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REGULAR PAPER – TAXONOMY/PHYLOGENETICS/EVOLUTIONARY BIOLOGY

Communities of mycorrhizal fungi in different trophic types of Asiatic *Pyrola japonica* sensu lato (Ericaceae)

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Abstract

Mixotrophic plants obtain carbon by their own photosynthetic activity and from their root-associated mycorrhizal fungi. Mixotrophy is deemed a pre-adaptation for evolution of mycoheterotrophic nutrition, where plants fully depend on fungi and lose their photosynthetic activity. The aim of this study was to clarify mycorrhizal dependency and heterotrophy level in various phenotypes of mixotrophic *Pyrola japonica* (Ericaceae), encompassing green individuals, rare achlorophyllous variants (albinos) and a form with minute leaves, *P. japonica* f. *subaphylla*. These three phenotypes were collected in two Japanese forests. Phylogenetic analysis of both plants and mycorrhizal fungi was conducted based on DNA barcoding. Enrichment in ^{13}C among organs (leaves, stems and roots) of the phenotypes with reference plants and fungal fruitbodies were compared by measuring stable carbon isotopic ratio. All plants were placed in the same clade, with f. *subaphylla* as a separate subclade. Leaf ^{13}C abundances of albinos were congruent with a fully mycoheterotrophic nutrition, suggesting that green *P. japonica* leaves are 36.8% heterotrophic, while rhizomes are 74.0% heterotrophic. There were no significant differences in δ^{13}C values among organs in both albino *P. japonica* and *P. japonica* f. *subaphylla*, suggesting full and high mycoheterotrophic nutrition, respectively. Among 55 molecular operational taxonomic units (OTUs) detected as symbionts, the genus *Russula* was the most abundant in each phenotype and its dominance was significantly higher in albino *P. japonica* and *P. japonica* f. *subaphylla*. *Russula* spp. detected in *P. japonica* f. *subaphylla* showed higher dissimilarity with other phenotypes. These results suggest that *P. japonica* sensu lato is prone to evolve mycoheterotrophic variants, in a process that changes its mycorrhizal preferences, especially towards the genus *Russula* for which this species has a marked preference.

Keywords Albino · DNA barcoding · Evolution · Mixotrophy · *Russula* · Stable isotope

Introduction

Non-parasitic achlorophyllous plants were once believed to grow as saprotrophs by decomposing soil organic matter (Leake 1994). However, during the last decades, such

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plants were found to acquire carbon, together with mineral nutrients, from the mycorrhizal fungi colonizing their roots, and are now called mycoheterotrophic plants (Leake 1994; Leake et al. 2004; Merckx 2013; Selosse and Roy 2009; Trudell et al. 2003). Mycoheterotrophic plants reverse the carbon flow usual for the mycorrhizal associations, where plant photosynthates are exchanged for mineral resources gained by fungal symbionts. Interestingly, mycoheterotrophy is not so rare among plants. In fact, autotrophic plants evolved independently into mycoheterotrophic plants many times, and this trophic type is present in around 880 extant species from 101 genera belonging to 17 families (Merckx et al. 2013). Moreover, the evolution and nutritional mode of mycoheterotrophic plants provide insight into the coevolution of interactions between plants and fungi (Jacquemyn and Merckx 2019).

In terms of evolution to mycoheterotrophic nutrition, certain forest green plants mixing both photosynthesis and mycoheterotrophic nutrition have lately excited considerable interest. This nutrition mode, called mixotrophy or partial mycoheterotrophy, has been especially well studied in the family Orchidaceae and in the subfamily Pyroloideae within Ericaceae (hereafter pyroloids) (Braukmann and Stefanović 2012; Gebauer and Meyer 2003; Julou et al. 2005; Selosse et al. 2017). Mixotrophic nutrition may even be more widespread in other families than hitherto expected (Gomes et al. 2019). Based on phylogenetic analyses (e.g. Lallemand et al. 2016; Ogura-Tsujita et al. 2012; Selosse and Roy 2009), mixotrophic nutrition is believed to be a pre-adaption to evolve mycoheterotrophy, and understanding mixotrophic physiology provides insights into the evolutionary shift to mycoheterotrophic nutrition (Roy et al. 2013).

In mycoheterotrophic and mixotrophic orchids and pyroloids, the use of resources from mycorrhizal fungi entails a spontaneous enrichment of ^{13}C and ^{15}N stable isotopes as compared to autotrophic plants, reflecting the ^{13}C and ^{15}N abundance of the mycorrhizal fungi themselves, which form ectomycorrhizas on surrounding trees (Gebauer and Meyer 2003; Hynson et al. 2013a; Trudell et al. 2003). Mycoheterotrophic plants, being entirely dependent on fungal carbon, have ^{13}C abundances similar to their fungal C source, while green, autotrophic plants have lower ^{13}C abundances due to carbon fractionation during photosynthesis. The ^{13}C and ^{15}N abundances in mixotrophic plants are intermediate between autotrophic and mycoheterotrophic plants and their ^{13}C abundance can be used to evaluate the dependency level on mycorrhizal fungi whenever references values are available for autotrophic and mycoheterotrophic plants (Gebauer and Meyer 2003; Hynson et al. 2013a; Julou et al. 2005; Selosse and Martos 2014). This dependency level can even be evaluated at the organ level and depending on the season (Gonneau et al. 2014; Roy et al. 2013). In addition, ^{13}C abundances in mixotrophic plants from different environments show that higher light availability can shift mixotrophic nutrition towards autotrophy, while lower light availability shifts it towards mycoheterotrophy (Gonneau et al. 2014; Matsuda et al. 2012; May et al. 2020; Preiss et al. 2010). When placed in shaded conditions, autotrophic plants shift to lower ^{13}C enrichment, because higher stomatal conductance and lower photosynthesis speed allow a better turn-over of leaf internal CO_2 and avoid an increase in $^{13}CO_2$ concentration during preferential $^{12}CO_2$ uptake (see mechanism in Farquhar et al. 1982). This entails a decreased ^{13}C abundance in biomass, while mixotrophic plants that rely more on fungal C in shaded conditions display a shift to higher ^{13}C abundance (e.g. Matsuda et al. 2012).

Behind physiological plasticity, a specificity for fungal symbionts is frequent in mycoheterotrophic and mixotrophic plants. Some species have a high genus and even species level specificity (Cullings et al. 1996; Girlanda et al. 2006; Gomes et al. 2017; Matsuda et al. 2011), while other (especially mixotrophic) plants are less specific (e.g. in Ericaceae: Hynson and Bruns 2009; Toftegaard et al. 2010; Vincenot et al. 2008, but see Uesugi et al. 2016 for the species used in the current work).

Knowledge of mixotrophic plant physiology and their transition to mycoheterotrophy has gained a lot of attention because of a fascinating natural phenomenon, namely the survival of achlorophyllous variants, also called albinos, among mixotrophic species (Julou et al. 2005; Lallemand et al. 2019; Selosse et al. 2004; Suetsugu et al. 2019, 2020). These albinos are mainly known from mixotrophic orchids, where they do not significantly differ in mycorrhizal fungi from green individuals, but, as expected for mycoheterotrophic organisms, are enriched in ^{13}C abundances compared to green individuals (Abadie et al. 2006; Gebauer and Meyer 2003; Julou et al. 2005; Suetsugu et al. 2019).

