| Title | Evidence for newly discovered albino mutants in a pyroloid: implication for the nutritional mode in the genus Pyrola |
|----------------------|----------------------------------------------------------------------------------------------------------|
| Author(s) | Shutoh, Kohtaroh / Tajima, Yuko / Matsubayashi, Jun / Tayasu, Ichiro / Kato, Syou / Shiga, Takashi / Suetsugu, Kenji |
| Citation | American Journal of Botany, 107(4):650-657 |
| Issue date | 2020-04 |
| Resource Type | Journal Article / 学術雑誌論文 |
| Resource Version | publisher |
| Rights | © 2020 The Authors. American Journal of Botany published by Wiley Periodicals LLC on behalf of Botanical Society of America. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes. |
| DOI | 10.1002/ajb2.1462 |
| URL | http://www.lib.kobe-u.ac.jp/handle_kernel/90007290 |

PDF issue: 2020-11-05
Evidence for newly discovered albino mutants in a pyroloid: implication for the nutritional mode in the genus *Pyrola*

Kohtaroh Shutoh1,7, Yuko Tajima2, Jun Matsubayashi3, Ichiro Tayasu4, Syou Kato5, Takashi Shiga5, and Kenji Suetsugu6,7

Manuscript received 20 September 2019; revision accepted 4 February 2020.

1 The Hokkaido University Museum, Hokkaido University, Kita 10, Nishi 8, Kita-ku, Sapporo, Hokkaido 060-0810, Japan
2 The Hokkaido University Museum, Hokkaido University, Kita 10, Nishi 8, Kita-ku, Sapporo, Hokkaido 060-0810, Japan
3 Department of Biogeochemistry, Japan Agency for Marine-Earth Science and Technology, 2-15 Natsumisima-cho, Yokosuka, Kanagawa 237-0061, Japan
4 Research Institute for Humanity and Nature, 457-4 Motoyama, Kamigamo, Kita-ku, Kyoto 603-8501, Japan
5 Faculty of Education, Niigata University, 2-8050, Ikarashi, Nishi-ku, Niigata 950-2181, Japan
6 Nishi-ku, Sapporo, Hokkaido 060-0810, Japan
7 Authors for correspondence (e-mail: shutoh@museum.hokudai.ac.jp; kenji-suetsugu@gmail.com)

Citation: Shutoh, K., Y. Tajima, J. Matsubayashi, I. Tayasu, S. Kato, T. Shiga, and K. Suetsugu. 2020. Evidence for newly discovered albino mutants in a pyroloid: implication for the nutritional mode in the genus *Pyrola*. American Journal of Botany 107(4): 650–657. doi:10.1002/ajb2.1462

**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 13C and 15N 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 a chlorophyllous shoots and the absence of ordinary leaves (Merckx, 2013). These extreme features complicate comparisons with closely related chlorophyllous plants (Merckx et al., 2013). Therefore, chlorophyllous (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., 2013).
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 concentrations, 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. (*Quercus 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 palatum* 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 oxypollus* Miq., *Misanthus 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 albino 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 *trn T*-*trn L* 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 Chl = 1.034 + 0.308(SPAD) + 0.110(SPAD$^2$) (Monje and Bugbee, 1992; Stoßel et al., 2011).

