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Parallel evolutionary paths to mycoheterotrophy in understorey Ericaceae and Orchidaceae: ecological evidence for mixotrophy in Pyroleae

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Abstract Several forest understorey achlorophyllous plants, termed mycoheterotrophs (MHs), obtain C from their mycorrhizal fungi. The latter in turn form ectomycorrhizas with trees, the ultimate C source of the entire system. A similar nutritional strategy occurs in some green forest orchids, phylogenetically close to MH species, that gain their C via a combination of MH and photosynthesis (mixotrophy). In orchid evolution, mixotrophy evolved in shaded habitats and preceded MH nutrition. By generalizing and applying this to Ericaceae, we hypothesized that green forest species phylogenetically close to MHs are mixotrophic. Using stable C isotope analysis with fungi, autotrophic, mixotrophic and MH plants as comparisons, we found the first quantitative evidence for substantial fungi-mediated mixotrophy in the Pyroleae, common ericaceous shrubs from boreal forests close to the MH Monotropoideae. *Orthilia secunda*, *Pyrola chlorantha*, *Pyrola rotundifolia* and *Chimaphila umbellata* acquired between 10.3 and 67.5% of their C from fungi. High N and ^{15}N contents also suggest that Pyroleae nutrition partly rely on fungi. Examination of root fungal internal transcribed spacer sequences at one site revealed that 39 species of mostly endophytic or ectomycorrhizal fungi, including abundant *Tricholoma* spp., were associated with *O. secunda*, *P. chlorantha* and *C. umbellata*. These fungi, particularly ectomycorrhizal associates, could thus link mixotrophic Pyroleae spp. to surrounding trees, allowing the C flows deduced from isotopic evidence. These data suggest that we need to reconsider ecological roles of understorey plants, which could influence the dynamics and composition of forest communities.

Keywords Mixotrophy · Pyroleae · Stable isotopes · Ectomycorrhizal fungi · Ericaceae

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Introduction

Light availability is a limiting factor for many photosynthetic autotrophs, and some of them also acquire organic compounds from environmental sources to supplement C obtained by photosynthesis, in a strategy called mixotrophy. In aquatic ecosystems, mixotrophy via prey phagocytosis is widespread among planktonic algae (up to 49% of phototrophic biomass, Havskum and Riemann 1996). Ingested cells improve mineral and C nutrition (Stibor and Sommer 2003) and may account for up to 90% of the C budget in mixotrophs (Havskum and Riemann 1996), depending on algal species and light availability (Jakobsen et al. 2000). Mixotrophic algae considerably affect the density of

grazed populations and alter competitive balance between grazed species (Havskum and Riemann 1996; Jeong et al. 2005).

In terrestrial ecosystems, most vascular plants obtain their mineral nutrition from a living source, i.e. the mycorrhizal fungi associated with their roots (Smith and Read 1997). However, there is limited evidence that under certain conditions, several green plants can be mixotrophic by recovering C from mycorrhizal fungi (Selosse et al. 2006). The possibility of C flow from a mycorrhizal fungus to a plant is clearly demonstrated in the particular case of forest achlorophyllous plants that rely solely on C provided by their mycorrhizal fungi, the so-called mycoheterotrophs (MH; Leake 2004). Fungi colonizing these MH plants are also mycorrhizal with surrounding green plants, from which they obtain C (McKendrick et al. 2000; Selosse et al. 2002). But can some green photosynthetic plants also exploit fungal C? Neighbouring plants often share common mycorrhizal fungi and thus create opportunities for using C from the resulting fungal network, a strategy that would be especially relevant to shaded individuals in forests (Simard and Durall 2004; Selosse et al. 2006). Labelling experiments showed C transfers from overstorey to understorey plants (Simard et al. 1997; Carey et al. 2004), but due to some negative results (Wu et al. 2001; Pfeffer et al. 2004) and methodological biases of labelling experiments (Simard and Durall 2004), the existence, ecological relevance and contribution to plant C budget of interplant C transfer is strongly debated for autotrophic plants.

However, studies of natural abundances of stable isotopes (^{13}C and ^{15}N) provide strong evidence for mixotrophy in some green, terrestrial orchids from temperate and boreal forests. Stable isotopes allow tracking of nutrient sources and fluxes in ecosystems, since fractionation against heavy isotopes is common in physical and metabolic processes (Dawson et al. 2002). Therefore, higher trophic levels of food webs preferentially accumulate heavy isotopes, especially ^{15}N (Post 2002). Compared with photosynthetic plants, ^{13}C and ^{15}N are more abundant in mycorrhizal fungi (Taylor et al. 2003; Trudell et al. 2003) and therefore also in MH plants (Delwiche et al. 1978; Gebauer and Meyer 2003; Trudell et al. 2003). Studies of stable isotopes demonstrated mixotrophy in green forest orchids that displayed ^{13}C and ^{15}N enrichments intermediate between MH and photosynthetic plants. It was estimated that up to 80% of the orchids' C was of fungal origin (Gebauer and Meyer 2003; Bidartondo et al. 2004; Julou et al. 2005). These orchids associate with fungi that form ectomycorrhizas (EcM) on surrounding trees (Selosse et al. 2004; Julou et al. 2005) that are likely their C source. Congruently, studies on gas exchange revealed low photosynthesis/respiration balance due to shade (Julou et al. 2005) or intrinsically low photosynthetic abilities (Girlanda et al. 2006). So far, direct evidence for such mycorrhiza-mediated mixotrophy is limited to some forest orchids that are phylogenetically related to MH species. This supports the hypotheses that during evolution of some forest understorey plants, transition to mixotrophy by sharing EcM fungi with surrounding trees: (1) counterbalanced low light conditions (Bidartondo et al. 2004; Selosse et al. 2004), and (2) allowed the rise of MH plants (Selosse et al. 2004; Julou et al. 2005).