For pyroloids, which encompass several mixotrophic (Johansson et al. 2017; Matsuda et al. 2012; Tedersoo et al. 2007) and two mycoheterotrophic species (Hynson et al. 2009; Lallemand et al. 2016; Shutoh et al. 2020), evidence of mixotrophic nutrition remains mixed. Some putatively mixotrophic pyroloids do not typically respond to shade by increasing ^{13}C abundances (Lallemand et al. 2017). Yet, Zimmer et al. (2007) reported increased ^{13}C abundance for *Orthilia secunda* under low irradiance and the East Asian *Pyrola japonica* (Takahashi 1991) responds to decreased light level by increasing ^{13}C abundance (Matsuda et al. 2012). The later species differs from other *Pyrola* species by a higher fungal specificity toward Russulaceae (Uesugi et al. 2016), and interestingly, the abundance of this symbiont is increased in shaded conditions (Matsuda et al. 2012). In addition, although albinos are very rare in pyroloids, an albino *P. japonica* was recently investigated by Shutoh et al. (2020), revealing an absence of photosynthetic pigments

and high ^{13}C abundance, as expected for a mycoheterotrophic plant. However, the mycorrhizal fungal community of such albinos remains unclear.

Finally, two subgroups of *Pyrola* contain varieties or even species that are close to mycoheterotrophy (Liu et al. 2010, 2014): these two geographically vicariant, sister groups are the American *P. picta* section that includes the mycoheterotrophic *P. aphylla* (Jolles and Wolfe 2012) and the above-mentioned Asiatic *P. japonica* section that contains *P. japonica* f. *subaphylla*, a taxon with degenerated leaves (Liu et al. 2010, 2014). *Pyrola japonica* f. *subaphylla* is sometimes treated as a separate species, *P. subaphylla* (Shutoh et al. 2017). Thus, the two phenotypes of *P. japonica*, namely albinos and f. *subaphylla*, can represent evolutionary pathways to mycoheterotrophic nutrition in pyroloids.

The aim of this study was to characterize the mycorrhizal communities and nutritional modes of *P. japonica* sensu lato in a comparative design between the typical green individuals and the two derived phenotypes, albinos and f. *subaphylla*. For this purpose, we sampled green *P. japonica* and neighbouring albinos or f. *subaphylla* and compared the ^{13}C enrichments of their different organs as well as their mycorrhizal communities. We hypothesized that (1) a higher mycoheterotrophy is achieved in the albinos and f. *subaphylla* phenotypes, and (2) exactly as in green *P. japonica* growing in shaded conditions, we expected an increased dominance of *Russula* spp. in these two phenotypes as compared to green individuals.

Materials and methods

Study sites and plant collections

We sampled two Japanese *P. japonica* populations, Chiba in 2019 (CH, 35°36´ N, 140°24’W), and Hokkaido in 2014 (HK, 42°69´ N, 141°72´ W; Fig. S1). The sites were ca. 800 km from each other. The CH site is a mixed forest in a warm-temperate zone where ectomycorrhizal *Castanopsis sieboldii* and arbuscular mycorrhizal *Cryptomeria japonica* dominate and, as understorey plants, seedlings of the dominant trees, sporadic *Trachelospermum asiaticum* (Apocynaceae) and *Pleioblastus chino* bamboos grow. The HK site is a deciduous broad-leaved forest in a cool-temperate zone dominated by *Quercus crispula*, *Q. serrata*, *Q. dentata* and *Betula platyphylla* (all ectomycorrhizal) without understorey. During the sampling year, the mean annual temperature and precipitation level were 15.9 °C and 1821.5 mm at CH, and 7.3 °C and 890.0 mm at HK.

In addition to typical green *P. japonica* individuals, albinos occurred in the CH population (Fig. 1a). Five individuals of each phenotype were collected within a 10 × 10 m plot. As autotrophic references for ^{13}C measurements, we collected leaves of *T. asiaticum* and *P. chino* ($n = 3$ each), *C. sieboldii* seedlings ($n = 2$) and fruitbodies of *Russula* spp. ($n = 3$). At HK, *P. japonica* f. *subaphylla* also occurred with typical green *P. japonica* individuals (Fig. 1b, c) and both phenotypes were collected at three plots (10 × 10 m) distanced ca. 50 m from each other (one representative of each phenotype per plot).

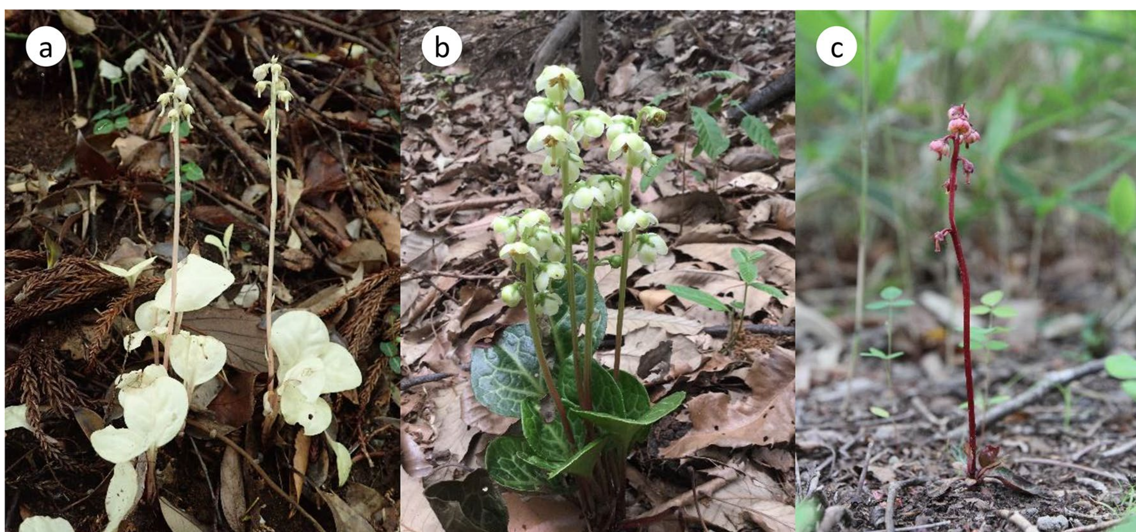

Fig. 1 *Pyrola japonica* phenotypes in naturally regenerated forests in Japan. **a** Fruiting albino *P. japonica* at Chiba; **b** flowering green *P. japonica* and **c** fruiting *P. japonica* f. *subaphylla* at Hokkaido

For molecular identification of mycorrhizal fungi and pyroloid plants, we sampled six root segments (3 cm long) per pyroloid plant and the stem or leaves; all samples were stored at – 20 °C until DNA extraction. For ^{13}C measurements, different organs (leaves, rhizomes and mycorrhizal roots) were harvested from each pyroloid plant, together with the *Russula* fruitbodies and leaves of autotrophic plants. All leaf samples were collected at a similar light level and at a similar distance from the soil to avoid bias due to the CO_2 released from soil—for this reason, no autotrophic references were available at HK. All samples for ^{13}C analysis were dried in an oven for 48 h at 50 °C.