**Stable isotope analysis**

To collect samples for stable isotope analysis, we set up five 2 x 2 m quadrats around the putative albino plants or *P. japonica* (one quadrant 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. Albino 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 δ13N or δ13C = (Rsample / Rstandard − 1) × 1000 [‰], where Rsample is the 13C/12C or 15N/14N ratio of the sample, and Rstandard is the 13C/12C ratio of Vienna Pee Dee Belemnite (VPDD) or the 15N/14N ratio of atmospheric nitrogen. We calibrated the isotope ratios of C and N against three laboratory standards: dl-alanine (δ13C = −25.36‰, δ15N = −2.89‰), l-alanine (δ13C = −19.04‰, δ15N = 22.71‰), and glycine (δ13C = −34.92‰, δ15N = 2.18‰), which are traceable back to international standards (Tayasu et al., 2011). The analytical standard deviations (SD) of these standards were 0.17‰ (δ13N, n = 6) for dl-alanine, 0.07‰ (δ13C, n = 13) and 0.14‰ (δ15N, n = 13) for l-alanine, and 0.05‰ (δ13C, n = 8) for glycine. We tested for homogeneity of variances for δ13C or δ15N 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 δ13C and P = 0.06 for δ15N), and differences were tested for significance using Tukey’s test after ANOVA (δ13C, F4,13 = 40.36, P < 0.001; δ15N, F4,13 = 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 δ13C and δ15N 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 13C and P = 0.03 in 15N), their differences were tested for significance using Tukey’s test after ANOVA for ε13C (F13,13 = 21.94, P < 0.001) or Steel–Dwass test (using the Monte Carlo method with 10000 iterations) after Kruskal–Wallis test for 15N (χ2 = 12.88, 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) as described by Gebauer and Meyer (2003), with slight modifications: %C = [(δCsample − δCplant) / (δCplant − δCsoil)] × 100, where δCsample, δCplant, and δCsoil are 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 ± SD = 0.9 ± 0.3; mean blade length ± SD = 2.2 ± 0.6 cm, range = 1.4–3.5 cm; mean blade width ± 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 ± SD = 2.3 ± 1.8; Mann–Whitney U-test, Z = −2.85, P = 0.006), although the leaf sizes (mean blade length ± SD = 2.5 ± 1.1 cm, range = 0.7–4.8 cm; mean blade width ± 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, df = 47, P = 0.51; width, t = −0.21, df = 47, P = 0.83) (Table 1). Furthermore, fewer flowers and shorter scapes were produced by the albino population (mean flower number ± SD = 2.8 ± 1.0, range 2–4; mean scape length ± SD = 11.9 ± 3.8 cm, range 7.7–16.4 cm) compared with those of P. japonica (mean flower number ± SD = 6.1 ± 2.3, range 2–9; mean scape length ± SD = 16.3 ± 2.3 cm, range 11.8–21.0 cm; flower number, t = −2.75, df = 18, P = 0.01; scape length, t = −2.97, 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         | n     | Lb (cm) | Bl (cm) | Fb (cm) | Fl (cm) | Lf (cm) | Sf (cm) |
|--------------|-------|---------|---------|---------|---------|---------|---------|
| Albino       | 9 (4,8) | 0.9 ± 0.3 | 2.2 ± 0.6 | 2.1 ± 0.7 | 2.8 ± 1.0 | 119 ± 3.8 |
| Wild type    | 18 (16,41) | 2.3 ± 1.8 | 2.5 ± 1.1 | 2.2 ± 1.2 | 6.1 ± 2.3 | 163 ± 2.3 |

Notes: n, number of shoots (scapes, leaves) measured; Lb, mean blade length (cm) ± SD (range); Bl, mean blade width (cm) ± SD (range); Fb, mean flower number per shoot ± SD (range); Fl, mean scape length (cm) ± 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 ± SD (mg m⁻²) |
|--------------------------|----|-----|-------------------|
| *P. japonica* (albino)   | 3  | 0.01±0.01 | undetectable     |
| *P. japonica* (wild type)| 3  | 0.76±0.05  | 173.0±46.2       |
| *Quercus* sp.            | 1  | 0.79 | 154.7±5.2         |

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

Stable isotope analyses

When comparing leaf samples collected from the quadrats, we found the δ¹³C values of albinos (mean δ¹³C ± 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 δ¹³C 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 δ¹³C values (−28.2 ± 0.6‰) than leaf samples of the same species (P = 0.01) but not significantly different from those 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 δ¹⁵N values in leaf samples of albinos (mean δ¹⁵N ± 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 δ¹³C values, δ¹⁵N values of these albino samples were not much higher than those of other analyzed pyroloid samples. The highest δ¹⁵N values were found 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 δ¹³C values for flower samples of *P. japonica* were slightly higher than in leaf samples of the same

TABLE 3. δ¹³C and δ¹⁵N abundance (δ¹³C or δ¹⁵N) and enrichment factor (ε¹³C or ε¹⁵N) 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. aphyllya* obtained from other studies for comparison.

| Sample                  | Mean δ¹³C ± SD (%) | Mean δ¹⁵N ± SD (%) | Mean ε¹³C ± SD (%) | Mean ε¹⁵N ± SD (%) |
|-------------------------|-------------------|-------------------|-------------------|-------------------|
| *Pyrola japonica* (albino) leaves (6) | −27.1 ± 1.1 A    | 3.0 ± 1.0 A       | 48.1 ± 1.2 A      | 8.0 ± 0.9 A       |
| *P. japonica* (albino) flowers (2) | −27.6             | 1.5               | 4.5               | 6.2              |
| *P. japonica* (normal) leaves (3) | −30.9 ± 1.1 B    | 2.5 ± 0.6 A       | 1.5 ± 1.0 B       | 6.0 ± 0.7 AB     |
| *P. japonica* (normal) flowers (3) | −28.2 ± 0.6 A    | 3.1 ± 0.3 A       | 4.2 ± 0.9 A       | 6.7 ± 0.1 AB     |
| *C. japonica* leaves (5) | −32.5 ± 1.0 B    | 7.0 ± 2.0 B       | −0.3 ± 1.2 B      | 11.3 ± 1.7 B     |
| Autotrophic reference species leaves (15) | −32.2 ± 0.9 B    | −4.4 ± 2.0 C     | -                 | -                |
| *P. subaphylla* flowers (2) | −26.6             | 8.4               | 5.0               | 12.0             |
| *P. aphyllya* stalk or flowers (37) | −25.7 to −22.2 A | 10.1–17.9 A      | 6.9 ± 0.9         | 18.0 ± 2.2       |
| *P. aphyllya* leaves (2) | −27.1             | 9.0               | 3.8               | 12.8             |

