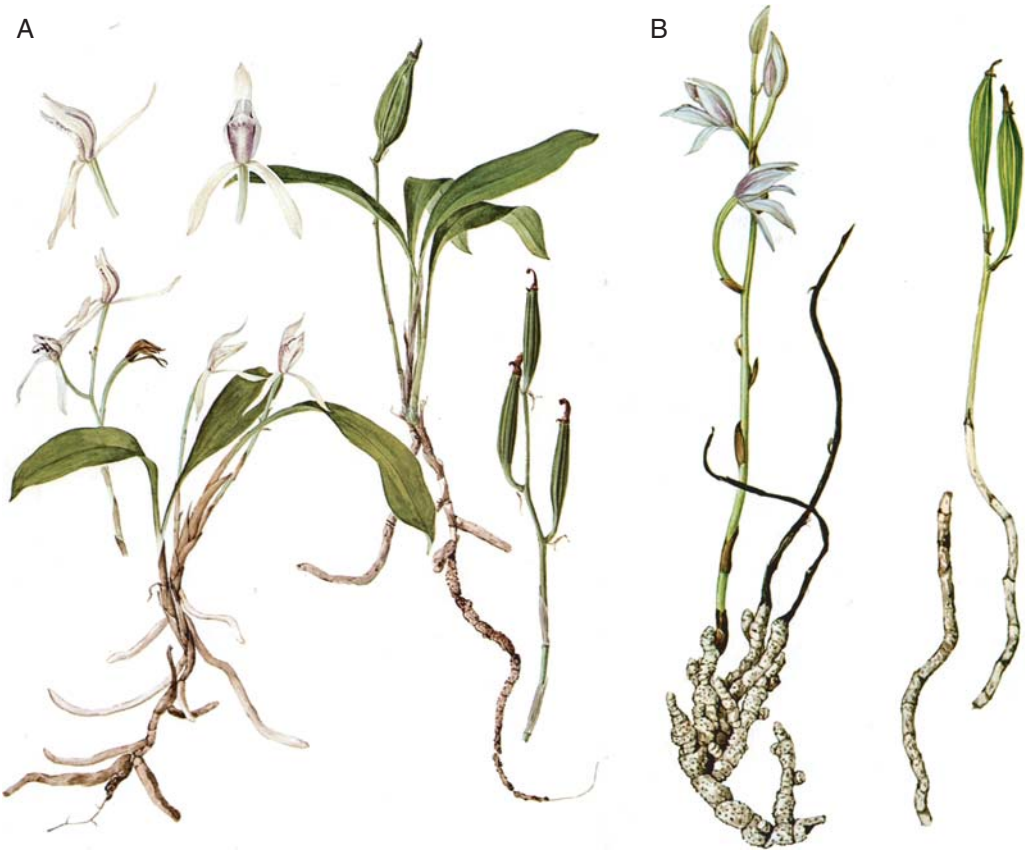

FIG. 1. *Cymbidium lancifolium*, a leafy and chlorophyllous species (A), and *C. macrorhizon*, a leafless and achlorophyllous species (B). *Cymbidium lancifolium* is the closest relative of the achlorophyllous *Cymbidium* species (see Fig. 2). Reproduced from Maekawa (1971), del. Yoai Ohta.

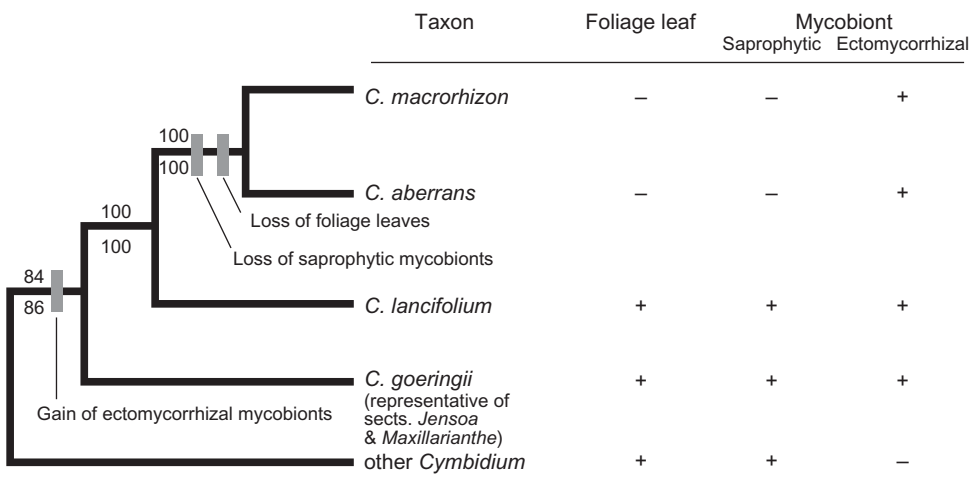

FIG. 2. Reconstruction of character evolution related to nutritional properties in *Cymbidium*. The phylogram of *Cymbidium* is summarized from the results of molecular phylogenetic analyses by Yukawa *et al.* (2002). Numbers above and below internodes indicate bootstrap values from 1000 replicates of Fitch parsimony analysis and neighbor-joining analysis, respectively.