Stable isotope analyses

Dried samples were ground to fine powder with 3 mm ceramic balls using a Beads Cell Disrupter (Micro Smash, MS-100, TOMY, Tokyo). We measured ^{13}C abundance using a Flash 2000 elemental analyzer linked online via a Conflo IV interface to a Delta V Advantage continuous flow isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). We calculated the relative abundances of the stable isotopes as $\delta^{13}C$ or $\delta^{15}N = (R_{sample} / R_{standard} - 1) \times 1000$ (‰), where R_{sample} is the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio of the sample, and $R_{standard}$ is the $^{13}C/^{12}C$ ratio of Vienna Pee Dee Belemnite or $^{15}N/^{14}N$ ratio of atmospheric N_2. In the CH site, we also estimated the percentage of mycoheterotrophic C gain in biomass of green mixotrophic *P. japonica*, *x*, using a linear mixing ratio (Gebauer and Meyer 2003; Shutoh et al. 2020): $x = [(\delta C_P - \delta C_R) / (\delta C_A - \delta C_R)] \times 100\%$, where δC_P, δC_A, and δC_R are the mean $\delta^{13}C$ values of the leaves of green *P. japonica*, leaves of albino *P. japonica*, and surrounding autotrophic reference plants, respectively. Due to lack of achlorophyllous reference plant species at the site CH, we used albino *P. japonica* as achlorophyllous reference. This was calculated for non-colonized organs, i.e. leaves and rhizomes, as for colonized ones the results would be biased (roots are mycorrhizal).

DNA identifications of fungi and plant taxa

DNA was extracted from five 2-mm root segments per plant and from three dried *Russula* fruitbodies with the DNeasy Plant Mini Kit (QIAGEN, Tokyo), and used for polymerase chain reaction (PCR). From individual root segments, we amplified the internal transcribed spacer (ITS) region of fungal ribosomal DNA with TaKaRa ExTaq™ (Takara, Otsu, Japan) using the primer set ITS1F (Gardes and Bruns 1993) and TW13 (Tedersoo et al. 2006) and the fruitbodies were amplified with ITS1F and ITS4 (White et al. 1990). PCR reactions were carried out with a thermal cycler (TaKaRa PCR Thermal Cycler Dice, Model TP600, Ohtsu, Japan) under the following conditions: initial cycle of 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 2 min, and the last period of 72 °C for 10 min, with negative controls. After PCR reactions, five successful amplicons from each plant individual were pooled as one sample for cloning analyses (one cloning analysis per plants) using the TOPO® TA Cloning Kit (Invitrogen, CA, USA) or the TA-Enhancer Cloning Kit (Nippon gene, Tokyo, Japan). Twenty-four colonies were used for colony PCR using the EmeraldAmp® MAX PCR Master Mix kit (TaKaRa, Ohtsu, Japan) with the same primer set as above. The PCR products were purified with ExoSAP-IT™ (Affymetrix, USA) and prepared for sequencing with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA), before analysis with ABI 3730 (Applied Biosystems, CA, USA). Ambiguous sites of obtained sequences were manually adjusted using MEGA v. 7.0.21 (Kumar et al. 2016) and fungal DNA sequences were aligned using the MAFFT online service (Katoh and Standley 2013) under the L-ins-i strategy, and further assigned to molecular operational taxonomic units (OTUs) at a 97% cutoff level using Mothur v.1.36 (Schloss et al. 2009). Each representative sequence was submitted to BLAST search (Altschul et al. 1997) in order to determine the closest known fungal taxa from the public available database DDBJ/EMBL/GenBank. After chimeric and contaminant sequences were removed, the clone number in OTUs were treated as abundance data for following community analyses. Since russulacean fungi occurred abundantly in all pyroloid phenotypes (See "Results"), six representative *Russula* OTUs originating from root samples and from the three fruitbodies in this study with OTUs from our previous study conducted in central Japan (Uesugi et al. 2016) were aligned with other similar *Russula* sequences in the International Nucleotide Sequence Database Collaboration by MAFFT with the L-ins-I strategy, and otherwise with default settings (Katoh and Standley 2013). A maximum likelihood tree was constructed with 1000 bootstrap replications based on the Kimura 2 parameter + G distribution model selected by the function implemented in MEGA7 (Kumar et al. 2016). No outgroups were used to construct the tree.

To infer taxonomic identity of pyroloid plants in the study, sequences of both ITS and *mat*K regions were obtained. We used *circa* 30 mg of albino, green *P. japonica*, or *P. japonica* f. *subaphylla* tissues for DNA extraction with the DNeasy Plant Mini Kit. PCR reactions were performed as stated above for mycorrhizal roots, except for primer sets: the ITS and *mat*K regions were amplified with the primer pairs ITS1 and ITS4 (White et al. 1990) and 3F_KIM and 1R_KIM (CBOL Plant Working Group 2009), respectively. Thermal cycles for the ITS were also done as above (see for fungal ITS), and for *mat*K an initial cycle of 94 °C for 3 min was followed by 30 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min, with a last cycle of 72 °C for 10 min.

A molecular phylogenetic tree of the genus *Pyrola* was constructed including *Pyrola* sequences retrieved from DDBJ/EMBL/GenBank. *Pyrola* sequences of both ITS and *mat*K regions were retrieved following Liu et al. (2014) adding two neighbouring taxa; *Moneses uniflora* and *Chimaphila japonica* as outgroup for the genus *Pyrola* (Lallemand et al. 2016). For the reference *P. japonica*, we concatenated sequences from two different origins, namely an ITS sequence from South Korea (AB758656) and a *mat*K sequence from Japan (KX290484; Lallemand et al. 2016). The concatenated alignment of ca. 1400 bp was built with MAFFT online (Katoh and Standley 2013). A maximum likelihood tree was then constructed with 1,000 bootstrap replications based on the Kimura 3 parameter + G distribution model selected by the function implemented in MEGA7 (Kumar et al. 2016). The DNA sequences obtained were deposited at DDBJ under accession numbers LC534717–LC534771 for mycorrhizal fungi, LC547862– LC547864 for *Russula* fruitbodies, and LC535078–LC535109 for plants.

Data analyses

To evaluate differences in $\delta^{13}C$ and $\delta^{15}N$ values between measurements we used one-way analysis of variance (ANOVA) followed by the Tukey HSD multiple comparison test for data with normal distribution and the Kruskal–Wallis test followed by a post-hoc Steel–Dwass test for data which did not have normal distribution using the pSDCFlig function in the NSM3 package (Schneider et al. 2020) in R 4.0.2 (R Core Team 2020).

