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Evidence for newly discovered albino mutants in a pyroloid: implication for the nutritional mode in the genus *Pyrola*

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PREMISE: Difficulties in comparing extremely divergent features in fully mycoheterotrophic plants with those in closely related chlorophyllous plants have complicated attempts to reveal the evolutionary patterns and processes of fully mycoheterotrophic plants. Albino mutants of partially mycoheterotrophic plants, generally observed in Orchidaceae, have provided an ideal model for investigating the evolution of mycoheterotrophy within similar genetic backgrounds. In 2018, we found a putative albino population of *Pyrola* (Ericaceae). Here we aimed to reveal the identity of the albino pyroloid and confirm its fully mycoheterotrophic status.

METHODS: To reveal the putative albino pyroloid's identity, we examined its morphology and sequenced its chloroplast DNA. In addition, we assessed the trophic status of the putative albino pyroloid by analyzing chlorophyll fluorescence, chlorophyll concentration, and natural ¹³C and ¹⁵N abundances.

RESULTS: We identified albino individuals as *P. japonica*—otherwise a partially mycoheterotrophic species. We confirmed their albino status by their considerably lower chlorophyll fluorescence and concentrations than those of sympatrically occurring chlorophyllous plants. ¹³C abundance in the albino individuals was significantly higher than in the green individuals of *P. japonica*.

CONCLUSIONS: This first report of albino mutants from partially mycoheterotrophic species in angiosperms other than orchids will play a valuable role in further studies focused on mycoheterotrophy. For instance, their $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values represent a reference for fully mycoheterotrophic plants in *Pyrola*. Our findings also indicate the strong dependence of some leafy *Pyrola* species on fungal C during their entire life cycle.

KEY WORDS *Chimaphila*; Ericaceae; flora; Japan; mixotrophy; *Pyrola aphylla*; *Pyrola japonica* species complex; *Pyrola subaphylla*; stable isotope.

The evolution of fully mycoheterotrophic plants is one of the most interesting and challenging topics of plant evolution (Merckx, 2013). They obtain their nutrients not from autotrophy (photosynthesis), but from heterotrophy with their associated mycorrhizal fungal networks and generally exhibit extreme morphology or ecology, such as achlorophyllous shoots and the absence of ordinary leaves (Merckx, 2013). These extreme features complicate comparisons with closely related chlorophyllous plants (Merckx et al., 2013). Therefore, achlorophyllous (albino) mutants of otherwise partially mycoheterotrophic plants have often been used to understand the evolutionary process of fully mycoheterotrophic plants because they simplify the comparison between photosynthetic plants and fully mycoheterotrophic plants (Merckx et al., 2013; Suetsugu et al.,

2017). In this study, we use the term “partial mycoheterotrophy (partially mycoheterotrophic)” rather than “mixotrophy (mixotrophic)” as recommended by Merckx (2013).

Although partially mycoheterotrophic species, which sometimes produce albino mutants, obtain nutrients through both photosynthesis and mycoheterotrophy regardless of their respective proportions, albino mutants rely solely on mycoheterotrophy (Selosse et al., 2016). Such mutants are known mainly in Orchidaceae, especially in *Epipactis* (e.g., Salmia, 1989) and *Cephalanthera* (e.g., Julou et al., 2005). The discovery of such albino orchids has provided an ideal model for investigating the evolution of mycoheterotrophy within similar genetic backgrounds (e.g., Abadie et al., 2006; Roy et al., 2013; Suetsugu et al.,

2019). Recently, they were also used for studies focusing on genomics or metabolomics (Suetsugu et al., 2017; Lallemand et al., 2019).

In July 2018, we found a population comprising putative albino mutants of *Pyrola* (Pyroleae, Ericaceae); such albino mutants are rarely known outside the family Orchidaceae (Fig. 1). All shoots were white or pale reddish and never green. The genus *Pyrola*, along with Orchidaceae, has attracted attention as an ideal model to examine mycoheterotrophic plants because it includes both putative fully and partially mycoheterotrophic species (Selosse and Roy, 2009; Shutoh et al., 2018). However, to date, albino mutants have never been reported in this genus (Lallemand et al., 2017). They are perennial herbs generally growing in forests and have entomophilous flowers, capsule fruits, and dust seeds like many orchid species (Takahashi, 1993). In addition, recent phylogenetic analysis revealed that their mycoheterotrophic status was independently evolved from fully mycoheterotrophic Monotropaeae within Ericaceae (Lallemand et al., 2016).

Here, we aimed to (1) reveal the identity of the putative albinos based on morphological observation and chloroplast DNA sequences and (2) confirm their fully mycoheterotrophic status based on chlorophyll fluorescence, chlorophyll concentration, and ^{13}C and ^{15}N abundances. Consequently, we report the albino mutants of *P. japonica* Klenze ex Alef. (taxonomically defined by Shutoh et al., 2018) for the first time as those of partially mycoheterotrophic species in Ericaceae. In addition, we discuss the nutritional mode in the plant tribe Pyroleae (Ericaceae) based on our novel discovery.

MATERIALS AND METHODS

Population description

We conducted a field survey in Sapporo-shi, Hokkaido, Japan, on 17 and 18 July 2018. The population comprised three patches, including nine shoots. Among them, two patches, including three shoots (one flowering) and five shoots (two flowering), respectively, were approximately 2 m from each other. The remaining patch, including only one vegetative shoot, occurred in a patch of *P. japonica* about 20 m from the other two patches. Therefore, the population probably included at least three genetic individuals. The population was found for the first time in 2016 by coauthor Y. Tajima and has been observed every year until 2019. Therefore, the population has survived for at least 4 years.

The population is in a young broad-leaved deciduous forest along a road in a graveyard where *Quercus* sp. (*Q. serrata* Murray or its hybrid with *Q. crispula* Blume) was dominant. *Cephalanthera erecta* (Thunb.) Blume and *P. japonica* also occurred as partially mycoheterotrophic species (Matsuda et al., 2012; Sakamoto et al., 2016). Notably, *P. japonica* exhibits plasticity in its heterotrophic levels on fungal C correlated with light availability (Matsuda et al., 2012). In addition, *Anthoxanthum odoratum* L., *Acer palmatum* Thunb. (probably derived from cultivation), *Aria alnifolia* (Siebold & Zucc.) Decne., *Artemisia indica* Willd. var. *maximowiczii* (Nakai) H.Hara, *Carex microtricha* Franch., *Celastrus orbiculatus* Thunb., *Chimaphila japonica* Miq., *Euonymus oxyphyllus* Miq., *Miscanthus sinensis* Andersson, *Sorbus commixta* Hedl., and *Plantago lanceolata* L. occurred sympatrically with the population.