In this study, spontaneous stable isotopic contents (^{13}C and ^{15}N) of aerial parts from the aforementioned *Cymbidium* species are used to estimate their level of heterotrophy and to draw conclusions about the evolution of MH, comparing an achlorophyllous clade with its closest chlorophyllous relatives.

MATERIALS AND METHODS

Sampling

Samples were collected in August 2006 from two forest sites in the eastern part of Honshû island, Japan: Minamibouso, Chiba (site A: 35°03′22″N, 140°01′35″E) and Mitaka, Tokyo (site B: 35°41′55″N, 139°34′22″E). At site A, the *Cymbidium* populations were growing under warm-temperate evergreen broadleaved forest in which *Castanopsis sieboldii* (Fagaceae), *Cinnamomum tenuifolium* (Lauraceae), *Lithocarpus edulis* (Fagaceae), *Neolitsea sericea* (Lauraceae) and *Machilus thunbergii* (Lauraceae) dominate. Site B harbours warm-temperate deciduous broadleaved forest in which *Aphananthe aspera* (Ulmaceae) and *Carpinus tschonoskii* (Betulaceae) dominate. The canopy begins to develop in May and leaves are shed in November.

In areas adjacent to site B, the mean annual precipitation from 1977 to 2004 was 1496 mm and mean temperature ranged from 13·9 °C to 16·7 °C. More humid conditions are observed in areas adjacent to site A where the mean annual precipitation from 1977 to 2004 was 1809 mm and mean temperature ranged from 14·4 °C to 16·6 °C (data from the Japan Meteorological Agency).

Shoots of achlorophyllous orchids (*Cyrtosia septentrionalis*, *Lecanorchis nigricans*, *Cymbidium macrorhizon* and *C. aberrans*) and achlorophyllous Ericaceae (*Monotropa uniflora*), and leaves of chlorophyllous *C. lancifolium* and *C. goeringii* (Table 1) were collected. As references, at site A, leaves of 40 non-orchid chlorophyllous species belonging to 26 plant families and four chlorophyllous orchids (*Cephalanthera erecta*, *Liparis nervosa*, *Zeuxine agyokua-na* and *Goodyera schlechtendaliana*) were collected. At site B, eight non-orchid chlorophyllous species belonging to seven plant families were collected. Fruit bodies of ectomycorrhizal fungi growing near the *Cymbidium* populations were also collected at both sites and their genera were identified using morphology and molecular identification on the basis of nucleotide sequences of internal transcribed spacer regions in ribosomal DNA as described in Selosse *et al.* (2002). The sequences obtained were deposited in GenBank (accession numbers GQ359817–GQ359821; Tables S1 and S2 available online).

Except for fungi, all samples were collected at 10–30 cm above the soil and in the same light conditions to avoid carbon isotope distortion due, respectively, to CO_2 resulting from soil respiration and different photosynthetic rates (slow rates enhance higher ^{13}C discrimination during CO_2 assimilation; Julou *et al.*, 2005). To ensure independence of the data, all samples were from different individuals; for each species, the number of replicates was up to six whenever possible, but in some cases the number of available individuals limited the repetition number.

Isotopic analysis

Samples were dried at 60 °C for 4 d before grinding with a steel ball mill (Wig-L-Bug Model 30; International Crystal Laboratories, Garfield, NJ, USA). ^{14}N and ^{15}N contents were measured using 4 mg of each ground sample and ^{12}C and ^{13}C contents using 2 mg. Stable isotope ratios were analysed using a combined system of an elemental analyser (NC-2500; CE Instruments, Milan, Italy) and an isotope ratio mass spectrometer (MAT-252; Thermo Electron, Bremen, Germany), as described by Motomura *et al.* (2008). The isotope ratios in the delta notation in per mil units (‰) were expressed using Pee Dee belemnite and atmospheric N_2 as standards:

$$\delta^{15}N \text{ or } \delta^{13}C = (R_{sample}/R_{standard} - 1) \times 1000[‰] \quad (1)$$

where R is the molar ratio, i.e. $^{15}N/^{14}N$ or $^{13}C/^{12}C$. The standard deviations for replicate combustions of the internal standards (DL-alanine) were 0·11 ‰ for $\delta^{15}N$ and 0·07 ‰ for $\delta^{13}C$.