In order to compare the fungal sequences obtained from mycorrhizal roots, rarefaction curves of the number of OTUs were processed using vegan package v. 2.5-2. (Oksanen et al. 2018) implemented in R with 1000 time replications without replacement. OTU diversity was quantified by the Shannon–Wiener index, Simpson's diversity index and Fisher's alpha index implemented in the vegan package. On each site, OTU composition and shared fungi between plant phenotypes were visualized by Venn diagrams. Differences in the composition between sites were evaluated using the Fisher's exact test. Moreover, we calculated the Bray–Curtis dissimilarity matrix based on clone abundance of fungal OTUs for each pair of plants using the "designdist" function in the vegan package. The matrix was used as input for hierarchical clustering with hclust (method = ward.D2) in stats package in R. To compare mycorrhizal communities among pyroloid populations, non-metrical multidimensional scaling (NMDS) using the Chao distance was performed at each site and the segregation among them was examined with function "adonis", in the vegan package to perform permutational multivariate analysis of variance (PERMANOVA, permutations = 9999). The same analysis was also conducted for *Russula* spp. communities.

To examine the taxonomic differences in mycorrhizal fungal taxa associated with each phenotype of pyroloids, i.e. albino or green *P. japonica* and *P. japonica* f. *subaphylla*, the indicator species analysis was conducted with the function Multipatt in the R package of indicspecies, using 9999 permutations (De Cáceres and Legendre 2009). Thus, data on green *P. japonica* collected at both CH and HK sites were set as the same group in this test. The type I error, α, was fixed at 5%, unless otherwise stated.

Results

Phenotype identity in *Pyrola japonica*

ITS and *mat*K sequences of pyroloid plants were all successfully obtained and best matched, according to a BLAST search, to those from *P. japonica* (i.e. AB758656 for ITS and KX290484 for *mat*K; Table S1). In a phylogenetic tree (Fig. 2), albino and green *P. japonica* and *P. japonica* f. *subaphylla* from both sites clustered together with reference *P. japonica* individuals from GenBank, and clearly differed from other *Pyrola* taxa supported by 99% of bootstrap values. Among the examined pyroloids, *P. japonica* f. *subaphylla* formed a subclade distinct from available samples, with 63% bootstrap support; its ITS and matK sequences differed by 0.2% and 0.1%, respectively, from the consensus sequence for green *P. japonica* from HK.

Stable isotopic abundances of pyroloid plants

At CH, green leaves of *P. japonica* were significantly richer in $\delta^{13}C$ than autotrophic plant leaves and albino *P. japonica* leaves were even more enriched (Fig. 3). A linear mixing model with autotrophic plants and albino *P. japonica* as autotrophic and mycoheterotrophic references, respectively, indicates that green *P. japonica* leaves were $x = 36.8\%$ heterotrophic. For $\delta^{15}N$ values, the green and albino leaves of *P. japonica* had a significantly higher value than autotrophic plants, but did not differ significantly from the *Russula* fruitbodies. Besides, leaves of green *P. japonica* from CH site were significantly more depleted in ^{13}C than rhizomes and roots (Fig. 4): these two underground organs were thus more heterotrophic, but did not significantly differ between each other. There were no significant $\delta^{13}C$ variations among all organs in albino *P. japonica*, showing equal heterotrophy levels. Moreover, none of the albino samples differed from ectomycorrhizal *Russula* spp. fruitbodies (Figs. 3 and 4).

At HK, $\delta^{13}C$ and $\delta^{15}N$ values in green *P. japonica* leaves were significantly lower than those in *P. japonica* f. *subaphylla* leaves (Fig. 5). The different organs of both *P. japonica* and *P. japonica* f. *subaphylla* did not differ in ^{13}C and ^{15}N abundances (Tukey HSD test, $P > 0.05$). However, ^{13}C

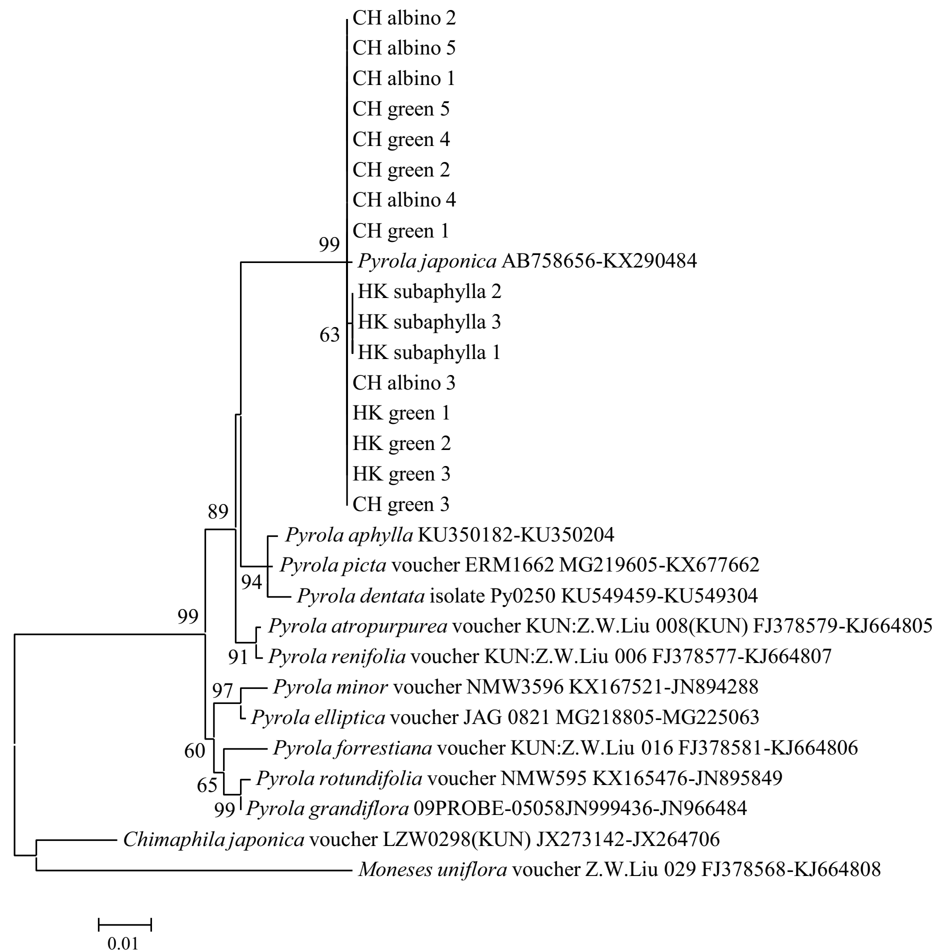

Fig. 2 A maximum likelihood tree of the genus *Pyrola* based on ITS2 and *mat*K regions, including the samples of this study: green and albino *P. japonica* collected at Chiba (CH green and CH albino, respectively), and green *P. japonica* and *P. japonica* f. *subaphylla* collected at Hokkaido (HK green and HK f. subaphylla, respectively). Bootstrap values higher than 60% are shown on branches