Species identification of putative albinos

To reveal the identity of putative albino mutants, we examined their morphological characteristics and sequenced three noncoding regions of chloroplast DNA. For morphological identification, we recorded qualitative characteristics in flowers and leaves, counted the number of leaves, and measured the length and width of all leaves in all shoots of the putative albino mutants and co-occurring *P. japonica*. The key to Japanese *Pyrola* species by Shutoh et al. (2017) was used for identification. In addition to identification, scape length was measured and flower number on each scape was counted. We looked for any morphological differences between the albinos and the green plants and tested differences using the Mann–Whitney *U* test (leaf number) or Student's *t* test (other traits) in R ver. 3.4.4 (R Core Team, 2018). For molecular identification, we sampled two patches per putative albino mutant and *P. japonica* and sequenced their *trnT-trnL* intergenic spacer, *rpl16* intron, and *ndhA* intron, based on the results of Shutoh et al. (2016), using protocols from DNA extraction to molecular identification by sequence comparisons as described previously (Shutoh et al., 2019), except we used the three primer pairs and annealing temperatures of Shutoh et al. (2016). We collected two putative albino shoots as voucher specimens and stored them in the herbarium of the Hokkaido University Museum (K. Shutoh & Y. Tajima 3000, SAPS53508).

Chlorophyll fluorescence and concentration

To confirm the albino status of the population, we evaluated their chlorophyll fluorescence and chlorophyll concentrations. We measured three shoots of potential albino individuals, one from each of the three patches. In addition, we measured one individual of *Quercus* sp. and three shoots of *P. japonica* as the reference for an autotrophic plant and partially mycoheterotrophic plants (Matsuda et al., 2012), respectively. For measuring chlorophyll fluorescence, we dark-adapted samples for 15 min and then measured their steady-state quantum yield of photosystem II (QY) using FluorPen FP100 (Photon Systems Instruments, Brno, Czech Republic). We defined this value as the ratio between the actual fluorescence yield (F_v) and the maximum fluorescence (F_m) when samples were dark-adapted before measurements. In chlorophyll concentrations, we used a chlorophyll meter (SPAD-502; Konica Minolta Sensing Inc., Osaka, Japan) and measured the soil plant analysis development (SPAD) values. We repeated the SPAD measurements three times per sample. We calculated chlorophyll concentrations (Chl, mg m^{-2}) from the SPAD values according as $\text{Chl} = 1.034 + 0.308(\text{SPAD}) + 0.110(\text{SPAD}^2)$ (Monje and Bugbee, 1992; Stöckel et al., 2011).

Stable isotope analysis

To collect samples for stable isotope analysis, we set up five 2×2 m quadrats around the putative albino plants or *P. japonica* (one quadrat included both species; Appendix S1). Within each quadrat, we sampled putative albino plants and/or *P. japonica* and several surrounding understory plant species as reference plants. We used this strategy to limit the influence of environmental factors, such as atmospheric CO_2 isotope composition, microscale light climate (which could affect C isotope values), and soil type (which could affect N isotope values) on

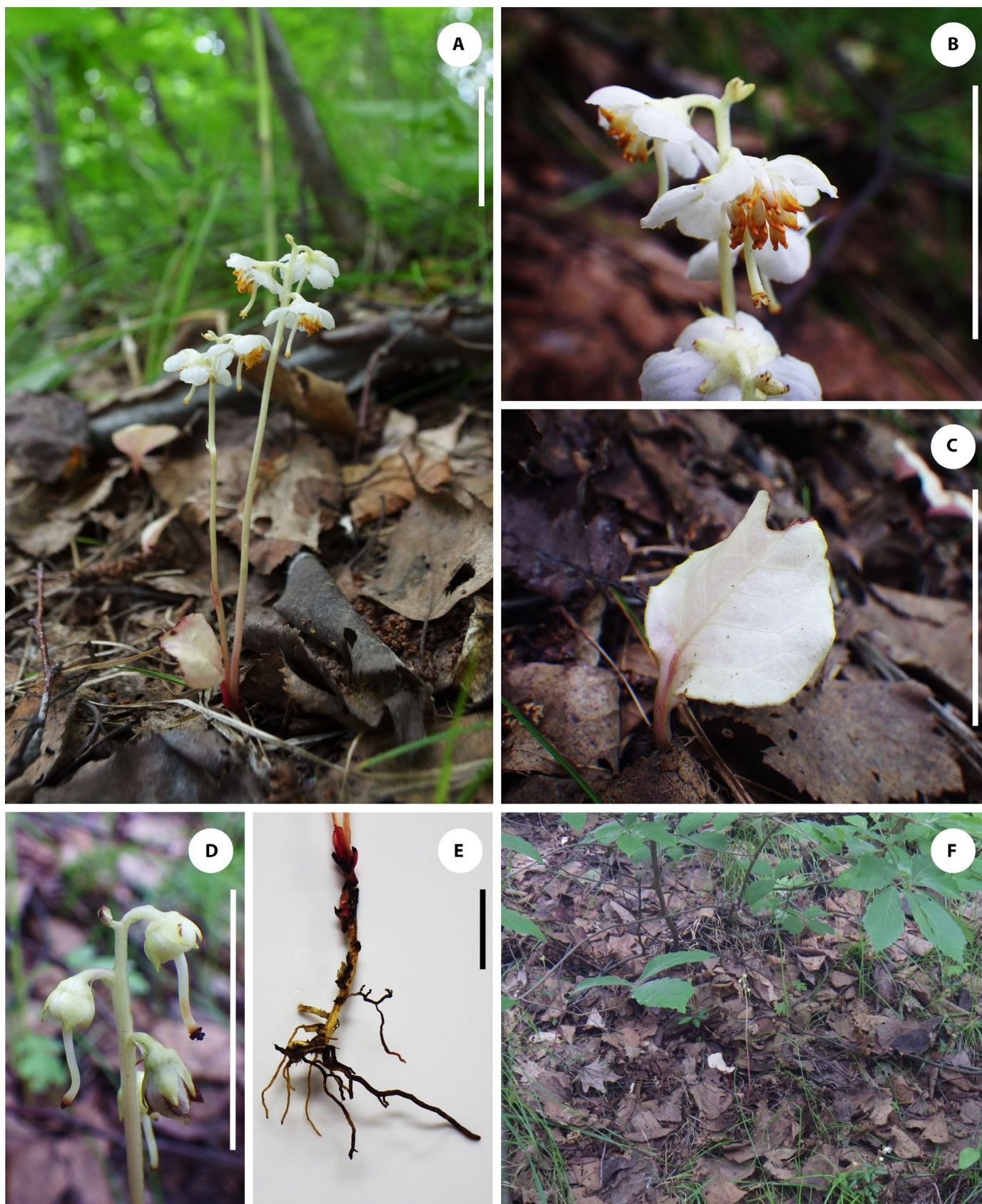


FIGURE 1. Albedo mutants of *Pyrola japonica* at Sapporo-shi, Hokkaido, Japan on 18 July 2018. A, Habit; B, flower; C, developed leaf; D, young fruits; E, roots; F, habitat. Scale bars = 2 cm.