We predict that other MH taxa evolved in parallel to orchids. Therefore, other green forest understorey plants closely related to MH species may have retained mixotrophic nutrition. In this study, we focused on the pyroloids (Ericaceae)—shade-tolerant perennial subshrubs that sometimes dominate oligotrophic boreal and temperate forests of the Northern Hemisphere (Fig. 1; Hunt and Hope-Simpson 1990; Freudenstein 1999). Pyroloids differ from mixotrophic orchids by their abundance and evergreenness, and could therefore have a much greater ecological impact by affecting forest community functioning. We predicted their mixotrophy for three reasons. First, the Pyroleae tribe forms a sister clade to two MH tribes, Monotropeae and Pterosporeae (Fig. 1; Kron et al. 2002). Second, several pyroloid species include aphyllous forms (Freudenstein 1999) suggesting that a non-photosynthetic C source allows survival, reminiscent of the subterranean or achlorophyllous individuals occurring in mixotrophic orchids (Selosse et al. 2004; Julou et al.

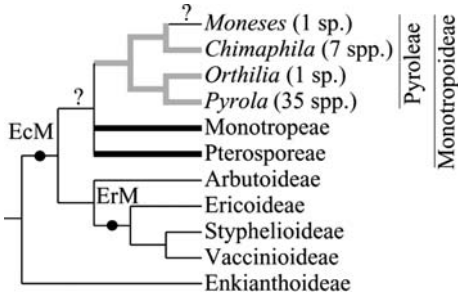

Fig. 1 Phylogeny of the Ericaceae and Pyroleae after Kron et al. (2002) and Freudenstein (1999). *Thin branches* indicate autotrophic taxa, *thick black lines* represent mycoheterotroph (MH) taxa and *grey lines* indicate mixotrophic taxa, assuming generalization of our results to all the genera in the Pyroleae. *Question marks* indicate missing data or uncertain ancestral states of mixotrophy. The ancestral mycorrhizal association in Ericaceae involves arbuscular mycorrhizal fungi, which still persist in Enkianthoideae (Abe 2005). The shift of association to ectomycorrhizal (EcM) fungi is indicated by *EcM*, and the shift to ericoid mycorrhizal fungi by *ErM*

2005). Third, although there are no detailed studies on their identity, pyroloid mycorrhizal fungi include asco- and basidiomycetes that form EcM on surrounding trees (Robertson and Robertson 1985; Smith and Read 1997; Bidartondo 2005), similarly to mixotrophic orchids. Indeed, a report of a ^{13}C-labelling pot experiment suggests C transfer from co-cultivated *Larix kaempferi* to *Pyrola incarnata* (Kunishi et al. 2004). However, the quantitative contribution of C transferred to the pyroloid nutrition remains unknown.

We aimed at uncovering the fungal associates, which potentially link pyroloids to surrounding autotrophic green plants. This study also addressed in situ C nutrition of four pyroloid species, *Chimaphila umbellata* Nutt., *Pyrola chlorantha* Sw., *Pyrola rotundifolia* L. and *Orthilia secunda* House in two boreal coniferous forests. We analysed the N content, ^{15}N and ^{13}C natural abundances to reveal the level of heterotrophy among these pyroloids. Further, we used molecular tools to identify the root-associated fungi at one of the sites.

Materials and methods

Study sites

Mycorrhizal studies were performed at Kärla, Saaremaa Island (4 ha; north-west Estonia; 58°20′N, 22°18′E). Isotopic studies were performed at Kärla and at Värska (2 ha; south-east Estonia; 57°57′N, 27°40′E). These sites were selected because they harboured: (1) a dense population of at least three pyroloid species, (2) abundant MH *Monotropa hypopithys* L. and several mixotrophic orchids that could be used as controls, (3) a canopy covering <70% of the area. The latter criterion was considered, because heterotrophy in mixotrophic orchids negatively responds to light availability (Gebauer 2005). Kärla and Värska had average Ellenberg light indicator values of 6.11 and 4.70, respectively, calculated on the basis of vegetation [the Ellenberg indicator values represent the preferences of individual species, based on empirical field observations, and range from 1 (deep shade) to 9 (full sunlight); Ellenberg et al. 1991]. The Kärla site was covered by a 100- to 120-year-old Scots pine (*Pinus sylvestris* L.) forest with sparse Norway spruce [*Picea abies* (L.) Karst.] undergrowth. The ericaceous species *Chimaphila umbellata*, *Orthilia secunda*, *Arctostaphylos uva-ursi* Spreng., *Monotropa hypopithys*, and *Epipactis atrorubens* Rostk. ex Spreng. dominated the shrub/herb layer. The Värska site was covered by a 60- to 80-year-old Scots pine–silver birch (*Betula pendula* Roth) forest with *Pyrola rotundifolia* L., *C. umbellata*, *O. secunda*, *Vaccinium myrtillus* L. dominating the shrub layer. *Pleurozium schreberi* (Brid.) Mitt. and *Hylocomium splendens* (Hewd.) BSG. were the dominant mosses at both sites. The soils were haplic podzols on sand dunes (<30 cm depth). The O-, A-, E- and B-horizons were respectively ca. 3, 3, 2 and 20 cm in thickness at both sites. The sites experience mean annual temperature of +5.5–6.5°C and rainfall of 700 mm year^{-1}.

Identification of mycorrhizal fungi

In early September 2003, root systems of the pyroloids *C. umbellata*, *O. secunda* and *Pyrola chlorantha* from Kärla were manually separated from five 20 × 40-cm (depth = 20 cm) soil cores. Soil cores were taken non-randomly, at least 10 m apart, to include roots of all three species. Roots of pyroloids were identified by attached leaf and rhizome morphology. Mycorrhizas occurred in long lateral roots that were sometimes covered by dense wefts of hyphae. Seven 2–4 mm root fragments were randomly selected from each species and core. The resulting 105 root samples were transferred to 100 ml CTAB lysis buffer [100 mM TRIS–HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 2% CTAB].