TABLE 1. *Plants examined in this study; number of individuals collected at each site and trophic status are provided*

Species	Family	Collection site A	Collection site B	Trophic status*
Amphicarpaea bracteata (L.) Fernald subsp. *edgeworthii* (Benth.) H.Ohashi	Fabaceae	2	–	FIX
Aphananthe aspera (Thunb.) Planch.	Ulmaceae	–	3	AM/ECM
Arachniodes standishii (T.Moore) Ohwi	Dryopteridaceae	3	–	AM/NON
Ardisia crenata Sims	Primulaceae	–	1	AM
Ardisia japonica (Thunb.) Blume	Primulaceae	6	–	AM
Arisaema aequinoctiale Nakai & F.Maek.	Araceae	4	–	AM
Aucuba japonica Thunb.	Cornaceae	3	–	AM
Carex conica Boott	Cyperaceae	2	–	AM/NON
Carex siderosticta Hance	Cyperaceae	3	–	AM/NON
Carpinus tschonoskii Maxim.	Betulaceae	–	3	ECM
Castanea crenata Siebold et Zucc.	Fagaceae	1	–	ECM
Castanopsis sieboldii (Makino) Hatus. ex T.Yamaz. & Mashiba	Fagaceae	6	–	ECM
Cephalanthera erecta (Thunb.) Blume	Orchidaceae	3	–	OM
Cephalotaxus harringtonia (Knight ex Forbes) K.Koch	Taxaceae	2	–	AM
Chamaecyparis obtusa (Siebold & Zucc.) Endl.	Cupressaceae	1	–	AM
Cinnamomum tenuifolium (Makino) Sugim. ex H.Hara	Lauraceae	2	–	AM
Cryptomeria japonica (L.f.) D.Don	Taxodiaceae	1	–	AM
Cymbidium goeringii (Rchb.f.) Rchb.f.	Orchidaceae	6	1	OM
Cymbidium macrorhizon Lindl.†	Orchidaceae	4	2	OM
Cymbidium lancifolium Hook.	Orchidaceae	6	–	OM
Cymbidium aberrans Finet†	Orchidaceae	–	3	OM
Cyrtosia septentrionalis (Rchb.f.) Garay†	Orchidaceae	1	–	OM
Damnacanthus indicus Gaertn.f.	Rubiaceae	6	–	AM
Dendropanax trifidus (Thunb.) Makino ex H.Hara	Araliaceae	3	–	AM/NON
Desmodium laxum DC.	Fabaceae	1	–	FIX
Deutzia scabra Thunb.	Hydrangeaceae	1	–	AM/NON
Dioscorea japonica Thunb.	Dioscoreaceae	1	1	AM/NON
Dryopteris pacifica (Nakai) Tagawa	Dryopteridaceae	1	–	AM/NON
Elaeagnus macrophylla Thunb.	Elaeagnaceae	1	–	AM
Eriobotrya japonica (Thunb.) Lindl.	Rosaceae	1	–	AM
Eurya japonica Thunb.	Theaceae	4	–	AM
Ficus erecta Thunb.	Moraceae	3	–	AM
Goodyera schlechtendaliana Rchb.f.	Orchidaceae	1	–	OM
Hedera rhombea (Miq.) Bean	Araliaceae	6	–	AM/NON
Ilex crenata Thunb.	Aquifoliaceae	–	1	AM
Ilex serrata Thunb. f. *argutidens* (Miq.) Satomi	Aquifoliaceae	3	–	AM
Lecanorchis nigricans Honda†	Orchidaceae	3	–	OM
Ligustrum lucidum Aiton	Oleaceae	–	2	AM
Lilium auratum Lindl.	Liliaceae	1	–	AM
Liparis nervosa (Thunb.) Lindl.	Orchidaceae	3	–	OM
Lithocarpus edulis (Makino) Nakai	Fagaceae	6	–	ECM
Machilus thunbergii Siebold & Zucc.	Lauraceae	2	–	AM
Monotropa uniflora L.†	Ericaceae	3	–	ECM
Neolitsea sericea (Blume) Koidz.	Lauraceae	2	–	AM
Ophiopogon japonicus (Thunb.) Ker Gawl.	Asparagaceae	2	–	AM
Ophiopogon japonicus (Thunb.) Ker Gawl. var. *umbrosus* Maxim.	Asparagaceae	3	–	AM
Oplismenus undulatifolius (Ard.) Roem. & Schult.	Poaceae	3	1	AM
Padus grayana (Maxim.) C.K.Schneid.	Rosaceae	1	–	AM
Piper kadsura (Choisy) Ohwi	Piperaceae	3	–	AM/NON
Pleioblastus chino (Franch. & Sav.) Makino	Poaceae	6	2	AM
Pteris cretica L.	Pteridaceae	1	–	AM/NON
Smilax china L.	Smilacaceae	1	–	AM
Stegnogramma pozoi (Lag.) K.Iwats. subsp. *mollissima* (Fisch. ex Kunze) K.Iwats.	Thelypteridaceae	3	–	AM/NON
Trachelospermum asiaticum (Siebold & Zucc.) Nakai	Apocynaceae	6	–	AM/NON
Wisteria floribunda (Willd.) DC.	Fabaceae	1	–	FIX
Zeuxine agyokuana Fukuy.	Orchidaceae	1	–	OM

* Trophic status based on general assumption: AM, arbuscular mycorrhizal plants; AM/NON: arbuscular mycorrhizal or non-mycorrhizal plants; ECM, ectomycorrhizal plants; FIX, nitrogen-fixing plants; OM, orchid mycorrhizal plants.