stable isotope ratios (Gebauer and Schulze, 1991). We recognized samples of *Acer palmatum*, *Artemisia indica* var. *maximowiczii*, *Quercus* sp., and *Sorbus commixta* as autotrophic reference species. We sampled flowers, if present, of putative albino plants and/or *P. japonica* and analyzed the flowers and leaves to compare their isotope abundances and provide reference values for flowers of albino plants. The carbon isotope ratio of leaves is known to be lower than that of non-assimilating organs (e.g., Badeck et al., 2005), also reported in *Pyrola* (Hynson et al., 2009). The samples had been dried with silica gel after storage at -30°C until analysis.

We dried the collected plants at 60°C for 4 d and then measured the stable C and N isotopes using a Delta plus XP mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled with a Flash EA 1112 elemental analyzer (Thermo Fisher Scientific) via a ConFlo III interface (Thermo Fisher Scientific). We then calculated the relative abundance of the stable isotopes as $\delta^{15}\text{N}$ or $\delta^{13}\text{C} = (R_{\text{sample}} / R_{\text{standard}} - 1) \times 1000$ [‰], where R_{sample} is the $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$ ratio of the sample, and R_{standard} is the $^{13}\text{C}/^{12}\text{C}$ ratio of Vienna Pee Dee Belemnite (VPDB) or the $^{15}\text{N}/^{14}\text{N}$ ratio of atmospheric nitrogen. We calibrated the isotope ratios of C and N against three laboratory standards: DL-alanine ($\delta^{13}\text{C} = -25.36\text{‰}$, $\delta^{15}\text{N} = -2.89\text{‰}$), L-alanine ($\delta^{13}\text{C} = -19.04\text{‰}$, $\delta^{15}\text{N} = 22.71\text{‰}$), and glycine ($\delta^{13}\text{C} = -34.92\text{‰}$, $\delta^{15}\text{N} = 2.18\text{‰}$), which are traceable back to international standards (Tayasu et al., 2011). The analytical standard deviations (SD) of these standards were 0.17‰ ($\delta^{15}\text{N}$, $n = 6$) for DL-alanine, 0.07‰ ($\delta^{13}\text{C}$, $n = 13$) and 0.14‰ ($\delta^{15}\text{N}$, $n = 13$) for L-alanine, and 0.05‰ ($\delta^{13}\text{C}$, $n = 8$) for glycine. We tested for homogeneity of variances of values for $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ among leaves of the putative albino plants and leaves and flowers of *P. japonica*, *C. japonica*, and autotrophic plant species using Bartlett's test ($P = 0.87$ for $\delta^{13}\text{C}$ and $P = 0.06$ for $\delta^{15}\text{N}$), and differences were tested for significance using Tukey's test after ANOVA ($\delta^{13}\text{C}$, $F_{4,27} = 40.36$, $P < 0.001$; $\delta^{15}\text{N}$, $F_{4,27} = 53.79$, $P < 0.001$) using R ver. 3.4.4. We excluded flowers of the putative albino plants from these statistical analyses because we had fewer than three samples.

For the values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in each quadrat, we also calculated enrichment factors (ϵ) for the putative albino plants, *P. japonica*, and *C. japonica* according to the method by Preiss and Gebauer (2008). After tests of homogeneity of variances for enrichment factors as mentioned above ($P = 0.96$ in ^{13}C and $P = 0.03$ in ^{15}N), their differences were tested for significance using Tukey's test after ANOVA for $\epsilon^{13}\text{C}$ ($F_{3,13} = 21.94$, $P < 0.001$) or Steel–Dwass test (using the Monte Carlo method with 10000 iterations) after Kruskal–Wallis test for ^{15}N ($\chi^2 = 12.88$, $\text{df} = 3$, $P = 0.005$) in R ver. 3.4.4. In addition, we obtained enrichment factors for *P. aphylla*—a fully mycoheterotrophic *Pyrola* distributed in North America—and *P. subaphylla*—a putative nearly fully mycoheterotrophic plant with limited photosynthetic ability (having no or relatively small leaves; Shutoh et al., 2017, 2018)—from previous studies (Hynson et al., 2009; Shutoh et al., 2016) and compared them with those of putative albino plants. Because Shutoh et al. (2016) did not calculate an

enrichment factor, we newly calculated the enrichment factor of *P. subaphylla* based on the raw data set of the study. Care is needed regarding the enrichment factors; they cannot be used simply to compare heterotrophic levels because the values can vary depending on the heterotrophic levels and sampling sites (e.g., Hynson et al., 2009). We also calculated the relative contribution of fungal associations to C gain in *P. japonica* ($\%C_x$) as described by Gebauer and Meyer (2003), with slight modifications: $\%C_x = [(\delta C_p - \delta C_r) / (\delta C_A - \delta C_r)] \times 100$, where δC_p , δC_A , and δC_r is the mean value of the leaves of *P. japonica*, leaves of the putative albino plants, and leaves of the surrounding autotrophic reference plants (within each quadrat), respectively.

RESULTS

Species identification of albinos

The albino population had radially symmetrical flowers, orbicular to elliptic ordinary leaves, and leaf-like scales. All shoots lacked leaves or had one small leaf (mean leaf number $\pm$ SD = 0.9 ± 0.3 ; mean blade length $\pm$ SD = 2.2 ± 0.6 cm, range = 1.4–3.5 cm; mean blade width $\pm$ SD = 2.1 ± 0.7 cm, range = 1.3–3.6 cm) (Table 1). Its scape color was neither red nor green but instead, white with pale reddish, due to albinism (Fig. 1). *Pyrola japonica* had significantly more leaves than in the albino population (mean leaf number $\pm$ SD = 2.3 ± 1.8 ; Mann–Whitney U test, $Z = -2.85$, $P = 0.006$), although the leaf sizes (mean blade length $\pm$ SD = 2.5 ± 1.1 cm, range = 0.7–4.8 cm; mean blade width $\pm$ SD = 2.2 ± 1.2 cm, range = 0.3–4.1 cm) were similar to each other (blade length, Student's t test, $t = -0.67$, $\text{df} = 47$, $P = 0.51$; width, $t = -0.21$, $\text{df} = 47$, $P = 0.83$) (Table 1). Furthermore, fewer flowers and shorter scapes were produced by the albino population (mean flower number $\pm$ SD = 2.8 ± 1.0 , range 2–4; mean scape length $\pm$ SD = 11.9 ± 3.8 cm, range = 7.7–16.4 cm) compared with those of *P. japonica* (mean flower number $\pm$ SD = 6.1 ± 2.3 , range 2–9; mean scape length $\pm$ SD = 16.3 ± 2.3 cm, range = 11.8–21.0 cm; flower number, $t = -2.75$, $\text{df} = 18$, $P = 0.01$; scape length, $t = -2.97$, $\text{df} = 18$, $P = 0.008$) (Table 1). Chloroplast sequences of the albino (LC498633 [*trnT-trnL* spacer], LC498635 [*rpl16* intron], and LC498637 [*ndhA* intron]) were identical to those of *P. japonica* (LC498632, LC498634, and LC498636), corresponding to haplotype C of the *P. japonica* species complex of Shutoh et al. (2016).