The root samples were further subjected to DNA extraction and polymerase chain reaction (PCR) of the internal transcribed spacer (ITS) using primers ITSIF and ITS4 as described in Selosse et al. (2002). Single PCR products were directly sequenced applying the same primers on an ABI3130xl sequencer (Applied Biosystems, Courtaboeuf) using the Big Dye Terminator kit. In the case of multiple PCR products or whenever direct sequencing failed, the products were cloned as in Selosse et al. (2004), amplified and sequenced as above. Raw sequences were checked for possible machine errors and trimmed to include only the ITS1, 5.8S and ITS2 rDNA regions using Sequencher 4.5 (GeneCodes., Ann Arbor, Mich.). Sequences were grouped based on >98.0% sequence identity over the whole ITS region, which we found the most suitable molecular species criterion. To identify the fungi, the most common individual sequence from each species was queried against GenBank and EMBL, and the EcM fungal sequence database UNITE (Kõljalg et al. 2005) using blastN or fasta3 algorithms. Possible PCR chimaeras, resulting from pairing of the conserved central 5.8S sequence, were detected by subjecting ITS1 and ITS2 regions separately to blastN searches against GenBank. Out of 126 sequences, three chimaeras were detected. These were used to estimate the proportion of each species. All reported sequence

identities are based on full-length pairwise alignments unless otherwise stated. Putative trophic status of the detected species is that of the reported lifestyle of the closest matching taxa.

Isotope analysis

For each plant species, one to three leaves were collected from individuals (i.e. replicates) situated (1) ca. 20 m apart from each other; (2) at 10–30 cm above soil, to avoid isotope distortion due to CO_2 resulting from soil respiration; and (3) in the same light conditions, because $\delta^{13}C$ is negatively correlated with leaf intracellular CO_2 concentration (decreased light supply result in slower photosynthesis, and higher ^{13}C discrimination during CO_2 assimilation; Julou et al. 2005). At Kärla, leaves of ten plant species, including mixotrophic orchids, autotrophic and MH plants (Fig. 2a–d), and fruit-bodies of nine EcM or saprotrophic fungal species (Fig. 3a) were collected in mid-July 2004. Since the criterion used did not allow sampling of more autotrophs at Kärla, an additional sampling was performed at Värska in mid-July 2006. This sampling comprised four pyroloids, seven autotrophic plants and a single MH (Fig. 2e–h).

All samples were transported to the laboratory within 3 h, dried at 30°C for 48 h and ground in 1.5-ml Eppendorf tubes using 1.1-mm- diameter Tungsten carbide balls (Biospec Products, Bartlesville, Okla.) in a Retch MM301 vortexer (Retch, Haan, Germany). Total N, C/N and abundances of ^{13}C and ^{15}N were measured using an on-line continuous flow CN analyser coupled to an isotope ratio mass spectrometer (Ohlsson and Wallmark 1999). Isotope abundances are expressed in $\delta^{13}C$ and $\delta^{15}N$ values in parts per thousand relative to international standards Vienna-Pee-Dee Belemnite and atmospheric N_2:

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

where R is the molar ratio, i.e. $^{13}C/^{12}C$ or $^{15}N/^{14}N$. The SD of the replicated standard samples was 0.034 for ^{13}C and 0.253 for ^{15}N.

Statistics

Total N concentrations, C/N ratio, $\delta^{13}C$ and $\delta^{15}N$ values of plant and fungal taxa were tested for normality and homogeneity of variances using a Wilks–Shapiro W-test and a Levene test, respectively. Accordingly, the C/N ratio was log-transformed to meet the assumptions of parametric tests. One-way ANOVAs were separately performed for each variable and site, followed by Tukey–Kramer tests for unequal sample size to separate significantly different groups at $\alpha = 0.05$.

The relative contribution of C of the putative mixotrophic taxa derived from autotrophic plants via EcM fungi was calculated using a linear two-source mixing model (Phillips and Gregg 2001; Gebauer and Meyer 2003) based on the mean δ-values and SDs of putative mixotrophic, autotrophic and MH plants. *M. hypopithys* was used as reference for a MH plant at both sites, while *A. uva-ursi* (the only ericaceous species at Kärla) and *V. myrtillus* (the least ^{13}C-depleted ericaceous species at Värska) were used as autotrophic references. Only ericaceous species were used as references, because they are close relatives to pyroloids (significant isotopic differences do occur among plant families, at least in $\delta^{15}N$, Delwiche et al. 1978). The means, SEs and 95% confidence intervals were calculated using a spreadsheet program as implemented in Phillips and Gregg (2001). χ^2 tests were used to reveal significant differences in colonization by different fungi on the pyroloid plant roots.

Results

Differences in $\delta^{13}C$, $\delta^{15}N$, C/N ratio and N concentration at Kärla

There were significant differences among plant species in $\delta^{13}C$ ($F_{9,51} = 45.2$, $P < 0.001$), $\delta^{15}N$ ($F_{9,51} = 48.5$, $P < 0.001$), N concentration ($F_{9,51} = 65.8$, $P < 0.001$) and C/N ratio ($F_{9,51} = 69.7$, $P < 0.001$; Fig. 2a–d). MH plants, mixotrophic orchids, fungi and pyroloids (except *Chimaphila umbellata*) were enriched in ^{15}N and ^{13}C, and had higher leaf N concentrations and lower C/N ratio compared to hemiparasitic and autotrophic plants. Both plant and fungal species belonging to different trophic groups were separated based on $\delta^{13}C$ and $\delta^{15}N$ values (Fig. 3). Saprotrophic fungi were enriched in ^{13}C, but depleted in ^{15}N compared to EcM fungi.