† Achlorophyllous species.

^{13}C and ^{15}N values were tested for normality and for homogeneity of variances using a Shapiro–Wilk test and a Levene test, respectively.

Nutritional modes of the *Cymbidium* species were tested in comparison with other AT and MH plants using $\delta^{13}C$ and $\delta^{15}N$ values and one-way ANOVA performed for each variable and

each site, followed by pairwise *t*-tests (Bonferroni correction) to calculate pairwise comparisons between group levels at $\alpha = 0{\cdot}01$. The percentage of carbon acquired in a MH way from fungi was estimated by using a linear two-source mixing model (Phillips and Gregg, 2001; Gebauer and Meyer, 2003; Tedersoo *et al.*, 2007):

$$C = (\delta C_{MX} - \delta C_{MH})/(\delta C_R - \delta C_{MH}) \times 100[\%] \qquad (2)$$

where δC_R and δC_{MH} are the mean values of AT and MH references, respectively, and δC_{MX} is the mean value of the putative MX species. All chlorophyllous plants except *Cymbidium goeringii*, *C. lancifolium* and *Cephalanthera erecta* were assumed to be AT (see below). At site A, the mean values for the chlorophyllous non-orchids and the chlorophyllous orchids were used as references for AT; since chlorophyllous orchid species were absent from site B, the mean value for all chlorophyllous non-orchids was used as the reference for AT. The relative contribution of carbon derived from fungi was estimated from mean values, with approximate standard errors and 95 % confidence intervals as used in Phillips and Gregg (2001). Statistical analyses and graphics were computed using R 2·7·1 (R Foundation, Vienna, Austria).

RESULTS

Tables S1 and S2 (available online) show the $\delta^{15}N$ and $\delta^{13}C$ values in plants and fungi collected at sites A and B and morphological and molecular identifications of fungal samples collected at these sites that proved to belong to the ectomycorrhizal genera *Amanita*, *Boletus*, *Lactarius* and *Russula* (GenBank accession numbers GQ359817–GQ359821; Tables S1 and S2).

Figure 3 shows that high $\delta^{15}N$ and $\delta^{13}C$ values were recorded in all *Cymbidium* species (*C. macrorhizon*, *C. aberrans*, *C. lancifolium* and *C. goeringii*) at both sites and achlorophyllous plants at site A (Orchidaceae: *Cyrtosia septentrionalis* and *Lecanorchis nigricans*; Ericaceae: *Monotropa uniflora*). At site A, *C. macrorhizon* did not significantly differ from the other MH species in $\delta^{13}C$ and $\delta^{15}N$ (Fig. 4A and B). At sites A and B, *C. macrorhizon* and *C. aberrans* had higher $\delta^{15}N$ but identical $\delta^{13}C$ values compared with co-occurring ectomycorrhizal fungi (Fig. 4), including Russulaceae which are mycorrhizal with these *Cymbidium* species. At site A, *C. lancifolium* and *C. goeringii* had significantly higher $\delta^{13}C$ and $\delta^{15}N$ values than the AT plants (orchids or non-orchids; Fig. 4A and B), and the same trend was observed for *C. goeringii* at site B (Fig. 4C and D). At both sites, $\delta^{13}C$ values of the