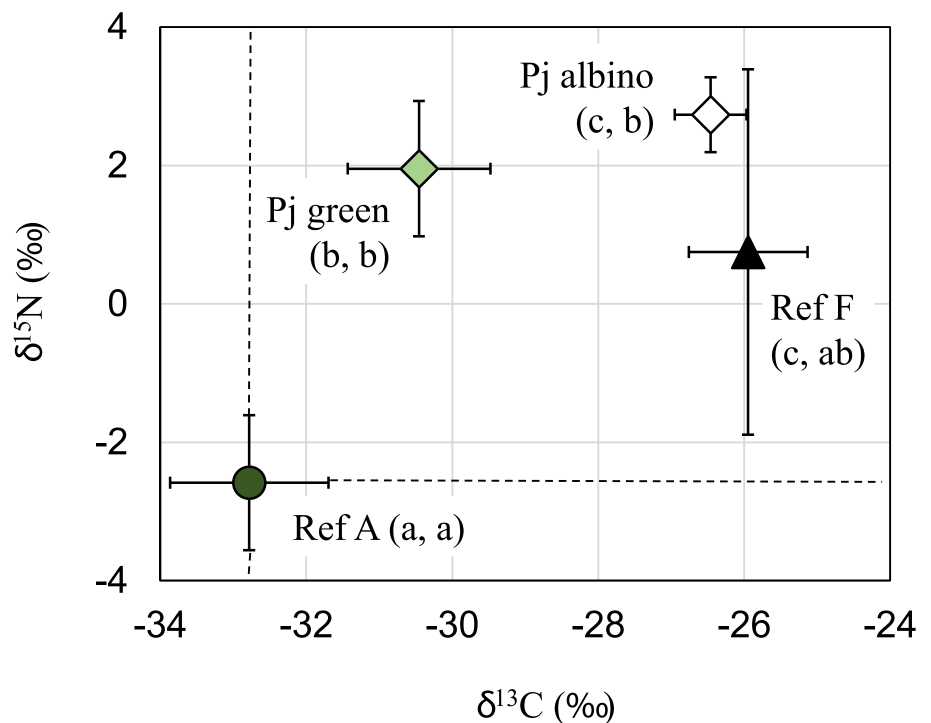

Fig. 3 δ^{13}C and δ^{15}N abundance in *Pyrola japonica* leaves at Chiba. Green (green diamond, Pj green) and albino (white diamond, Pj albino) *Pyrola japonica* ($n=5$), autotrophic plants (dark green circle, Ref A) pool of *Trachelospermum asiaticum* ($n=3$), *Pleioblastus chino* ($n=3$) and *Castanopsis sieboldii* ($n=2$), and *Russula* fruitibodies (black tiriangle, Ref F, $n=3$). Different letters in brackets (the first letter for δ^{13}C and the second one for δ^{15}N) indicate significant differences between samples (Kruskal-Wallis test with a post-hoc Steel-Dwass test; $P<0.05$). Dotted lines indicate mean δ^{13}C and δ^{15}N of autotrophic references

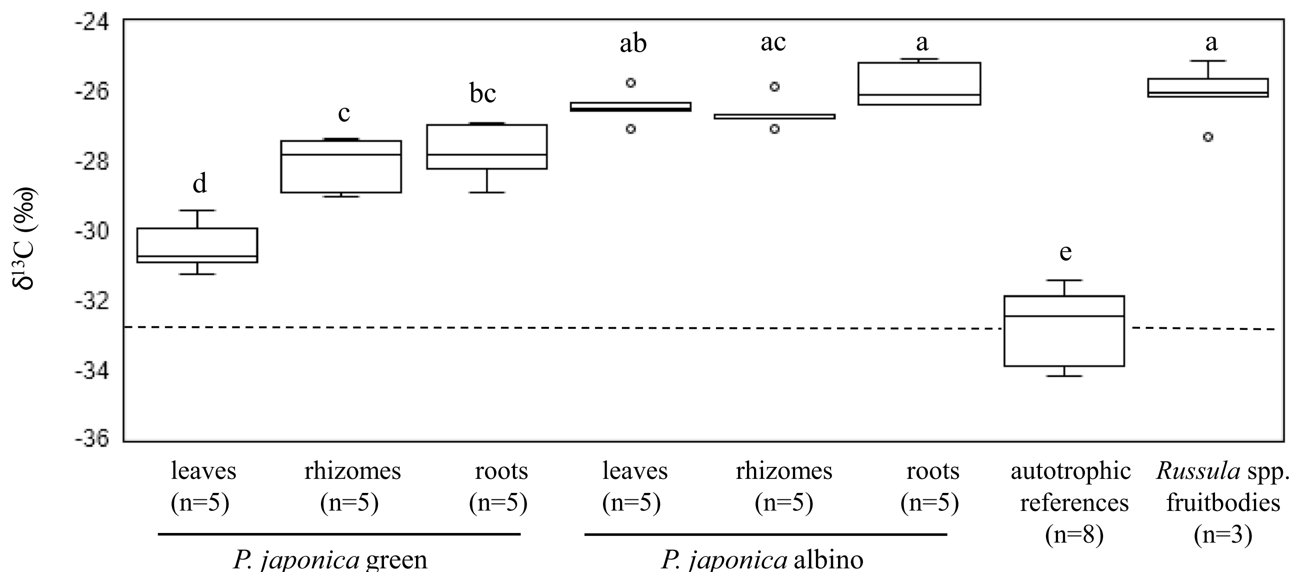

Fig. 4 ^{13}C abundance in different organs of *Pyrola japonica* at Chiba. Green and albino *Pyrola japonica* ($n=5$ each), autotrophic plants pool *Trachelospermum asiaticum* ($n=3$), *Pleioblastus chino* ($n=3$) and *Castanopsis sieboldii* ($n=2$) and fruitbodies of *Russula* spp. ($n=3$). Different letters indicate significant differences between samples (one-way ANOVA followed by multiple comparisons using the Tukey-HSD test; $P<0.05$). Boxplots indicate quartiles on both sides of the median and outliers, and a dotted line indicates mean δ^{13}C of autotrophic references