Pyroloids and mixotrophic orchids had $\delta^{13}C$ values intermediate between autotrophic and MH plants, suggesting exploitation of fungal C (Figs. 2a, 3). Statistically significant C gain from the fungal association was found in the pyroloids *Orthilia secunda* (49.9 ± 4.9%, SEM) and *Pyrola chlorantha* (37.6 ± 5.8%), as well as in the orchids *Platanthera bifolia* (60.1 ± 7.4%) and *Epipactis atrorubens* (62.6 ± 7.2%). *Listera ovata* and *C. umbellata* did not significantly gain C from the fungal association (Table 1). C gains, calculated using

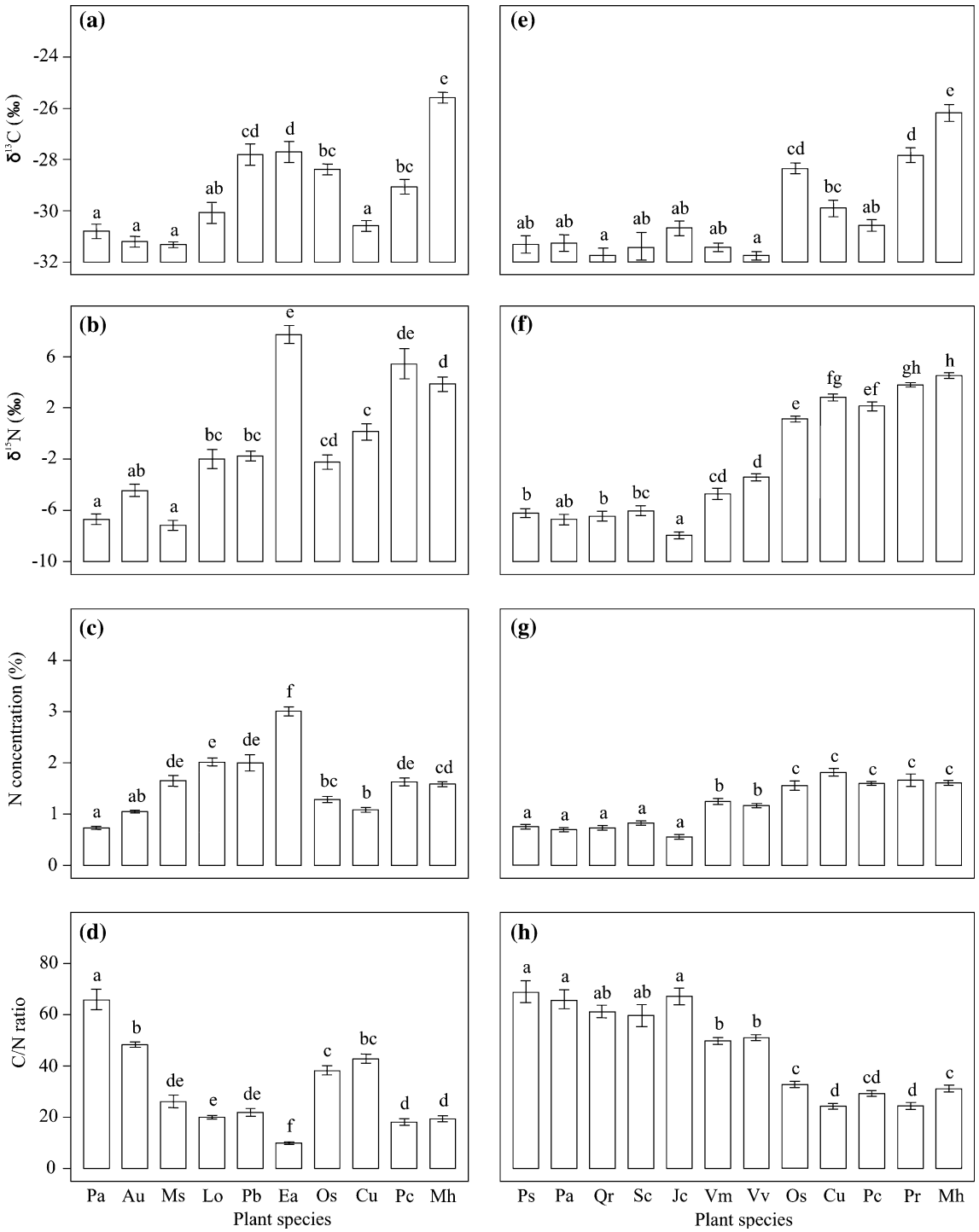

Fig. 2 $\delta^{13}C$, $\delta^{15}N$ values (‰), N concentrations and C/N ratios of plant species from the study sites (means ± SE), arranged according to putative functional groups at Kärla (**a**–**d**) and Värska (**e**–**h**). *Different letters* denote significant differences between species according to one-way ANOVA and Tukey–Kramer tests. Species at Kärla: autotrophic plants—*Picea abies* (*Pa*; $n = 6$) and *Arctostaphylos uva-ursi* (*Au*; 6); hemiparasitic—*Melampyrum sylvaticum* (*Ms*; 6); potentially mixotrophic orchids—*Listera ovata* (*Lo*; 4), *Platanthera bifolia* (*Pb*; 6) and *Epipactis atrorubens* (*Ea*; 6); pyroloids—*Orthilia secunda* (*Os*; 8), *Chimaphila umbellata* (*Cu*; 8) and *Pyrola chlorantha* (*Pc*; 8); mycoheterotrophic—*Monotropa hypopithys* (*Mh*; 6). Species at Värska ($n = 6$ for each species): autotrophic plants—*Juniperus communis* (*Jc*), *Picea abies* (*Pa*), *Pinus sylvestris* (*Ps*), *Quercus robur* (*Qr*), *Salix caprea* (*Sc*), *Vaccinium myrtillus* (*Vm*) and *Vaccinium vitis-idaea* (*Vv*); pyroloids—Os, Cu, Pc and *Pyrola rotundifolia* (Pr); mycoheterotrophic—Mh