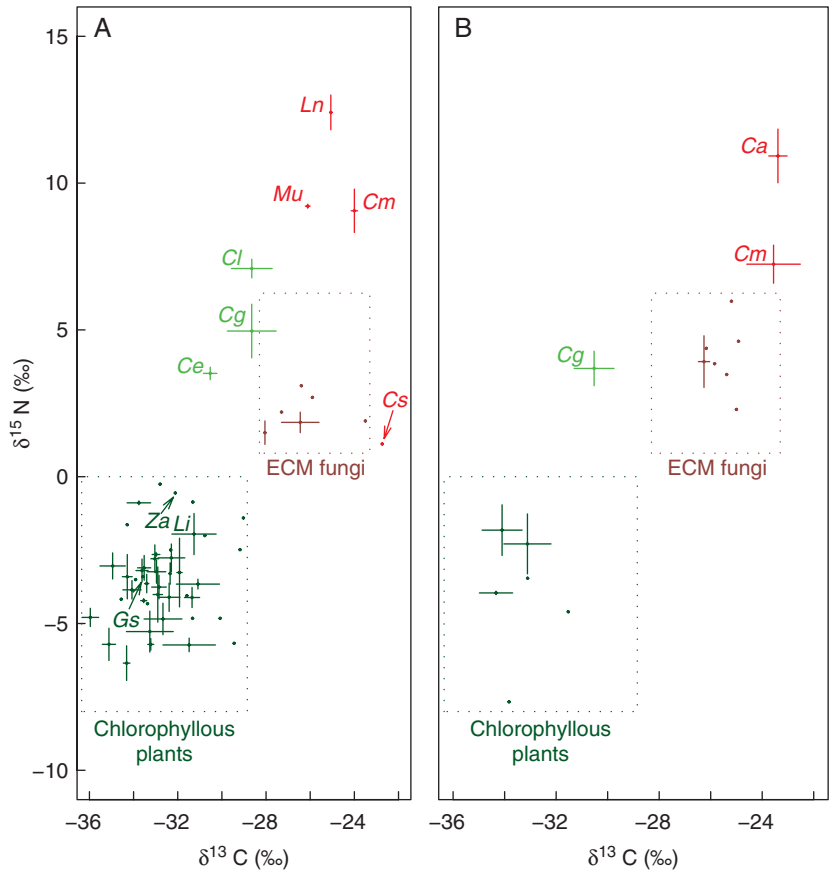

FIG. 3. $\delta^{13}C$ and $\delta^{15}N$ values in plants and ectomycorrhizal (ECM) fungi collected at site A (A) and site B (B). Bars indicate standard errors, and squares surrounded by dotted lines indicate values for chlorophyllous plants and ECM fungi (see Tables S1 and S2). Chlorophyllous plants, achlorophyllous plants, ECM fungi and green, mixotrophic plants are shown dark green, red, brown–red and light green, respectively. Abbreviations for Orchidaceae: *Ca*, *Cymbidium aberrans*; *Ce*, *Cephalanthera erecta*; *Cm*, *Cymbidium macrorhizon*; *Cl*, *Cymbidium lancifolium*; *Cs*, *Cyrtosia septentrionalis*; *Gs*, *Goodyera schlechtendaliana*; *Ln*, *Lecanorchis nigricans*; *Li*, *Liparis nervosa*; *Za*, *Zeuxine agyokuana*. Abbreviation for Ericaceae: *Mu*, *Monotropa uniflora*.