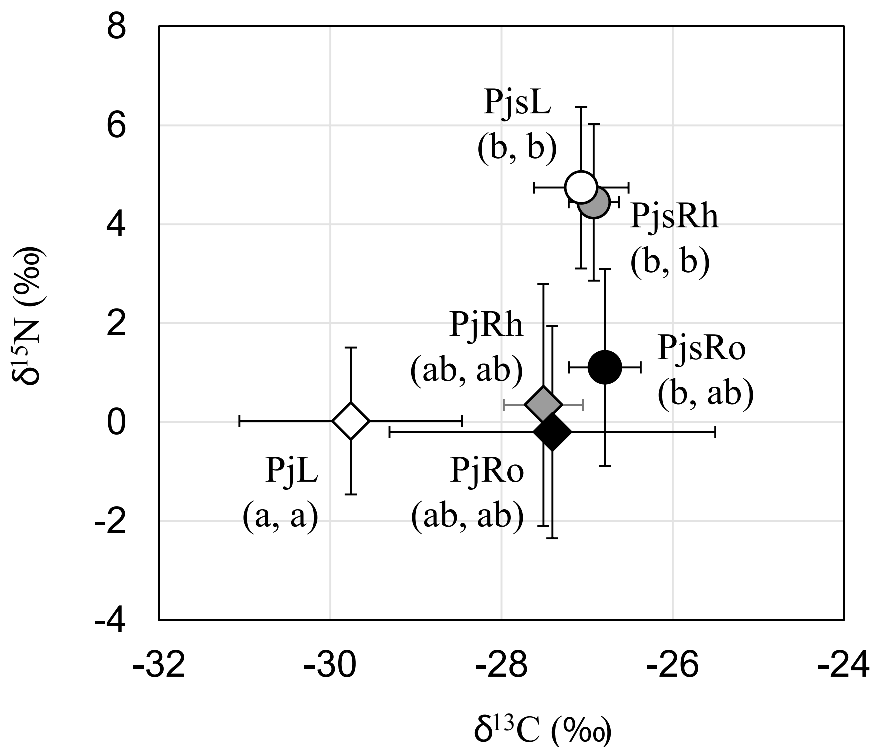

Fig. 5 δ^{13}C and δ^{15}N abundance in different organs of *Pyrola japonica* and neighbouring *P. japonica* f. *subaphylla* at Hokkaido. Symbols show leaves (white diamond, PjL), rhizome (grey diamond, PjRh) and roots (black diamond, PjRo) of *Pyrola japonica* and leaves (white circle, PjsL), rhizome (grey circle, PjsRh) and roots (black circle, PjsRo) of *P. japonica* f. *subaphylla*. Different letters in brackets (the first letter for δ^{13}C and the second one for δ^{15}N) indicate significant differences (one-way ANOVA followed by multiple comparisons using the Tukey-HSD test; $P<0.05$). Neither autotrophs nor fruitbodies available for this site

abundance of green *P. japonica* tended to be lower than its rhizomes and roots. Unfortunately, neither understorey reference plants nor fruitbodies of ectomycorrhizal fungi were available at HK.

Fungal community composition in pyroloid plants

Sequences obtained from 228 to 132 clones from CH and HK, respectively, were grouped into 55 OTUs (Table S2). Rarefaction curves did not saturate with the number of plants investigated (Fig. S2), but (1) accumulation curves of OTUs for albinos at CH and for *P. japonica* f. *subaphylla* at HK were lower compared to that of green *P. japonica*, and (2) accumulation curves at CH were higher than at HK. Yet, there were no significant differences in diversity indexes, i.e. Shannon, Simpson and Fisher's alpha, between phenotypes at both sites (ANOVA, $P > 0.05$, Table S3).

In total, 90.9% of OTUs belonged to Basidiomycota (21 OTUs) and Ascomycota (29 OTUs). Basidiomycota was mostly ectomycorrhizal fungal genera such as *Russula*, *Inocybe, Tomentella* and *Cortinarius*, while Ascomycota was mostly endophytic taxa e.g. Helotiales (Fig. 6; Table S2). Based on clone abundance, *Russula* spp. dominated with 6 OTUs accounting for 49.2% (177/360) of the clones, which reached 78.3% (177/226) of the clones when considering only ectomycorrhizal groups (Table S2). At CH, *Russula* sp.1 was the most abundantly detected taxon (27.7% for green- and 55.2% for albino-derived clones) and its sequence best matched in a BLAST search with mycorrhizal fungi from *P. japonica* collected in Mie prefecture (Central Japan, LC096795; Figs. S1, S3). At HK, *Russula* sp. 2 was the most abundant, being exclusively detected from *P. japonica* f. *subaphylla* accounting for 69.0% of clones: this taxon was also detected in pyroloids from CH and best matched in a BLAST search with *P. japonica* root collected in Aichi prefecture (Central Japan; LC096388; Figs. S1, S3). In addition, other *Russula* sp.4, sp.5 and sp.6 also best matched with fungi identified from *P. japonica* collected at the Mie or Aichi prefecture (Table S2). The representative *Russula* OTUs found in *P. japonica* roots and the three

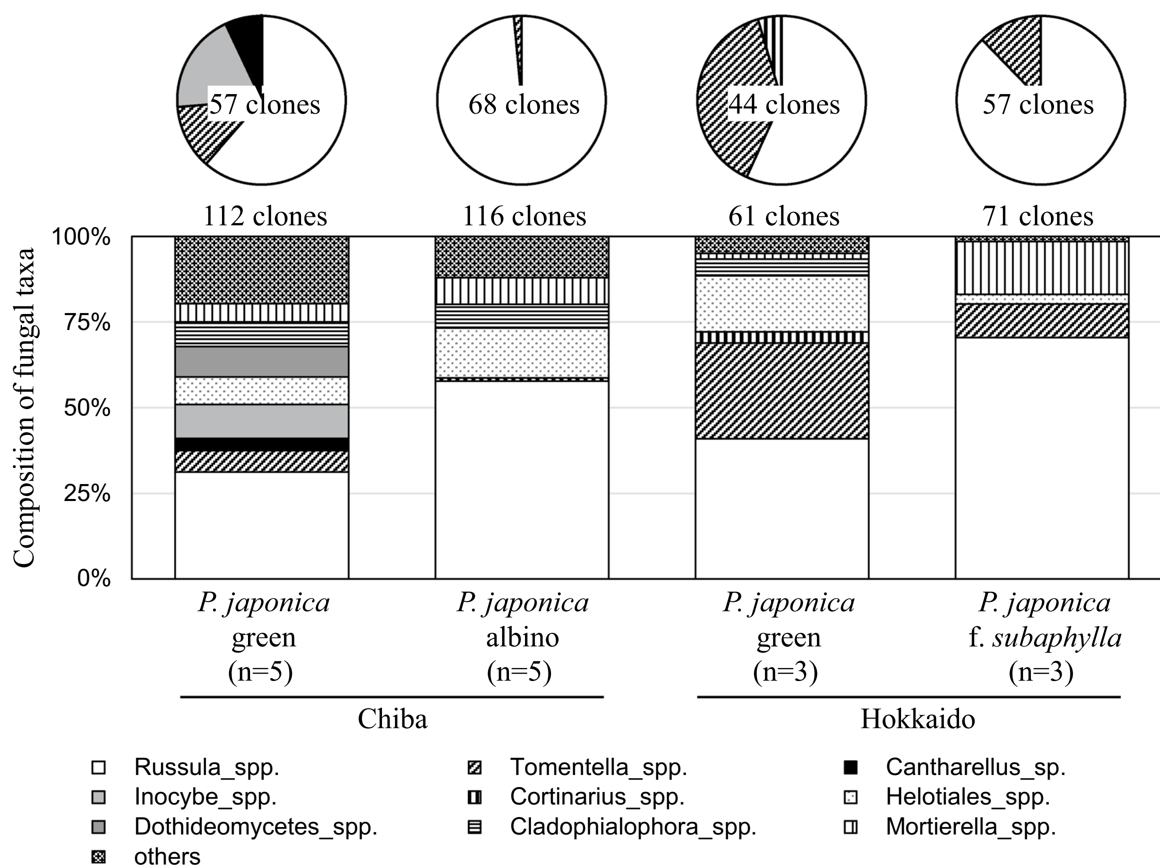

Fig. 6 Percentages of fungal taxa detected from pyroloid mycorrhizas: albino and green *Pyrola japonica* at Chiba, as well as green *P. japonica* and *P. japonica* f. *subaphylla* at Hokkaido. Number of clones investigated shown at the top. Pie charts above the graph indicate percentages of fungal taxa considering ecomycorrhizal fungi only, with number of clones displaying ecomycorrhizal OTUs. For detailed list of taxa, see Table S2

Russula fruitbodies used for the isotopic analysis in this study were distributed throughout the phylogenetic diversity of the genus (Fig. S3). Although no *Russula* fruitbody matched with any OTUs detected from *P. japonica* roots in this study, *Russula* sp.1, 4, 5 and 6 clustered with *P. japonica* mycorrhizal fungi detected by Uesugi et al. (2016) with 100% bootstrap supports.