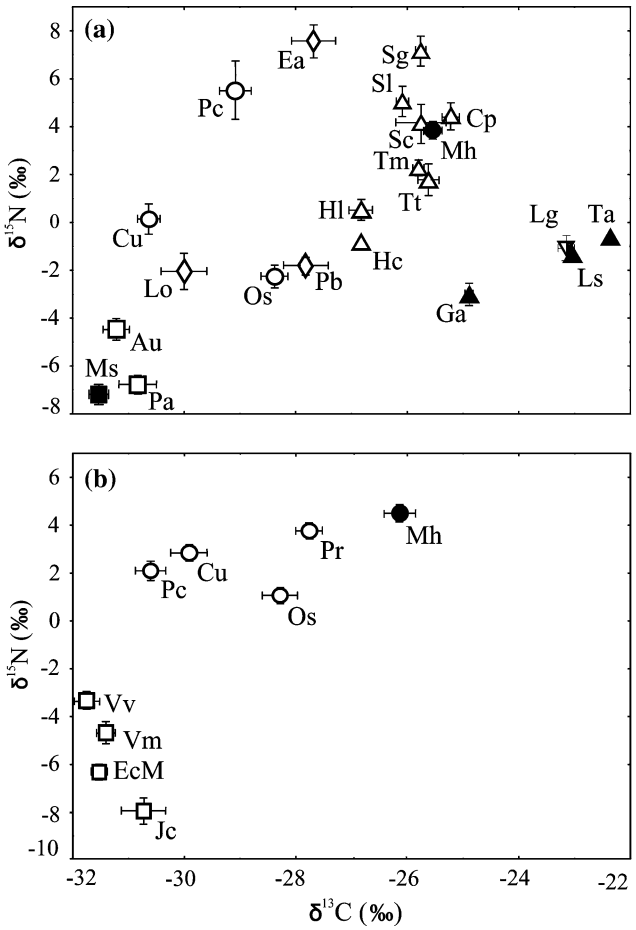

Fig. 3 C versus N stable isotope values (‰) of plants and fungi at **a** Kärla and **b** Värska (means ± SE). Species at Kärla—Ms (n = 6), Pa (6), Au (6), Cu (8), Lo (4), Pc (8), Os (8), Pb (3), Mh (6), Ea (6), *Suillus granulatus* (*Sg*; 5), *Suillus luteus* (Sl; 5), *Coltricia perennis* (*Cp*; 4), *Sarcosphaera coronaria* (*Sc*; 2), *Tricholoma myomyces* (*Tm*; 2), *Thelephora terrestris* (*Tt*; 2), *Helvella lacunosa* (*Hl*; 6), *Helvella crispa* (*Hc*; 1), *Gymnopus acervatus* (*Ga*; 2), *Lepista sordida* (*Ls*; 1), *Limacella glioderma* (*Lg*; 6), *Tapinella atrotomentosa* (*Ta*; 1). Species at Värska—Jc (6), Vm (6), Vv (6), Os (6), Cu (6), Pc (6), Pr (6), Mh (6), *EcM* EcM trees [Pa (6), Ps (6), Qr (6), Sc (6)]. *Error bars* represent SEM for the mean values of each four species. *Open squares* Autotrophic plants, *closed squares* hemiparasitic plants, *diamonds* green orchids, *open circles* pyroloids, *closed circles* mycoheterotrophic plants, *open triangles* ectomycorrhizal fungi, *closed triangles* saprotrophic fungi, *black and white inverted triangles* fungi with uncertain trophic status. For other abbreviations, see Figs. 1 and 2

leaves of *P. abies* as an autotrophic reference, revealed similar amounts (not shown). All putative mixotrophic plants displayed δ^{15}N values intermediate between autotrophic and MH plants or higher (for *P. chlorantha* and *E. atrorubens*). For orchids and pyroloids δ^{13}C and δ^{15}N values ($R^2 = 0.082$, $n = 6$, $P = 0.570$) were not correlated, suggesting that C and N are obtained through different biochemical pathways.

Differences in δ^{13}C, δ^{15}N, C/N ratio and N concentration at Värska

There were significant differences among plant species in δ^{13}C ($F_{11,60} = 30.8$, $P < 0.001$), δ^{15}N ($F_{11,60} = 212$, $P < 0.001$), N concentration ($F_{11,60} = 41.0$, $P < 0.001$) and C/N ratio ($F_{11,60} = 75.7$, $P < 0.001$) (Fig. 2e–h). MH plants and pyroloids were enriched in ^{15}N and ^{13}C and had significantly higher leaf N concentrations and lower C/N ratio compared to autotrophic plants. *V. myrtillus* and *V. vitis-idaea* had δ^{15}N values, N concentration and C/N ratio intermediate between trees and pyroloids, suggesting differences in N nutrition in these Ericaceae (Fig. 2e–h). Based on δ^{13}C values, and using *V. myrtillus* as a reference for autotrophic biomass (Table 1), statistically significant C gain from the fungal association was calculated in all four pyroloids, *C. umbellata* (29.0 ± 6.6%, SEM), *O. secunda* (58.2 ± 5.2%), *P. chlorantha* (15.5 ± 5.8%) and *P. rotundifolia* (67.5 ± 6.4%). Calculation of C gains using the least ^{13}C-depleted autotroph, *J. communis*, as a reference also revealed significant mixotrophy in *P. rotundifolia* and *O. secunda* (not shown).

Diversity of root fungi at Kärla

For each pyroloid species, 35 mycorrhizal root fragments were analysed for identification of fungi. In all, 57.5% of the root samples provided a single PCR product that was directly sequenced, indicating root colonization by a single fungus. In addition, 25.6% of the root samples produced multiple PCR products that were sequenced after cloning. Sequencing revealed a total of 39 species of fungi (Table 2), with an average of 4.67 (range 1–11) species in each plant species per soil core. Cloning revealed up to five species in a single root fragment. Putative EcM fungi (18 species) and endophytes (15 species), which are all biotrophic, accounted for 89.8% of the retrieved sequences (Fig. 4). A single putative saprobe was found, indicating that samples were well preserved. Direct sequencing of PCR products resulted in 60.0% EcM species, whereas sequencing after cloning resulted in 75.0% endophytic species (significant difference: $\chi^2 = 10.5$, $df = 1$, $P = 0.001$), suggesting that the presence of multiple endophytes was the main obstacle to direct sequencing. Basidiomycetes and ascomycetes accounted for 51.3 and 48.7% of the fungal species, respectively. Only 43.6% of the root-inhabiting fungal taxa could be assigned to species (Table 2).