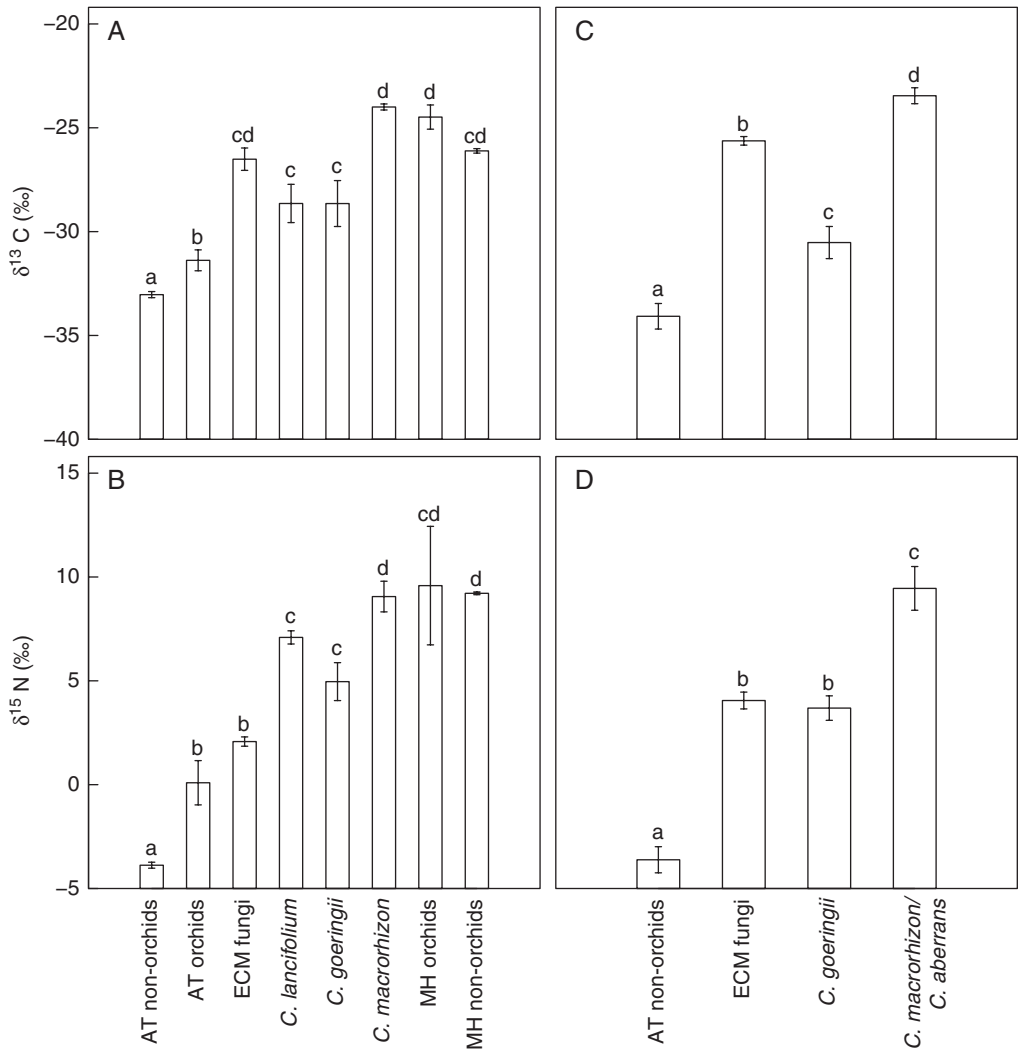

FIG. 4. δ^{13}C and δ^{15}N values (mean ± s.e.) of plants and ectomycorrhizal (ECM) fungi grouped in different species and/or nutritional modes at site A (A, B) and site B (C, D). Different letters denote significant differences between species and functional groups according to Bonferroni corrected pairwise *t*-tests ($P < 0{\cdot}01$). *Cymbidium goeringii* and *C. lancifolium* are chlorophyllous; *C. macrorhizon* and *C. aberrans* are achlorophyllous. Mycoheterotrophic (MH) orchids include *Cyrtosia septentrionalis* and *Lecanorchis nigricans*; and MH non-orchids are represented by *Monotropa uniflora*. For details on autotrophic (AT) non-orchids, AT orchids and ECM fungi, see Tables 1, S1 and S2.

chlorophyllous *Cymbidium* species were significantly lower than for the achlorophyllous *Cymbidium* species; similarly, δ^{15}N values of the chlorophyllous *Cymbidium* species tended to be lower, although this was not significant at site A (Fig. 4). Among the remaining chlorophyllous species, *Cephalanthera erecta* at site A showed significantly higher δ^{15}N and non-significantly higher δ^{13}C values as compared with the other AT species ($P < 0{\cdot}0001$ and $P = 0{\cdot}014$, respectively, according to a pairwise *t*-test).

To estimate the percentage of carbon acquired heterotrophically from fungi, references for δ^{13}C were determined in full MH and full AT nutrition, focusing on phylogenetically close lineages. Given the results above, *C. macrorhizon* and *C. aberrans* were hypothesized to be full MH reference. At site A, the mean values for the chlorophyllous non-orchids and the chlorophyllous orchids were used as a reference for AT; the possibly MX *Cephalanthera erecta* was omitted from baseline calculations. Since chlorophyllous orchid species were absent from site B, only the mean value for all chlorophyllous non-orchids was available as reference for AT. Table 2 showed that a significant contribution of fungal C was found in *Cymbidium lancifolium* at site A (between 41·2 % and 48·3 % in the mean value among the different references for AT) and *C. goeringii* at the two sites (between 33·4 % and 48·3 % in the mean value among the sites or the different references for AT). This result was not much changed by using non-*Cymbidium* achlorophyllous orchids as full MH reference at site A (not shown), since these orchids showed δ^{13}C values similar to those of the achlorophyllous *Cymbidium*, whereas using the ericaceous *Monotropa uniflora* (which has lower δ^{13}C) and all chlorophyllous plants as baselines, fungal carbon contribution reached 63 % both for *C. lancifolium* and *C. goeringii*. A two-way analysis of variance showed no effect of either the species ($P = 0{\cdot}88$) or the site for *C. goeringii* ($P = 0{\cdot}27$) on the fungal contribution. For *Cephalanthera erecta* at site A,

TABLE 2. *Net carbon gain from fungi in* Cymbidium *species and* Cephalanthera erecta *at sites A and B (mean ± s.e.), based on a linear mixing model*

Species	Carbon gain, using all autotrophic plants as 0 % baseline[†]	Carbon gain, using all non-*Cymbidium* autotrophic orchids as 0 % baseline[‡]
At site A		
Cymbidium macrorhizon	100 % (baseline)	100 % (baseline)
C.goeringii	48·3 ± 12·3 %*	41·1 ± 14·0 %*
C.lancifolium	48·3 ± 10·2 %*	41·2 ± 11·7 %*
Cephalanthera erecta	27·3 ± 3·4 %	17·3 ± 3·8 %*
At site B		
C. macrorhizon + *C. aberrans*	100 % (baseline)	–
C. goeringii	33·4 ± 7·3 %*	–

* Significant difference based on 95 % confidence intervals following Phillips and Gregg (2001).

[†] In this calculation, the $\delta^{13}C$ of autotrophic plants (0 % gain from fungi) is supposed to be the mean $\delta^{13}C$ value of all chlorophyllous plants (including non-*Cymbidium* orchids, with the exception of the possible mixotrophic *Cephalanthera erecta*).

[‡] In this calculation, the $\delta^{13}C$ of autotrophic plants (0 % gain from fungi) is supposed to be the mean $\delta^{13}C$ value of all chlorophyllous orchids (with the exception of *Cymbidium* and the possible mixotrophic *Cephalanthera erecta*).

the carbon gain from fungi was lower (17–27 %) and only significant when estimated with the chlorophyllous orchids as baseline (Table 2).