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 (δ¹³C, δ¹⁵N, and ε¹³C) or the Steel–Dwass test (ε¹⁵N; 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 \(^{13}\text{C}\) in albinos \((\text{mean} \pm \text{SD} = 4.8 \pm 1.2\%)\) was either similar to or lower than for \(P. \text{subaphylla} \quad (5.0\%–5.9\%)\) and \(P. \text{aphylla} \quad (6.9 \pm 0.9\%)\), excluding one leaf sample of \(P. \text{aphylla} \quad (3.8\%\); Table 3). The other leaf sample of \(P. \text{aphylla} \quad (6.3\%)\) had a value similar to those of flower samples of the same species and of \(P. \text{subaphylla}\) \((\text{Hynson et al., 2009})\). For flower samples, \(P. \text{aphylla}\) had higher values than \(P. \text{subaphylla}\). For the enrichment factor for \(^{15}\text{N}\), albinos \((\text{mean} \pm \text{SD} = 8.0 \pm 0.9\%)\) had considerably lower values than those of \(P. \text{subaphylla} \quad (12.0\%)\) and \(P. \text{aphylla} \quad (12.8–15.8\%)\); the enrichment factor for \(^{13}\text{C}\) in albinos \((12.0\%)\) had somewhat smaller leaves than found on plants with \(P. \text{subaphylla}\), with no or relatively small leaves \((\text{taxonomically defined by Shutoh et al., 2018})\). Chloroplast haplotype \(C\), described by Shutoh et al. \((2016)\), is considered to be one of the haplotypes of \(P. \text{japonica}\) \((\text{Shutoh et al., 2016, 2017})\) as also determined for the other \(P. \text{japonica}\) samples in the current study. Although the population is similar to \(P. \text{subaphylla}\) in its smaller leaves, the leaf size of haplotype \(C\) remains unknown owing to the scarcity of samples \((\text{Shutoh et al., 2017})\); a population having this haplotype had somewhat smaller leaves than found on plants with the main haplotypes of \(P. \text{japonica} \quad (A\) 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 \((\text{Table 1})\). 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 \(^{13}\text{C}\) and \(^{15}\text{N}\) abundances. Here we report, for the first time, the occurrence of albino mutants from partially mycoheterotrophic angiosperms outside the family Orchidaceae \((\text{Bruce and Beitel, 1979; Johnson-Groh and Lee, 2002; Selosse et al., 2016; Lallemand et al., 2017})\). 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 \((\text{e.g., Abadie et al., 2006; Roy et al., 2013; Suetsugu et al., 2017, 2019; Lallemand et al., 2019})\). Further, the albino mutants were fortunately found within the \(P. \text{japonica}\) species complex, which exhibits morphological similarities and continuous leaf size variation between partially mycoheterotrophic and nearly fully mycoheterotrophic species \((\text{Shutoh et al., 2017, 2018})\). The mutants should also play a significant role as a standard with fully mycoheterotrophic status in further studies using the \(P. \text{japonica}\) species complex \((\text{or other Pyrola species})\).

**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 \(P. \text{japonica}\) species complex taken in 2010 at Fukuoka Prefecture, Japan \((\text{http://keiko65.sakura.ne.jp/yamaaruki2/fukuoka/itiyakuso_w100525.htm, accessed on 25 October 2018})\), leaf size variation in the population or among multiple shoots is unclear; thus, it is difficult to determine whether these plants belong to either \(P. \text{japonica}\) or \(P. \text{subaphylla}\) \((\text{Shutoh et al., 2017})\). 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 \(P. \text{eliptica}\) \((\text{Nutt. on 3 June 2017 (@wisconsinflora, https://twitter.com/wisconsinflora, accessed on 25 October 2018})\). These photos thus raise the possibility that albino mutants have been produced not only in the \(P. \text{japonica}\) 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 \(P. \text{subaphylla}\), thought to strongly depend on fungal \(C\), but \(P. \text{japonica}\), typically with potentially developed leaves. These findings suggest that at least some species of the genus \(Pyrola\) \((\text{including the} P. \text{japonica} \text{species complex—rely on mycoheterotrophy to such degrees that they can grow even when lacking photosynthetic ability. Fungal} \text{C would significantly contribute to their growth even in the wild, having developed leaves, while a net flow of} \text{C from fungus to plant has been questioned in some pyroloids (Lallemand et al., 2017})\).