Chlorophyll fluorescence and concentration

Chlorophyll fluorescence and chlorophyll concentrations of albinos were considerably lower than those of *Quercus* sp. and *P. japonica* (Table 2). For chlorophyll fluorescence, QY values of the population were 0.00–0.01, and 0.76 ± 0.05 for *P. japonica* and 0.79 for *Quercus* sp. We measured SPAD values of *Quercus* sp. and *Pyrola*

TABLE 1. Leaf number, blade length, blade width, flower number, and scape length of albino *Pyrola japonica* and wild-type *P. japonica* in Sapporo, Hokkaido, Japan.

Type	<i>n</i>	L_N	B_L	B_W	F_N	S_L
Albino	9 (4, 8)	0.9 ± 0.3 A (0–1)	2.2 ± 0.6 A (1.4–3.5)	2.1 ± 0.7 A (1.3–3.6)	2.8 ± 1.0 A (2–4)	11.9 ± 3.8 A (7.7–16.4)
Wild type	18 (16, 41)	2.3 ± 1.8 B (0–8)	2.5 ± 1.1 A (0.7–4.8)	2.2 ± 1.2 A (0.3–4.1)	6.1 ± 2.3 B (2–9)	16.3 ± 2.3 B (11.8–21.0)

Notes: *n*, number of shoots (scapes, leaves) measured; L_N , mean leaf number $\pm$ SD (range); B_L , mean blade length (cm) $\pm$ SD (range); B_W , mean blade width (cm) $\pm$ SD (range); F_N , mean flower number per shoot $\pm$ SD (range); S_L , mean scape length (cm) $\pm$ SD (range). Different letters after values for a trait indicate a significant difference according to Mann–Whitney U test (leaf number) or Student's t test (others; $P < 0.05$).

TABLE 2. Chlorophyll fluorescence and concentration among albino *Pyrola japonica*, wild-type *P. japonica*, and *Quercus* sp. in Sapporo, Hokkaido, Japan.

Species	n	QY	Chl
<i>P. japonica</i> (albino)	3	0.01 ± 0.01	undetectable
<i>P. japonica</i> (wild type)	3	0.76 ± 0.05	173.0 ± 46.2
<i>Quercus</i> sp.	1	0.79	154.7 ± 5.2

Notes: n, number of shoots measured; QY, mean steady-state quantum yield of photosystem II (Φ_{PSII}) ± SD; Chl, mean chlorophyll concentration (mg m⁻²) ± SD, measured three times per sample.

japonica and converted them into chlorophyll concentrations (mean *Quercus* sp. Chl ± SD = 154.7 ± 5.2 mg m⁻²; mean *P. japonica* Chl ± SD = 173.0 ± 46.2 mg m⁻²). However, all samples of albinos showed “error” because their values were less than the minimum limit of detection (SPAD value < 0.1).

Stable isotope analyses

When comparing leaf samples collected from the quadrats, we found the $\delta^{13}\text{C}$ values of albinos (mean $\delta^{13}\text{C}$ ± SD = -27.1 ± 1.1‰) to be significantly higher than those of autotrophic reference plants (-32.2 ± 0.9‰; P < 0.001), green *P. japonica* (-30.9 ± 1.1‰; P < 0.001), and *C. japonica* (-32.5 ± 1.0‰; P < 0.001) (Table 3, Fig. 2; Appendix S1). Meanwhile, we observed no significant differences in the $\delta^{13}\text{C}$ values among *P. japonica*, *C. japonica*, and the autotrophic reference plants (P = 0.19–0.97). In addition, flower samples of *P. japonica* had significantly higher $\delta^{13}\text{C}$ values (-28.2 ± 0.6‰) than leaf samples of the same species (P = 0.01) but not significantly different from those from leaf samples of albinos (P = 0.54). Such a trend was not observed in the albino samples: two flower samples of albinos (-27.6‰) had slightly higher values than flower samples of *P. japonica*, but lower than leaf samples of albinos.

The $\delta^{15}\text{N}$ values in leaf samples of albinos (mean $\delta^{15}\text{N}$ ± SD = 3.0 ± 1.0‰) were significantly higher than those of the autotrophic reference plants (-4.4 ± 2.0‰; P < 0.001; Table 3 and Fig. 2; Appendix S1). However, unlike $\delta^{13}\text{C}$ values, $\delta^{15}\text{N}$ values of these albino samples were not much higher than those of other analyzed pyroloid samples. The highest $\delta^{15}\text{N}$ values were found

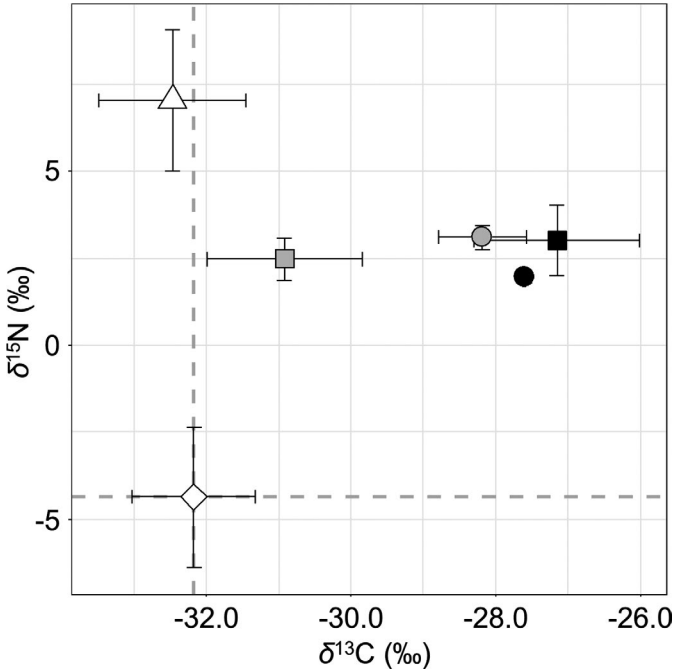


FIGURE 2. Abundance of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in a population of *Pyrola japonica* albino mutants. Black square, albino mutants (leaf); black circle, albino mutants (flower); gray square, wild-type *P. japonica* (leaf); gray circle, wild-type *P. japonica* (flower); white triangle, *Chimaphila japonica* (leaf); white rhombus, autotrophic reference plants (leaf). Plots, error bars, and dashed gray lines indicate, respectively, mean value, standard deviation ($n > 2$), and mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of autotrophic reference plants.