The genus *Russula* was the most dominant taxon in each phenotype, ranging from 31.3% in green *P. japonica* at CH to 70.4% in *P. japonica* f. *subaphylla* at HK (Fig. 6). A dominance of *Russula* was higher for albinos (57.5 ± 32.8%, mean ± SD, $n = 5$) and even more for *P. japonica* f. *subaphylla* (70.4 ± 16.7%, $n = 3$), than for green individuals (29.1 ± 34.4% at CH and 35.8 ± 49.0% at HK; Fig. S4). The relative abundance of *Russula* spp. was significant between different phenotypes in both sites (Fisher's exact test, $\chi^2 = 15.142$, df = 1, $P < 0.01$ at CH and $\chi^2 = 10.421$, df = 1, $P < 0.01$ at HK). The predominance of *Russula* spp. was much more striking when only ectomycorrhizal fungi were considered (Fig. 6); the relative occurrences in green *P. japonica* at CH and HK were then 61.4 and 56.8%, respectively, and 98.5 and 87.7% in albinos and *P. japonica* f. *subaphylla*, respectively. The second most shared taxon was Helotiales (shared by 8 out of 16 plants). Indicator species analysis showed that two OTUs were statistically significantly associated with albino individuals at CH (*Russula* sp.1, $P = 0.006$, and *Sordariomycetes* sp., $P = 0.045$) and one with *P. japonica* f. *subaphylla* at HK (*Russula* sp.2, $P = 0.001$; Table S2).

A hierarchical clustering demonstrated some overlaps among green and albino *P. japonica*, and between sites for these phenotypes, but displayed a strong separation of *P. japonica* f. *subaphylla* (Fig. S5). Sharing of detected OTUs were depicted by a Venn diagram in each site, and there was no significant difference in patterns between sites (Fisher's exact test, $P > 0.05$). On each site, no significant segregation of mycorrhizal communities was found by NMDS scattering plots (data not shown). When *Russula* spp. were only chosen to detect segregations among different pyroloids in each site, the community structures of the taxa significantly differed from each other (PERMANOVA, $P < 0.01$, Fig. S7).

Discussion

This study takes advantage of a recently discovered site where *P. japonica* albinos, which are rarely observed, or *P. japonica* f. *subaphylla* coexist with typical, green *P. japonica* individuals. Of course, these rare situations entail a limited sampling, to protect the populations, and thus result a low repetition number. We examined both heterotrophy level and mycorrhizal fungal communities of these plants. Phylogenetic analyses of ITS and *mat*K regions suggest that albinos at CH are conspecific with neighbouring and HK green *P. japonica*; this applies also to *P. japonica* f. *subaphylla* at HK, although the latter were slightly more polymorphic. Thus, our data are congruent with the taxonomic assignment of *P. japonica* f. *subaphylla* as a sub-clade of *P. japonica* sensu lato (Liu et al. 2014; Shutoh et al. 2017), a species in which an evolutionary trend to reduced leaf size likely happened several times (Shutoh et al. 2018), probably due to a predisposition for mixotrophy.

Carbon resources of the various *P. japonica* phenotypes

Until recently, reports on ^{13}C abundances in albinos were only available for mixotrophic orchids (e.g. Abadie et al. 2006; Gonneau et al. 2014; Julou et al. 2005; Roy et al. 2013; Sakamoto et al. 2016; Suetsugu et al. 2017). Our results, together with those of Shutoh et al. (2020), show that albino *P. japonica* leaves are significantly enriched in ^{13}C compared to green phenotypes and even more when compared to neighbouring autotrophic plants, as expected due to the use of C from mycorrhizal fungi. Indeed, albinos leaves had ^{13}C abundances similar to those of *Russula* fruitbodies, which makes use of the latter relevant in roughly approximating the mycoheterotrophy level '*x*' when albinos are not available.

Green *P. japonica* leaves showed $\delta^{13}C$ values intermediate between albino *P. japonica* leaves and green reference plants at CH, allowing calculation of $x = 36.8\%$, which is in the range observed by Shutoh et al. (2020), 30.2 ± 19.9%, but lower than in Matsuda et al. (2012; 48.7%). Such a high variability is expected since x varies with different shading levels (Matsuda et al. 2012). Fungal-derived carbon may be used in variable amounts among different organs in mixotrophic orchids (Lallemand et al. 2019), and the same is observed at CH and HK. Roots and rhizomes of green *P. japonica* showed higher $\delta^{13}C$ values compared with the green leaves, and higher mycoheterotrophy level (estimated $x = 74.0\%$ for rhizomes at CH), as also supported by higher $\delta^{15}N$ values for green *P. aphylla*. Similar evidence was also found for *P. chlorantha*, whose $\delta^{13}C$ values in leaves were lower than in roots (Johansson et al. 2015), while albinos did not show difference between organs, again supporting systemic mycoheterotrophic nutrition in their case.

Interestingly, the roots and rhizomes of green individuals were less enriched in ^{13}C than those of albinos, which can be explained by import of photosynthates with lower ^{13}C abundance. In this respect, *P. japonica* may differ from mixotrophic orchids of the genera *Cephalanthera* and *Epipactis*, where underground nutrition is mostly of fungal origin and $\delta^{13}C$ values are similar to those of albinos (Gonneau et al. 2014; Lallemand et al. 2019; Těšitel et al. 2018). A systemic contribution of photosynthates to support underground, perennial structures in pyroloids could explain why

albinos are rarer than in mixotrophic orchids, because survival of vegetative underground parts is more impacted by loss of photosynthesis. However, the isotopic variation must be explained with caution since individual plants can show spatio-temporal variation of isotopic values among organs (Gebauer and Schulze 1991).

Leaves of *P. japonica* f. *subaphylla* showed higher ^{13}C and ^{15}N abundance than those of green *P. japonica* at HK, supporting a lower photosynthetic input. Suetsugu et al. (2020) also noted a higher δ^{13}C value for *P. japonica* f. *subaphylla* than in surrounding autotrophic *Quercus serrata*. Yet, due to the presence of green leaves, the plant may be somewhat photosynthetic, even if isotopic data supporting this are not available (absence of mycoheterotrophic plants supported by ectomycorrhizal fungi in this study and in Suetsugu et al. (2020) does not allow any exact quantification). However, the trend to higher abundance in ^{13}C for *P. japonica* f. *subaphylla* leaves at HK, may be biologically relevant, and indicative of a lower photosynthetic input, diluted in a large amount of fungal resources. Albinos or forms with minute leaves indicate a use of fungal carbon in all plant tissues, and green *P. japonica* may regulate this dual carbon source from photosynthesis and/or from associating fungi, possibly depending on light availability (Matsuda et al. 2012).