Seven fungal species were present on more than one pyroloid species. The endophytic *Phialocephala fortinii* sp. 1 was the only fungus identified in root systems of

Table 1 Net C gain from fungi in mixotrophic plants at Kärla and Värska (means ± SE), based on a linear mixing model

Species	C gain (%)
At Kärla	
Arctostaphylos uva-ursi	0 (Baseline)
Monotropa hypopithys	100 (Baseline)
Orthilia secunda	49.9 ± 4.9[a]
Pyrola chlorantha	37.6 ± 5.8[a]
Chimaphila umbellata	10.3 ± 5.1
Epipactis atrorubens	62.6 ± 7.2[a]
Platanthera bifolia	60.1 ± 7.4[a]
Listera ovata	19.9 ± 8.1
At Värska	
Vaccinium myrtillus	0 (Baseline)
Monotropa hypopithys	100 (Baseline)
Orthilia secunda	58.2 ± 5.2[a]
Chimaphila umbellata	29.0 ± 6.6[a]
Pyrola chlorantha	15.5 ± 5.8[a]
Pyrola rotundifolia	67.5 ± 6.4[a]

[a] Denotes significant difference from zero based on 95% confidence intervals following Phillips and Gregg (2001)

all three pyroloids. These shared fungal species were usually found in several root tips per plant. *P. chlorantha* and *O. secunda* had seven fungal species in common, while they shared only four and one fungal species with *C. umbellata*, respectively, but this may result from an unequal cloning effort (Table 2). Different fungi dominated in the three pyroloids. *P. fortinii* sp. 1 dominated the roots of *O. secunda*, inhabiting plants in all five soil cores. *Tricholoma imbricatum* was the most abundant in root systems of *P. chlorantha*, whereas *C. umbellata* hosted no clear dominants. EcM fungi were relatively more frequent in the least heterotrophic *C. umbellata* compared to *P. chlorantha* ($\chi^2 = 9.88$, $df = 1$, $P = 0.002$) and *O. secunda* ($\chi^2 = 8.02$, $df = 1$, $P = 0.005$), but this may be due to the fact that cloning contributed to only 12.5% of the different sequences amplified from *C. umbellata*, instead of 55 and 60% in *O. secunda* and *P. chlorantha*, respectively.

Discussion

Are pyroloids mixotrophic?

We verified the prediction, based on their phylogenetic position as sister group to MH lineages (Fig. 1), that pyroloids are mixotrophic. This is the first quantitative evidence for substantial mixotrophy mediated by mycorrhizal associations in non-orchid plants.

The $\delta^{13}C$ and $\delta^{15}N$ values of fungi, autotrophic and MH plants at our study sites were within the range reported for boreal forests (Taylor et al. 2003; Trudell et al. 2003). Moreover, there was low intraspecific dispersion of $\delta^{13}C$ and $\delta^{15}N$ values for each species, although different individuals were sampled, which allows us to draw conclusions from interspecific comparisons. In agreement with Trudell et al. (2003), the MH *M. hypopithys* displayed a similar $\delta^{13}C$ and 1.6‰ higher $\delta^{15}N$ value compared to its putative fungal C source at Kärla, *Tricholoma* spp. (see Bidartondo and Bruns 2002). This difference in $\delta^{15}N$ corresponds to the lower limit of trophic N fractionation in animal food chains (3.4 ± 1‰ SD; Post 2002).

Pyroloids displayed $\delta^{13}C$ values intermediate between the MH *M. hypopithys* and autotrophic plants at both study sites (Figs. 2, 3). Such values are unusual for green Ericaceae, as shown for other species in this and other studies (Delwiche et al. 1978), and suggest mixotrophy. Further supporting this, two of the potentially mixotrophic orchids, *Epipactis atrorubens* and *Platanthera bifolia*, had similar $\delta^{13}C$ values that are in the range expected for mixotrophic orchids (Gebauer and Meyer 2003; Julou et al. 2005). Noteworthy, *C. umbellata* was mixotrophic at Värska, but not significantly different in $\delta^{13}C$ from autotrophic plants at Kärla (Figs. 2, 3; Table 1). Similarly, no significant mixotrophy was observed for the hemiparasitic *Melampyrum sylvaticum* and the orchid *Listera ovata* at Kärla (Fig. 2a). The latter strongly ranges from mixotrophic to fully autotrophic, depending on the study site (Gebauer and Meyer 2003). Thus, *C. umbellata* could be either facultatively mixotrophic, or have a level of heterotrophy below our detection limits at Kärla.

Mixotrophic pyroloids and orchids had a high N content (Fig. 2), which can be explained by: (1) the N richness of fungal organic matter (2.5–4.1%, not shown), and (2) respiratory C losses that concentrate N in mixotrophs as compared to food source. Similarly, the hemiparasitic *M. sylvaticum* was somewhat N enriched (Fig. 2c, d). Mixotrophic pyroloids and orchids had high $\delta^{15}N$ values, also considered as indicators of MH nutrition (Trudell et al. 2003). However, the high $\delta^{15}N$ values of possibly autotrophic *C. umbellata* and *L. ovata* at Kärla suggest that ^{15}N concentration may vary between plant families with no clear relationship to mixotrophy.