DISCUSSION

The pattern of evolution of nutritional modes was examined in a *Cymbidium* clade comprising achlorophyllous *C. macrorhizon* and *C. aberrans* and chlorophyllous *C. lancifolium* and *C. goeringii* (Fig. 2). The following data showed that the two achlorophyllous *Cymbidium* species are MH. First, they had significantly higher $\delta^{15}N$ but similar (to slightly higher) $\delta^{13}C$ values compared with the co-occurring ectomycorrhizal fungi, including Russulaceae, which is mycorrhizal with these *Cymbidium* species (Yokoyama *et al.*, 2002; Y. Ogura-Tsujita and T. Yukawa, unpubl. res.). Trudell *et al.* (2003) showed the same trend in other MH plants relative to co-existing ectomycorrhizal fungi, as expected in trophic chains in which ^{15}N contents tend to increase from one level to another (Figs 3 and 4). Secondly, *C. macrorhizon* had similar $\delta^{15}N$ and $\delta^{13}C$ values relative to the other MH species at site A (Figs 3 and 4). Exceptionally, *Cyrtosia septentrionalis* had conspicuously higher $\delta^{13}C$ than the other MH species. The divergence is probably due to the fact that *C. septentrionalis* forms mycorrhizae with *Armillaria* (Hamada, 1939), a saprophytic fungus group living on dead or living wood. Zeller *et al.* (2007) showed that $\delta^{13}C$ values of fruiting bodies of *Armillaria* are much higher than those of ectomycorrhizal fungi, such as those with which *Cymbidium* and other MH species coexist (Table S1 and Fig. 3). Thirdly, ^{13}C enrichment in *C. macrorhizon* and *C. aberrans* in comparison with surrounding AT plants (8·9 ± 0·4 ‰ at site A and 10·6 ± 0·8 ‰ at site B) was higher than the range reported for MH plants from temperate regions (6·9 ± 1·5 ‰; Zimmer *et al.*, 2008) and for Japanese MH *Gastrodia confusa* (7·5 ± 0·8 ‰; Ogura-Tsujita *et al.*, 2009). However, ^{13}C enrichment was in the upper range observed for Thai MH orchids (6·8–9·9 ‰; Roy *et al.*, 2009) and for the MH *Gastrodia similis*, a Mascarene MH orchid (11·8 ‰; Martos *et al.*, 2009). $\delta^{15}N$ values for *C. macrorhizon* and *C. aberrans* were also above those of surrounding AT plants (12·9 ± 1·7 ‰ at site A and 13·0 ± 2·2 ‰ at site B) and were in the range reported for other MH plants from temperate regions (11·7 ± 2·3 ‰; Zimmer *et al.*, 2008). In accordance with the above-mentioned results, *C. macrorhizon* has degenerated stomata on its scale leaves, indicating a lack of gas exchange in this species (Yukawa and Stern, 2002).

Chlorophyllous *Cymbidium lancifolium* and *C. goeringii* exhibited higher $\delta^{15}N$ and $\delta^{13}C$ values than co-existing AT orchids and other AT plants and lower values than MH plants (Figs 3 and 4). The estimated level of MX (approx. one-third to one-half in the mean value depending on species and sites; Table 2) indicates that carbon derived from fungi is at least invested partially in aerial parts of the host plant. The values are within the wide range of previous studies (7–85 % of leaf mass depending on species and sites; Gebauer and Meyer, 2003; Bidartondo *et al.*, 2004; Julou *et al.*, 2005; Abadie *et al.*, 2006; Selosse *et al.*, 2006; Tedersoo *et al.*, 2007). The results are congruent with the life history of MX species: after germination, they exhibit an underground phase in which the rhizomes symbiotic with mycorrhizal fungi are the sole vegetative organ for several seasons (Ogura-Tsujita and Yukawa, 2008*a*, *b*). Subsequently, the leafy shoots appear above the ground, but they still maintain mycorrhizal rhizomes (T. Yukawa, unpubl. res.). A probable MX nutrition was also found for *Cephalanthera erecta*, which belongs to a genus rich in MH and MX species in Europe, Asia and America (Taylor and Bruns, 1997; Julou *et al.*, 2005; Abadie *et al.*, 2006; Roy *et al.*, 2009).

Some *Cymbidium* species operate crassulacean acid metabolism (CAM) as a photosynthetic pathway, entailing $\delta^{13}C$ values typically above −20 ‰ (Motomura *et al.*, 2008), i.e. higher than expected for MX and MH species. However, partial CAM photosynthesis is excluded as an explanation for the observed $\delta^{13}C$ values, because *C. lancifolium* and *C. goeringii* have negligible diurnal malate fluctuations and low enzymatic activities relative to those associated with CAM metabolism (Motomura *et al.*, 2008). Consequently, it is likely that $\delta^{13}C$ values in *C. lancifolium* and *C. goeringii* are due to MX nutrition.