for leaf samples of *C. japonica* (7.0 ± 2.0‰), and they were significantly higher than those of all other analyzed samples (P < 0.03). Values did not differ significantly among leaf samples of albinos, *P. japonica* leaves (2.5 ± 0.6‰; P = 0.99) and *P. japonica* flowers (3.1 ± 0.3‰; P = 0.99). Although $\delta^{15}\text{N}$ values for flower samples of *P. japonica* were slightly higher than in leaf samples of the same

TABLE 3. ^{13}C and ^{15}N abundance ($\delta^{13}\text{C}$ or $\delta^{15}\text{N}$) and enrichment factor ($\epsilon^{13}\text{C}$ or $\epsilon^{15}\text{N}$) of albino *Pyrola japonica*, wild-type *P. japonica*, *Chimaphila japonica*, and autotrophic reference species collected in Sapporo, Hokkaido, Japan and those for *P. subaphylla* and *P. aphylla* obtained from other studies for comparison.

Sample (n)	Mean $\delta^{13}\text{C}$ ± SD (‰)	Mean $\delta^{15}\text{N}$ ± SD (‰)	Mean $\epsilon^{13}\text{C}$ ± SD (‰)	Mean $\epsilon^{15}\text{N}$ ± SD (‰)
<i>Pyrola japonica</i> (albino) leaves (6) ^a	-27.1 ± 1.1 A	3.0 ± 1.0 A	4.8 ± 1.2 A	8.0 ± 0.9 A
<i>P. japonica</i> (albino) flowers (2) ^a	-27.6	1.5	4.5	6.2
<i>P. japonica</i> (normal) leaves (3) ^a	-30.9 ± 1.1 B	2.4	4.3	8.2
<i>P. japonica</i> (normal) flowers (3) ^a	-28.2 ± 0.6 A	2.5 ± 0.6 A	1.5 ± 1.0 B	6.0 ± 0.7 AB
<i>C. japonica</i> leaves (5) ^a	-32.5 ± 1.0 B	3.1 ± 0.3 A	4.2 ± 0.9 A	6.7 ± 0.1 AB
Autotrophic reference species leaves (15) ^a	-32.2 ± 0.9 B	7.0 ± 2.0 B	-0.3 ± 1.2 B	11.3 ± 1.7 B
<i>P. subaphylla</i> flowers (2) ^b	-26.6	-4.4 ± 2.0 C	-	-
<i>P. aphylla</i> stalk or flowers (37) ^c	-25.6	8.4	5.0	12.0
<i>P. aphylla</i> leaves (2) ^c	-25.7 to -22.2 ^d	8.5	5.9	12.0
		10.1–17.9 ^d	6.9 ± 0.9	18.0 ± 2.2
	-27.1	9.0	3.8	12.8
	-24.7	12.4	6.3	15.8

Notes: a, present study; b, Shutoh et al. (2017); c, Hynson et al. (2009); d, range of mean values among populations. Different capital letters after means within a column indicate a significant difference according to Tukey's test ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\epsilon^{13}\text{C}$) or the Steel–Dwass test ($\epsilon^{15}\text{N}$; P < 0.05).

species, the differences were not significant ($P = 0.99$). As with $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ values in flower samples (1.5–2.4‰) were not higher than in leaf samples within albinos.

The mean value of the enrichment factor for ^{13}C in albinos (mean $\pm$ SD = $4.8 \pm 1.2\text{‰}$) was either similar to or lower than for *P. subaphylla* (5.0‰–5.9‰) and *P. aphylla* ($6.9 \pm 0.9\text{‰}$), excluding one leaf sample of *P. aphylla* (3.8‰; Table 3). The other leaf sample of *P. aphylla* (6.3‰) had a value similar to those of flower samples of the same species and of *P. subaphylla* (Hynson et al., 2009). For flower samples, *P. aphylla* had higher values than *P. subaphylla*. For the enrichment factor for ^{15}N , albinos (mean $\pm$ SD = $8.0 \pm 0.9\text{‰}$) had considerably lower values than those of *P. subaphylla* (12.0‰) and *P. aphylla* (12.8–15.8‰ in leaves and $18.0 \pm 2.2\text{‰}$ in stalk or flowers), regardless of the organs analyzed. We evaluated the relative contribution of fungal associations to C gain in *P. japonica* as $30.2 \pm 19.9\%$ (mean $\pm$ SD).

DISCUSSION

Species identification, morphology, and the discovery value of albino plants in *Pyrola*

We identified the albino population based on its chloroplast haplotype and morphology as *P. japonica*, a partially mycoheterotrophic species in the wild. Qualitative morphology of flowers, leaves, and scales suggests that the population belongs, at least, to the *P. japonica* species complex, including *P. japonica* and the nearly fully mycoheterotrophic *P. subaphylla*, with no or relatively small leaves (taxonomically defined by Shutoh et al., 2018). Chloroplast haplotype C, described by Shutoh et al. (2016), is considered to be one of the haplotypes of *P. japonica* (Shutoh et al., 2016, 2017) as also determined for the other *P. japonica* samples in the current study. Although the population is similar to *P. subaphylla* in its smaller leaves, the leaf size of haplotype C remains unknown owing to the scarcity of samples (Shutoh et al., 2017); a population having this haplotype had somewhat smaller leaves than found on plants with the main haplotypes of *P. japonica* (A and D). Therefore, the considerably smaller leaves of the albino individuals does not conflict with their molecular identification.

For quantitative morphological traits other than leaf size, compared with wild-type individuals, albino individuals had significantly shorter scapes and fewer leaves and flowers (Table 1). These morphological differences may be caused by albinism, but more samples are needed to verify this possibility.