Based on $\delta^{13}C$ values, mixotrophic pyroloids obtained up to 67.5% of their C in a non-photosynthetic way similar to *M. hypopithys*, i.e. from fungi (Table 1). Low photosynthetic rates in pyroloids (Hunt and Hope-Simpson 1990) allow equilibration of $^{13}CO_2$ and $^{12}CO_2$ concentrations in stomatal chambers and the strongest isotopic fractionation can thus occur. We therefore do not overestimate heterotrophy in anabolism (biomass accumulation).

Table 2 Occurrence of fungal species associated with roots of pyroloid plants at Kärla. Data indicate the number of root tips harbouring the fungus (*in parentheses* the number of plant individuals with such roots). *ITS* Internal transcribed spacer, *EcM* ectomycorrhizal fungus, *Endoph* endophyte, *Unkn* unknown, *Sapr* saprotroph, *An par* animal parasite

Species	EMBL Accession no.	Putative ecology	*Orthilia secunda*	*Pyrola chlorantha*	*Chimaphila umbellata*	Best sequence match spanning the whole ITS region		
						Species/isolate	Accession no	Identity (%)
Phialocephala fortinii s.l. sp. 1	AM181379	Endoph	12 (5)[a, b]	2 (2)[a, b]	1 (1)[b]	*Phialocephala fortinii* cryptic species 3	AY347410	100.0
Wilcoxina rehmii	AM181380	EcM	6 (2)[a, b]			*Wilcoxina rehmii*	DQ069001	99.8
Phialocephala fortinii s.l. sp. 2	AM181381	Endoph	5 (3)[a, b]	2 (1)[b]		*Phialocephala fortinii* cryptic species 2b	AY347402	100.0
Helotiales sp. 1	AM181382	Endoph	2 (2)[b]	2 (2)[a, b]		*Phragmites* root-associated fungus (Helotiales)	AJ875364	96.9
Helotiales sp. 2	AM181383	Endoph	2 (2)[a]	1 (1)[a]		EcM-associated fungus (Helotiales)	AY880946	99.4
Inocybe sp. 1	AM181384	EcM	2 (2)[a, b]			*Inocybe relicina*	AF325664	81.7
Tomentella sp. 1	AM181385	EcM	2 (1)[a, b]		1 (1)[a]	*Tomentella coerulea*	UDB000951	95.4
Helotiales sp. 3	AM181386	Endoph	1 (1)[a]	2 (1)[b]		EcM-associated fungus (Helotiales)	AJ430414	99.6
Tomentella sp. 2	AM181387	EcM	1 (1)[b]	1 (1)[a]		*Tomentella fuscocinerea*	UDB000776	95.6
Tetracladium maxilliforme	AM181388	Endoph	1 (1)[a]	1 (1)[b]		*Tetracladium maxilliforme*	DQ068996	100.0
Tricholoma myomyces	AM181389	EcM	1 (1)[a]		3 (1)[a]	*Tricholoma myomyces*	AF377210	100.0
Basidiomycota sp1	AM181390	Unkn	1 (1)[b]		1 (1)[a]	Orchid root-associated fungus (*Epulorhiza*)	AJ313456	80.1
Inocybe sp. 2	AM181391	EcM	1 (1)[a]		1 (1)[a]	*Inocybe godeyi*	AJ889954	79.6
Helotiales sp. 4	AM181392	Endoph	1 (1)[b]			Uncultured Helotiales	AY969372	94.7
Stachybotrys diachroa	AM181393	Sapr	1 (1)[b]			*Stachybotrys diachroa*	AF081472	100.0
Malassezia restricta	AM181394	An par	1 (1)[b]			*Malassezia restricta*	AJ437695	100.0
Tomentella subclavigera	AM181395	EcM	1 (1)[b]			*Tomentella subclavigera*	UDB000259	99.8
Sebacinales sp. 1	AM181396	EcM	1 (1)[b]			Decaying EcM root-associated fungus (Sebacinales)	DQ093739	96.1
Cenococcum geophilum	AM181397	EcM	1 (1)[b]			*Cenococcum geophilum*[c]	AY940649	98.7
Tricholoma imbricatum	AM181398	EcM		7 (3)[a, b]	1 (1)[a]	*Tricholoma imbricatum*	AY573537	100.0
Helotiales sp. 5	AM181399	Endoph		3 (2)[a, b]	1 (1)[a]	EcM-associated fungus (Helotiales)	AJ430412	97.8
Suillus variegatus	AM181400	EcM		3 (1)[a]		*Suillus variegatus*	AJ971399	98.8
Sebacinales sp. 2	AM181401	Endoph		2 (1)[b]		Ericoid root-associated fungus (Sebacinales)[c]	AF300769	95.5
Basidiomycota sp. 2	AM181402	Unkn		2 (1)[b]		*Exidiopsis plumbescens*	AF395309	83.2
Laccaria amethystina	AM181403	EcM		1 (1)[a]	2 (2)[a]	*Laccaria amethystina*	AF440665	100.0
Basidiomycota sp. 3	AM181404	Unkn		1 (1)[a]		*Clavulina cinerea*[d]	AY456339	80.7
Humaria hemisphaerica	AM181405	EcM		1 (1)[b]		*Humaria hemisphaerica*	UDB000988	100.0
Sebacinales sp. 3	AM181406	Endoph		1 (1)[b]		Orchid root-associated fungus (Sebacinales)	AY634132	99.2
Helotiales sp. 6	AM181407	Endoph		1 (1)[b]		*Chalara microchona*	DQ093752	95.9
Atheliaceae sp. 1	AM181408	EcM		1 (1)[b]		*Amphinema byssoides*	AY219839	83.8
Phialocephala fortinii s.l. sp. 3	AM181409	Endoph		1 (1)[a]		*Phialocephala fortinii* cryptic species 2a	AY347395	99.6
Helotiales sp. 7	AM181410	Endoph		1 (1)[b]		EcM-associated fungus (Helotiales)	AY587280	98.1
Helotiales sp. 8	AM181411	Endoph		1 (1)[b]		Ericoid root-associated fungus (Helotiales)	AY046400	99.6