Orchid species ubiquitously show obligate heterotrophic associations with fungi during germination and the subsequent juvenile, underground stage (Bernard, 1899; Rasmussen, 1995; Rasmussen and Rasmussen, 2009). However, most species shift to AT with the development of photosynthetic organs, as supported by isotopic analyses (Gebauer and Meyer, 2003; Bidartondo *et al.*, 2004; Abadie *et al.*, 2006; Tedersoo *et al.*, 2007; this study). It is thus reasonable to postulate that the outgroups of the studied taxa are AT, the plesiomorphic condition in the adult stage. Therefore, the MX *Cymbidium lancifolium* and *C. goeringii* evolved from AT ancestors. Further, the phylogenetic relationships showed that MH *C. macrorhizon* and *C. aberrans* appeared within this MX clade (Fig. 2). These results indicate that MH in *Cymbidium* evolved after the establishment of MX rather than directly from it. This pattern of evolution is also likely to exist in several plant groups that include both chlorophyllous and achlorophyllous species, including *Cephalanthera* (Orchidaceae: Abadie *et al.*, 2006), the *Limodorum–Aphyllorchis* clade (Orchidaceae: Roy *et al.*, 2009), the *Corallorhiza–Oreorchis* clade (Orchidaceae: Zimmer *et al.*, 2008) and tribe Pyroleae (Ericaceae: Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007). In these groups, however, evolution of MH nutrition has been inferred from assumptions without using clades in which leaflessness evolved and/or lack of data from sister groups of achlorophyllous species.

Yokoyama *et al.* (2002) and subsequent investigation by Y. Ogura-Tsujita and T. Yukawa (unpubl. res.) found major shifts of mycobionts in *Cymbidium*. The outgroup species of this study generally have only saprophytic mycobionts (mainly Tulasnellaceae, common orchid partners belonging to the *Rhizoctonia* assemblage; Rasmussen, 1995; Yukawa *et al.*, 2009). The MX species (*C. lancifolium* and *C. goeringii*) harbour both Tulasnellaceae and ectomycorrhizal fungi (Russulaceae and others). The MH species *C. macrorhizon* and *C. aberrans* associate exclusively with the ectomycorrhizal fungi. These results indicate that the nutritional shift from AT to MH through MX in *Cymbidium* may correlate with shifts in mycobionts from saprophytic to ectomycorrhizal fungi. This scenario is in line with the evolution of considerable numbers of MH species within clades of chlorophyllous MX species associated with ectomycorrhizal fungi, such as *Cephalanthera* species (Taylor and Bruns, 1997; Bidartondo *et al.*, 2004; Abadie *et al.*, 2006) and Monotropoideae (Ericaceae; Tedersoo *et al.*, 2007).

Light availability is a major limiting factor for plant distribution. The *Cymbidium* species studied here grow on floors of evergreen broadleaved forests, and *C. goeringii*, *C. macrorhizon* and *C. aberrans* are also distributed in warm-temperate deciduous broadleaved or *Pinus* forests (Maekawa, 1971; Du Puy and Cribb, 2007). Light intensities in these habitats are dark to dim in shaded sites of forests, woodland or scrub, except for during winter in deciduous broadleaved forests. MX or MH abilities of these *Cymbidium* species may have enabled them to survive low light conditions. Indeed, the light level at site A is lower than at site B, and MX *C. goeringii* tended to be more heterotrophic at site A than at site B, perhaps adapting to (or suffering from) a lower level of photosynthesis (Table 2). Gebauer (2005) reviewed the same tendencies in other MX orchids.

Adaptation to low light conditions in MX and MH species may have led to niche expansions and radiation in Orchidaceae. As mentioned above, orchid species ubiquitously show obligate MH nutrition during the juvenile stage. Among the seeds dispersed at shady sites, seedlings that extend MH in later stages of growth and operate more efficient nutritional interactions with fungal partners are expected to survive and adapt better to such environments. This process may select for MX and MH species in many independent orchid lineages. The MX and MH species pairs in *Cymbidium* provide an excellent model for future studies on the adaptive mechanism of plants on the forest floor.

In this study, it is demonstrated for the first time that MH plants evolved after the establishment of MX nutrition rather than directly from AT ancestors, suggesting that this course would be one of the principal patterns in the evolution of MH species. Further, the results confirm that the establishment of symbiosis with ectomycorrhizal fungi in the lineage leading to MX *Cymbidium* is a pre-adaptation to the evolution of the species. In addition, the MX and MH species are well-adapted to environments with low light conditions. Similar processes of nutritional innovations probably occurred in several independent orchid groups and may have contributed to niche expansions and radiation in Orchidaceae, probably the largest plant family (approx. 25000 species; Dressler, 2005).

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org. and consist of the following tables. Table S1: $\delta^{15}N$ and $\delta^{13}C$ of fungi and plants growing at site A. Table S2: $\delta^{15}N$ and $\delta^{13}C$ of fungi and plants growing at site B.

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