In this study, we confirmed the fully mycoheterotrophic status of the population based on its morphology, white plant bodies, very low chlorophyll fluorescence and chlorophyll concentrations, and the higher ^{13}C and ^{15}N abundances. Here we report, for the first time, the occurrence of albino mutants from partially mycoheterotrophic angiosperms outside the family Orchidaceae (Bruce and Beitel, 1979; Johnson-Groh and Lee, 2002; Selosse et al., 2016; Lallemand et al., 2017). Our novel discovery will facilitate the progress of further studies focused on mycoheterotrophic evolution using albino mutants in not only Orchidaceae, but also now in Ericaceae. Such studies should provide valuable findings in terms of comparison with the many findings obtained from Orchidaceae (e.g., Abadie et al., 2006; Roy et al., 2013; Suetsugu et al., 2017, 2019; Lallemand et al., 2019). Further, the albino mutants were fortunately found within the *P. japonica* species complex, which exhibits morphological

similarities and continuous leaf size variation between partially mycoheterotrophic and nearly fully mycoheterotrophic species (Shutoh et al., 2017, 2018). The mutants should also play a significant role as a standard with fully mycoheterotrophic status in further studies using the *P. japonica* species complex (or other *Pyrola* species).

Other putative albino individuals in *Pyrola*

Although a few images of putative albino mutants of some *Pyrola* species can be found on the Internet, their mode of nutrition remains unknown. In two photos of the *P. japonica* species complex taken in 2010 at Fukuoka Prefecture, Japan (http://keiko65.sakura.ne.jp/yamaaruki2/fukuoka/itiyakuso_w100525.htm, accessed on 25 October 2018), leaf size variation in the population or among multiple shoots is unclear; thus, it is difficult to determine whether this mutant belongs to either *P. japonica* or *P. subaphylla* (Shutoh et al., 2017). In addition, a Twitter account that presents plant photographs taken in Wisconsin, USA, uploaded a photo of a white vegetative shoot, which could be an albino mutant of *P. elliptica* Nutt. on 3 June 2017 (@wisconsinflora, <https://twitter.com/wisconsinflora>, accessed on 25 October 2018). These photos thus raise the possibility that albino mutants have been produced not only in the *P. japonica* complex but also in other *Pyrola* species.

Intriguingly, on the basis of these photographs, the plants have a greater number and larger size of leaves than on our albino mutants. In addition, our albino mutants were not *P. subaphylla*, thought to strongly depend on fungal C, but *P. japonica*, typically with potentially developed leaves. These findings suggest that at least some species of the genus *Pyrola*—including the *P. japonica* species complex—rely on mycoheterotrophy to such degrees that they can grow even when lacking photosynthetic ability. Fungal C would significantly contribute to their growth even in the wild, having developed leaves, while a net flow of C from fungus to plant has been questioned in some pyrolids (Lallemand et al., 2017).

Lallemand et al. (2017) suggested the possibility that some pyrolids gain C from the fungus, but that fungal C contributes little to their growth; such plants rely on autotrophy rather than partial mycoheterotrophy (called “C-exchangers”). As evidence, they also noted that albino mutants had never been reported for pyrolids. They also referred to the presence of true partially mycoheterotrophic species based on previous studies and discussed that C-exchangers were predisposed to evolve into these species. It is noteworthy that albino mutants were discovered from *P. japonica*, which was demonstrated to be true partially mycoheterotrophic species by Matsuda et al. (2012). However, as discussed here, albino mutants can exist outside the *P. japonica* species complex. Although its identification needs to be confirmed, *P. elliptica* does not seem to be closely related to true partial mycoheterotrophs such as *P. japonica* (Liu et al., 2010; Lallemand et al., 2017). Any discovery of albino mutants in the genus would facilitate the search for species or lineages with high levels of dependence on fungal and our understanding of the actual status and evolution of mycoheterotrophy in the entire *Pyrola* genus.

Possibly, the emergence of the albino might be associated with the ability to have a flexible nutritional mode driven by the carbon demands related to its chlorophyll concentrations and light availability. Although Lallemand et al. (2017) could not detect any flexible exploitation of mycorrhizal fungi in the five pyrolids species investigated, according to light availability and tissue age, such responses have been reported in some species. Zimmer et al.

(2007) suggested such ability for *Orthilia secunda* (L.) House based on examinations of plants from multiple sites that varied in light availability. Matsuda et al. (2012) showed that *P. japonica* could change its use of fungal C, according to light availability even within the same population. In Orchidaceae, such balanced usage of carbon resources is well known in the genus *Epipactis* and the genus *Cephalanthera*, both containing many albino mutants (Preiss et al., 2010; Stöckel et al., 2011; Gonneau et al., 2014). Albino mutants were able to survive for at least 4 years and reach flowering. Therefore, the emergence of albinos can be related to flexible fungal exploitation. The discovery of albino mutants can facilitate the search for species or lineages exhibiting a flexible degree of mycoheterotrophy.

Heterotrophic levels of albino individuals and other analyzed species

Higher ^{13}C abundance in albino mutants than in wild-type *P. japonica* (partially mycoheterotrophic) and autotrophic reference plants within analyzed quadrats supported the fully mycoheterotrophic status of the albino mutants. In this study, we measured the stable isotope abundances of the mutants in *Pyrola* for the first time; these values can be used as a reference for fully mycoheterotrophic individuals in the genus. In addition, our results on carbon sources suggest an autotrophic status for adult individuals of *Chimaphila japonica*, a member of the tribe Pyroleae, as with *C. umbellata* (Hynson et al., 2012; Lallemand et al., 2016). The higher abundance of nitrogen in albino individuals than in autotrophic reference plants, and the lack of significant differences between albino and wild-type individuals of the same species may have been due not only to their heterotrophic status but also to their phylogeny (Hynson et al., 2016) and/or associated mycorrhizal fungi (Schiebold et al., 2017).

^{13}C was higher in flowers than in leaves in wild-type *P. japonica*, but not in the albino mutants. Assimilating organs, as opposed to other organs, have lower ^{13}C abundance (e.g., Gebauer and Schulze, 1991; Badeck et al., 2005), as measured in *P. aphylla* (Hynson et al., 2009). These results are likely due to leaf dysfunction of the albino mutants; the leaves do not photosynthesize but act as non-assimilating organs. Previously, such differences in $\delta^{13}\text{C}$ between assimilating and non-assimilating organs has been explained by differences in chemical composition such as lipids, proteins, and other secondary carbon metabolites resulting in lower ^{13}C abundance in photosynthetic tissues (Winkler et al., 1978; O'Leary, 1981; Gebauer and Schulze, 1991).