Lallemand et al. \((2017)\) suggested the possibility that some pyroloids gain \(C\) from the fungus, but that fungal \(C\) contributes little to their growth; such plants rely on autotrophy rather than partial mycoheterotrophy \((\text{called} \text{“C-exchangers”})\). As evidence, they also noted that albino mutants had never been reported for pyroloids. 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 \(P. \text{japonica}\), which was demonstrated to be true partially mycoheterotrophic species by Matsuda et al. \((2012)\). However, as discussed here, albino mutants can exist outside the \(P. \text{japonica}\) species complex. Although its identification needs to be confirmed, \(P. \text{elliptica}\) does not seem to be closely related to true partial mycoheterotrophs such as \(P. \text{japonica}\) \((\text{Liu et al., 2010; Lallemand et al., 2017})\). 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 pyroloids species investigated, according to light availability and tissue age, such responses have been reported in some species. Zimmer et al.
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 $^{13}$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 $^{13}$C and $^{15}$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.

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 ($^{13}$C or $^{15}$N) per sample among target and reference species collected in Sapporo, Hokkaido, Japan.

LITERATURE CITED

Abadie, J.-C., Ü. Püttspepp, 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. Ghachghaie. 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. 15N and 13C 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 hypo-
egeous 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 ecto-
mycorrhizal fungi. *Annals of Botany* 118: 467–479.

Johnson-Groh, C. L., and J. M. Lee. 2002. Phenology and demography of two species of *Bostrychium* (Ophioglossaceae). *American Journal of Botany* 89: 1624–1633.

Julou, T., B. Burghardt, G. Gebauer, D. Berveiller, C. Damesin, and M.-A. Selosse. 2019. Mixotrophy in mycorrhizal plants: extracting carbon from mycorrhizal net-
works. *In F. Martin [ed.], Molecular mycorrhizal symbiosis, 451–471. John Wiley, Hoboken, NJ, USA.

Shutoh, K., S. Kaneko, K. Suesutsu, Y. I. Naito, and T. Kurosa. 2016. Variation in vegetative morphology tracks the complex genetic diversification of the myco-
heterotrophic species *Pyrola japonica* sensu lato. *American Journal of Botany* 103: 1618–1629.

Shutoh, K., S. Kaneko, and T. Kurosa. 2017. Taxonomy and distribution of *Pyrola subaphylla* Maxim. (Pyroleae, Ericaceae). *Acta Phytotaxonomica et Geobotanica* 68: 181–192.

Shutoh, K., K. Suesutsu, S. Kaneko, and T. Kurosa. 2018. Comparative morpho-
logical analysis of two parallel mycorrhizotrophic 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 mycorheterotrophic carbon gain in green, variegated and vegetative albino individuals of *Cephalanthera damasonium* is related to leaf chlorophyll concentrations. *New Phytologist* 189: 790–796.

Suesutsu, 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 mycorrhizotrophic orchid *Epipactis helleyrione* on molecular iden-
tities of mycorrhizal fungi, nutritional modes and gene expression in mycor-
rhizal roots. *Molecular Ecology* 26: 1652–1669.

Suesutsu, K., M. Yamato, J. Matsubayashi, and I. Tayasu. 2019. Comparative study of nutritional mode and mycorrhizal fungi in green and albino variants of *Goodyera velatina*, an orchid mainly utilizing saprotrophic rhizomonia. *Molecular Ecology* 28: 4290–4299.

Takahashi, H. 1993. Pyroleaceae. In *K. Iwatsuki, T. Yamazaki, D. E. Boufford, and H. Oliva [eds.], Flora of Japan, vol. IIIa: Angiospermae dicotyledoneae sympeta-
liae (a), 64–70. Kodansha, Tokyo, Japan.

Tayasu, R. Hirasawa, N. O. Ogawa, N. Ohkouchi, and K. Yamada. 2011. New organic re-
ference 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. *Linnmology* 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 δ13C 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.