Mycorrhizal associations in the various phenotypes of *P. japonica*

Many mycoheterotrophic plants are specialists in terms of fungal symbionts (Bidartondo and Bruns 2001; Cullings et al. 1996; Matsuda et al. 2011; Selosse et al. 2002). Specificity is less frequent in mixotrophic plants, although recorded in the orchids *Limodorum abortivum* with *Russula* spp. (Girlanda et al. 2006) and *Corallorhiza trifida* with *Tomentella* spp. (Cameron et al. 2009; Zimmer et al. 2008). In the framework of the *Pyrola* genus, which is largely nonspecific (Hynson and Bruns 2009; Johansson et al. 2017; Tedersoo et al. 2007; Toftegaard et al. 2010; Vincenot et al. 2008), *P. japonica* has a preference for *Russula* spp. (Matsuda et al. 2012; Uesugi et al. 2016). We confirmed that most individuals, whatever the phenotype, were dominated by *Russula* spp. from various subclades of this genus (Fig. S3) and that albino *P. japonica* and *P. japonica* f. *subaphylla* have a much higher preference for *Russula* spp. than green *P. japonica*.

The most dominant fungus *Russula* sp.1 was shared between sympatric albino and green *P. japonica*, while the second most dominant fungus, *Russula* sp. 2, was detected only in *P. japonica* f. *subaphylla* and was never shared with sympatric green *P. japonica* (Fig. S6). Albino *P. japonica* may compensate for sudden loss of photosynthesis by associating tightly with fungal taxa existing nearby, while *P. japonica* f. *subaphylla*, genetically diverging from green *P. japonica*, has mycorrhizal affinity toward slightly different fungal taxa. The taxon phylogenetically closest to *Russula* sp. 2 was detected from *P. japonica* in a previous study (Uesugi et al. 2016), which may suggest that *P. japonica* f. *subaphylla* has higher symbiont preferences within the same partner range as typical *P. japonica*. This, together with slight divergences in ITS and *mat*K sequences, suggests a genetically isolated lineage, although the taxonomic status of *P. japonica* f. *subaphylla* deserves further study at other sites.

Quantitatively, the community tended to be less diverse in the two phenotypes that are limited in photosynthates, with more frequent *Russula* spp. This is reminiscent of the plasticity already reported in *P. japonica* in shaded conditions, which shift to increased preference for *Russula* spp. (Matsuda et al. 2012). The partner shift toward increasing preference for *Russula* may be a consequence of the loss of photosynthesis (e.g. affecting carbon nutrition of endophytes and parasites, and excluding them). Yet, we suspect that it is a trait more linked to mycoheterotrophic nutrition, allowing *P. japonica* to adapt to reduction or loss of photosynthesis by adapting its fungal associations and favouring higher mycoheterotrophic nutrition in the albinos and *P. japonica* f. *subaphylla*.

Evolutionary perspectives

Mixotrophic pyroloids produce very minute dust seeds with limited resources (Eriksson and Kainulainen 2011; Figura et al. 2019; Johansson et al. 2014) and thus their germination and subsequent underground seedling stages fully depend on mycorrhizal fungi, a strategy called initial mycoheterotrophy (Bidartondo and Read 2008; Johansson et al. 2017) that is likely a predisposition to be mixotrophic at the adult stage. Fungal specificity was detected at seedling stages for some mixotrophic pyroloids (Bidartondo et al. 2004; Hashimoto et al. 2012), but not all (Hynson et al. 2013b; Johansson et al. 2017), and they all harbour various fungal taxa later in their development. Indeed, monitoring fungal specificity of *P. japonica* during its germination and growth would add to our understanding of mycoheterotrophic evolution. However, since the American mycoheterotrophic *P. aphylla* is not specific (Hynson and Bruns 2009), it is difficult at present to strictly link mycoheterotrophic nutrition and specificity in pyroloids.

Pyrola japonica has a high heterotrophy level (this study and Matsuda et al. 2012) and displays trends to enhanced (in *P. japonica* f. *subaphylla*) or full mycoheterotrophic nutrition (albinos). Mixotrophic status is often considered as an evolutionary transition from autotrophic to mycoheterotrophic nutrition (Jacquemyn and Merckx

2019; Selosse and Roy 2009), with albinos as 'missing links' (Selosse et al. 2004), even if for fitness reasons most albinos observed are not very evolutionarily promising (Gonneau et al. 2014; Roy et al. 2013; Shefferson et al. 2016). Minute leaves of *P. japonica* f. *subaphylla* suggest another, less sudden evolutionarily pathway, where plants become mycoheterotrophic by reducing leaves and selecting for specific fungal taxa providing more carbon, i.e. within *Russula* spp. Such a gradual evolution may be more steady than a sudden shift to albinism, which remains rare and is likely counter-selected (Roy et al. 2013), as shown by the rarity of albinos in the wild. Yet, even green *P. japonica* has a high heterotrophy level, and the survival of albinos and leafless forms is a situation from which a mycoheterotrophic species can evolve in the genus *Pyrola* (see below the *P. picta* / *P. aphylla* section).

Our analysis can be placed in the evolutionary continuum of Lallemand et al. (2017; see their Fig. 4), where some basal species (called 'C-exchangers') gain a lot of carbon from their fungi, but still less than they give, without plasticity of use of this fungus depending on ecological conditions: this is the case of most *Pyrola* species whose heterotrophy does not respond to light (Lallemand et al. 2017). From this first status can eventually evolve true 'mixotrophic' plants that gain a lot of carbon from their fungi, more than they give, and even have some plasticity of use of this fungus depending on light level, such as *P. japonica* (Matsuda et al. 2012). In these true mixotrophic species, but not in C-exchangers, albinos can be observed. From this second status fully mycoheterotrophic plants can eventually evolve, possibly through forms with reduced or absent leaves. *P. japonica* is, in our current state of knowledge, the only true mixotrophic pyroloid (following the strict sense of Lallemand et al. 2017) described. Yet, the *Pyrola picta* section in North America, where many leafless forms occur and to which the mycoheterotrophic *P. aphylla* belongs (Jolles and Wolfe 2012), is another good candidate, although the potential ability of green *P. picta* to change mycoheterotrophic level depending on light level remains to be clarified (Hynson et al. 2012).

More studies of the *P. picta* and *P. aphylla* sections (two sister clades in the genus *Pyrola*; Liu et al. 2010, 2014) are now needed, since they look promising for understanding the origin of mycoheterotrophic nutrition (Shutoh et al. 2017, 2020). More studies of population genetics (together with identification of fungi) are especially awaited to clarify the rise of phenotypes with the reduced leaves of mycoheterotrophic nutrition, their status of species, number of evolutionary occurrences, and the existence of gene flow between the phenotypes.

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