Although we cannot compare heterotrophic levels simply using enrichment factors, it would be fruitful to discuss *P. subaphylla* and *P. aphylla* individually for their overall higher enrichment factors for $\epsilon^{13}\text{C}$ and $\epsilon^{15}\text{N}$ compared with those for the albino mutants. *Pyrola subaphylla* had values higher than or similar to those of the albino mutants, giving the impression that these results supported the fully mycoheterotrophic status of the species. However, these results may be affected by our use of flower samples, which often have higher stable carbon isotope values than in leaves (e.g., Badeck et al., 2005; Hynson et al., 2009), but nitrogen isotope values have never been published. Actually, values for the flower samples of *P. japonica* did not differ significantly from leaves and flowers of the albino mutants. Further stable isotope analyses using various organs are necessary to precisely evaluate the heterotrophic level of *P. subaphylla*.

Meanwhile, *P. aphylla* had considerably higher enrichment factors than our albino mutants, excluding one leaf sample. Although

a previous study evaluated enrichment factors for *P. aphylla* using mainly flowers or scapes (Hynson et al., 2009), these values were also higher than for flower samples of *P. subaphylla*, as mentioned above (Shutoh et al., 2016). We previously found that scape lengths of *P. aphylla* were significantly larger than those of *P. subaphylla* (Shutoh et al., 2018) and proposed the following reason: There might have been positive selection for smaller plants of *P. subaphylla* due to limitations in the carbon availability from fungi; *P. aphylla* might be able to increase its size by some unidentified mechanisms to overcome this limitation. This unidentified mechanism—for instance, *P. aphylla* might be able to obtain more carbon than *P. subaphylla* from the fungus—might explain the high enrichment factors in the species.

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AUTHOR CONTRIBUTIONS

K. Shutoh led the research and wrote the manuscript. Y. Tajima found the albino mutants. K. Suetsugu contributed to designing the research and writing the manuscript. All other authors contributed to data collection and interpretation and critically reviewed the manuscript. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

DATA ACCESSIBILITY

Stable isotope and sequence data are available in Appendix S1 in the online supporting information of this article and the DNA Data Bank of Japan (DDBJ), GenBank, the European Molecular Biology Laboratory (EMBL) database (LC498632–498637), respectively.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

APPENDIX S1. Abundance of ^{13}C and ^{15}N ($\delta^{13}\text{C}$ or $\delta^{15}\text{N}$) per sample among target and reference species collected in Sapporo, Hokkaido, Japan.

LITERATURE CITED

Abadie, J.-C., Ü. Püttsep, G. Gebauer, A. Faccio, P. Bonfante, and M.-A. Selosse. 2006. *Cephalanthera longifolia* (Neottieae, Orchidaceae) is mixotrophic:

- a comparative study between green and nonphotosynthetic individuals. *Canadian Journal of Botany* 84: 1462–1477.
- Badeck, F.-W., G. Tcherkez, S. Nogués, C. Piel, and J. Ghashghaie. 2005. Post-photosynthetic fractionation of stable carbon isotopes between plant organs—a widespread phenomenon. *Rapid Communications in Mass Spectrometry* 19: 1381–1391.
- Bruce, J. G., and J. M. Beitel. 1979. A community of *Lycopodium* gametophytes in Michigan. *American Fern Journal* 69: 33–41.
- Gebauer, G., and M. Meyer. 2003. ^{15}N and ^{13}C natural abundance of autotrophic and myco-heterotrophic orchids provides insight into nitrogen and carbon gain from fungal association. *New Phytologist* 160: 209–223.
- Gebauer, G., and E.-D. Schulze. 1991. Carbon and nitrogen isotope ratios in different compartments of a healthy and a declining *Picea abies* forest in the Fichtelgebirge, NE Bavaria. *Oecologia* 87: 198–207.
- Gonneau, C., J. Jersáková, E. de Tredern, I. Till-Bottraud, K. Saarinen, M. Sauve, M. Roy, et al. 2014. Photosynthesis in perennial mixotrophic *Epipactis* spp. (Orchidaceae) contributes more to shoot and fruit biomass than to hypogeous survival. *Journal of Ecology* 102: 1183–1194.
- Hynson, N. A., K. Preiss, G. Gebauer, and T. D. Bruns. 2009. Isotopic evidence of full and partial myco-heterotrophy in the plant tribe Pyroleae (Ericaceae). *New Phytologist* 182: 719–726.
- Hynson, N. A., S. Mambelli, A. S. Amend, and T. E. Dawson. 2012. Measuring carbon gains from fungal networks in understory plants from the tribe Pyroleae (Ericaceae): a field manipulation and stable isotope approach. *Oecologia* 169: 307–317.
- Hynson, N. A., J. M.-I. Schiebold, and G. Gebauer. 2016. Plant family identity distinguishes patterns of carbon and nitrogen stable isotope abundance and nitrogen concentration in mycoheterotrophic plants associated with ectomycorrhizal fungi. *Annals of Botany* 118: 467–479.
- Johnson-Groh, C. L., and J. M. Lee. 2002. Phenology and demography of two species of *Botrychium* (Ophioglossaceae). *American Journal of Botany* 89: 1624–1633.
- Julou, T., B. Burghardt, G. Gebauer, D. Berveiller, C. Damesin, and M.-A. Selsos. 2005. Mixotrophy in orchids: insights from a comparative study of green individuals and non-photosynthetic mutants of *Cephalanthera damasonium*. *New Phytologist* 166: 639–653.
- Lallemant, F., M. Gaudel, J. Lambourdière, Y. Matsuda, Y. Hashimoto, and M.-A. Selsos. 2016. The elusive predisposition to mycoheterotrophy in Ericaceae. *New Phytologist* 212: 314–319.
- Lallemant, F., Ü. Puttsepp, M. Lang, A. Luud, P.-E. Courty, C. Palancade, and M.-A. Selsos. 2017. Mixotrophy in Pyroleae (Ericaceae) from Estonian boreal forests does not vary with light or tissue age. *Annals of Botany* 120: 361–371.
- Lallemant, F., M.-L. Martin-Magniette, F. Gilard, B. Gakière, A. Launay-Avon, É. Delannoy, and M.-A. Selsos. 2019. *In situ* transcriptomic and metabolomic study of the loss of photosynthesis in the leaves of mixotrophic plants exploiting fungi. *Plant Journal* 98: 826–841.
- Liu, Z.-W., J. Zhou, E.-D. Liu, and H. Peng. 2010. A molecular phylogeny and a new classification of *Pyrola* (Pyroleae, Ericaceae). *Taxon* 59: 1690–1700.
- Matsuda, Y., S. Shimizu, M. Mori, S.-I. Ito, and M.-A. Selsos. 2012. Seasonal and environmental changes of mycorrhizal associations and heterotrophy levels in mixotrophic *Pyrola japonica* (Ericaceae) growing under different light environments. *American Journal of Botany* 99: 1177–1188.
- Merckx, V. S. F. T. 2013. Mycoheterotrophy: an introduction. In V. S. F. T. Merckx [ed.], *Mycoheterotrophy: the biology of plants living on fungi*, 1–17. Springer, New York, NY, USA.
- Merckx, V. S. F. T., C. B. Mennes, K. G. Peay, and J. Geml. 2013. Evolution and diversification. In V. S. F. T. Merckx [ed.], *Mycoheterotrophy: the biology of plants living on fungi*, 215–244. Springer, New York, NY, USA.
- Monje, O. A., and B. Bugbee. 1992. Inherent limitations of nondestructive chlorophyll meters: a comparison of two types of meters. *HortScience* 27: 69–71.
- O'Leary, M. H. 1981. Carbon isotope fractionation in plants. *Phytochemistry* 20: 553–567.
- Preiss, K., and G. Gebauer. 2008. A methodological approach to improve estimates of nutrient gains by partially myco-heterotrophic plants. *Isotopes in Environmental and Health Studies* 44: 393–401.
- Preiss, K., I. K. U. Adam, and G. Gebauer. 2010. Irradiance governs exploitation of fungi: fine-tuning of carbon gain by two partially myco-heterotrophic orchids. *Proceedings of the Royal Society, B, Biological Sciences* 277: 1333–1336.
- R Core Team. 2018. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
- Roy, M., C. Gonneau, A. Rocheteau, D. Berveiller, J.-C. Thomas, C. Damesin, and M.-A. Selsos. 2013. Why do mixotrophic plants stay green? A comparison between green and achlorophyllous orchid individuals in situ. *Ecological Monographs* 83: 95–117.
- Sakamoto, Y., Y. Ogura-Tsujita, K. Ito, K. Suetsugu, J. Yokoyama, J. Yamazaki, T. Yukawa, et al. 2016. The tiny-leaved orchid *Cephalanthera subaphylla* obtains most of its carbon via mycoheterotrophy. *Journal of Plant Research* 129: 1013–1020.
- Salmia, A. 1989. General morphology and anatomy of chlorophyll-free and green forms of *Epipactis helleborine* (Orchidaceae). *Annales Botanici Fennici* 26: 95–105.
- Schiebold, J. M.-I., M. I. Bidartondo, P. Karasch, B. Gravendeel, and G. Gebauer. 2017. You are what you get from your fungi: nitrogen stable isotope patterns in *Epipactis* species. *Annals of Botany* 119: 1085–1095.
- Selsos, M.-A., and M. Roy. 2009. Green plants that feed on fungi: facts and questions about mixotrophy. *Trends in Plant Science* 14: 64–70.
- Selsos, M.-A., M. F. Bocayuva, M. C. M. Kasuya, and P.-E. Courty. 2016. Mixotrophy in mycorrhizal plants: extracting carbon from mycorrhizal networks. In F. Martin [ed.], *Molecular mycorrhizal symbiosis*, 451–471. John Wiley, Hoboken, NJ, USA.
- Shutoh, K., S. Kaneko, K. Suetsugu, Y. I. Naito, and T. Kurosawa. 2016. Variation in vegetative morphology tracks the complex genetic diversification of the mycoheterotrophic species *Pyrola japonica* sensu lato. *American Journal of Botany* 103: 1618–1629.
- Shutoh, K., S. Kaneko, and T. Kurosawa. 2017. Taxonomy and distribution of *Pyrola subaphylla* Maxim. (Pyroleae, Ericaceae). *Acta Phytotaxonomica et Geobotanica* 68: 181–192.
- Shutoh, K., K. Suetsugu, S. Kaneko, and T. Kurosawa. 2018. Comparative morphological analysis of two parallel mycoheterotrophic transitions reveals divergent and convergent traits in the genus *Pyrola* (Pyroleae, Ericaceae). *Journal of Plant Research* 131: 589–597.
- Shutoh, K., T. Yamanouchi, S. Kato, H. Yamagishi, Y. Ueno, S. Hiramatsu, J. Nishihiro, et al. 2019. The aquatic macrophyte flora of a small pond revealing high species richness in the Aomori Prefecture, Japan. *Journal of Asia-Pacific Biodiversity* 12: 448–458.
- Stöckel, M., C. Meyer, and G. Gebauer. 2011. The degree of mycoheterotrophic carbon gain in green, variegated and vegetative albino individuals of *Cephalanthera damasonium* is related to leaf chlorophyll concentrations. *New Phytologist* 189: 790–796.
- Suetsugu, K., M. Yamato, C. Miura, K. Yamaguchi, K. Takahashi, Y. Ida, S. Shigenobu, et al. 2017. Comparison of green and albino individuals of the partially mycoheterotrophic orchid *Epipactis helleborine* on molecular identities of mycorrhizal fungi, nutritional modes and gene expression in mycorrhizal roots. *Molecular Ecology* 26: 1652–1669.
- Suetsugu, K., M. Yamato, J. Matsubayashi, and I. Tayasu. 2019. Comparative study of nutritional mode and mycorrhizal fungi in green and albino variants of *Goodyera velutina*, an orchid mainly utilizing saprotrophic rhizoctonia. *Molecular Ecology* 28: 4290–4299.
- Takahashi, H. 1993. Pyrolaceae. In K. Iwatsuki, T. Yamazaki, D. E. Boufford, and H. Ohba [eds.], *Flora of Japan*, vol. IIIa: Angiospermae dicotyledoneae sympetalae (a), 64–70. Kodansha, Tokyo, Japan.
- Tayasus, L., R. Hirasawa, N. O. Ogawa, N. Ohkouchi, and K. Yamada. 2011. New organic reference materials for carbon- and nitrogen-stable isotope ratio measurements provided by Center for Ecological Research, Kyoto University, and Institute of Biogeosciences, Japan Agency for Marine-Earth Science and Technology. *Limnology* 12: 261–266.
- Winkler, F. J., E. Wirth, E. Latzko, H.-L. Schmidt, W. Hoppe, and P. Wimmer. 1978. Influence of growth conditions and development on $\delta^{13}\text{C}$ values in different organs and constituents of wheat, oat and maize. *Zeitschrift für Pflanzenphysiologie* 87: 255–263.
- Zimmer, K., N. A. Hynson, G. Gebauer, E. B. Allen, M. F. Allen, and D. J. Read. 2007. Wide geographical and ecological distribution of nitrogen and carbon gains from fungi in pyroloids and monotropoids (Ericaceae) and in orchids. *New Phytologist* 175: 166–175.