Table 2 continued

Species	EMBL Accession no.	Putative ecology	*Orthilia secunda*	*Pyrola chlorantha*	*Chimaphila umbellata*	Best sequence match spanning the whole ITS region		
						Species/isolate	Accession no	Identity (%)
Helotiales sp. 9	AM181412	Endoph		1 (1)[b]		EcM-associated fungus (Helotiales)[c]	AJ292198	93.2
Tricholoma sp.	AM181413	EcM		1 (1)[b]		*Tricholoma myomyces*	AF377210	96.0
Atheliaceae sp. 2	AM181414	EcM			2 (1)[a]	*Amphinema byssoides*	AY219839	82.6
Atheliaceae sp. 3	AM181415	EcM			1 (1)[a]	*Piloderma fallax*[c]	AY010281	78.8
Hebeloma velutipes	AM181416	EcM			1 (1)[a]	*Hebeloma velutipes*	AY818351	100.0
Cadophora finlandica	AM181417	EcM			1 (1)[b]	*Cadophora finlandica*	AF486119	100.0

[a] Found by direct sequencing (and thus likely the sole colonist of the root)

[b] Found among other sequences after cloning

[c] Based on ITS2 match

[d] Based on partial ITS match

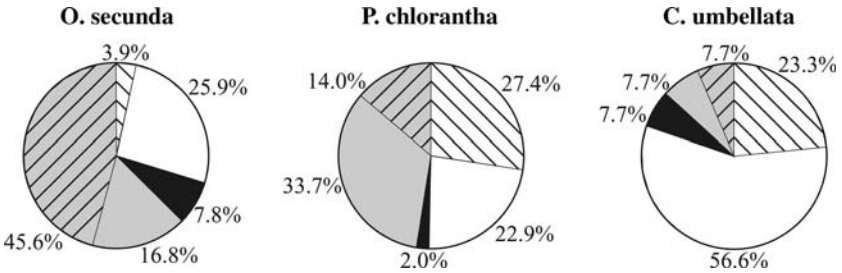

Fig. 4 Abundance of fungal trophic groups in the roots of the three investigated pyroloids at Kärla. Percentages show frequency of isolation per root tip. Whenever *n* fungi were retrieved from the same root fragment by cloning, the importance of each of these fungi was determined as 1/*n*. *Unshaded* EcM fungi (*hatched Tricholoma* spp.), *shaded* endophytic fungi (*hatched Phialocephala fortinii* spp.), *black* other fungi

What are the heterotrophic C sources and pathways for pyroloids?

Intracellular digestion of fungal hyphae is a shared feature of all MH plants (Smith and Read 1997) and mixotrophic orchids (Selosse et al. 2004) studied so far, during which C transfer may occur. In Monotropeae and Pterosporeae, small intracellular hyphal pegs release fungal cytosol by emitting membranaceous sacs (Robertson and Robertson 1982). This and the ^{15}N values suggest that hyphal contents, rather than cell wall materials, are transferred (Trudell et al. 2003). Hyphae form large intracellular coils in pyroloid roots (Read 1983; Robertson and Robertson 1985) but senescence of host cell cytoplasm always precedes that of the hyphae (Robertson and Robertson 1985; Smith and Read 1997), without any structural evidence of fungal lysis in living host cells. Pyroloids are therefore unusual among mixotrophic and MH plants. They suggest that either: (1) fungal lysis remains to be discovered in pyroloids, or (2) fungal lysis is unrelated to C transfer to MH plants, or (3) pyroloids exhibit different pathways of recovering fungal C. Extensive microscopic investigations carried out so far (Robertson and Robertson 1985; Smith and Read 1997) strongly favour the two latter explanations.

The EcM and endophytic fungi found in pyroloid roots (Fig. 4; Table 2) are potential C sources. We provide a preliminary description and the first molecular identification of their diversity in pyroloids. So far, only the EcM *Hysterangium* sp. has been identified on *O. secunda*, although many unidentified basidiomycetes were noted in pyroloids (Robertson and Robertson 1985; Smith and Read 1997). More fungal species could be documented with a more intensive and large-scale sampling. Unfortunately, our approach cannot distinguish between occasional colonists and C-supplying fungi. EcM fungi support

growth of ericaceous MHs (McKendrick et al. 2000; Leake 2004; Bidartondo 2005). Similarly, the identified EcM fungi may link pyroloids to surrounding trees, and allow indirect exploitation of tree C, as suggested for mixotrophic orchids (Bidartondo et al. 2004; Selosse et al. 2004; Julou et al. 2005; Girlanda et al. 2006). In a parallel study at the same site, two pyroloid EcM fungi, *Wilcoxina rehmii* and *Humaria hemisphaerica*, were identified on neighbouring tree roots (Tedersoo et al. 2006). Congruently, Japanese *Pyrola incarnata* and *Larix kaempferi* mycorrhizas displayed identical restriction fragment length polymorphism patterns of fungal ITS (Kunishi et al. 2004).

Moreover, endophytic fungi are diverse, abundant (Fig. 4), and usually co-occur with EcM fungi (Table 2). Indeed, unidentified root endophytes have already been isolated from several pyroloids (Lihnell 1942). Some endophytes, especially *Phialocephala fortinii*, have the potential to link roots of different plants, because of low host specificity and large persistent genets (Ahlich and Sieber 1996, Queloz et al. 2005). Root endophytes are considered commensalists, but there are a few reports of altered growth rate and/or improved nutrition of host plants, especially in nutrient-poor organic soils (Addy et al. 2005). Their contribution to pyroloid mixotrophy is thus an intriguing possibility. In any case, despite the fact that EcM fungi and helotialean root-associated fungi can obtain limited amounts of organic compounds from soil (Read et al. 2004), surrounding green plants are the most likely ultimate C source for pyroloids. Although Read (1983) failed to demonstrate C flow from *Salix* sp. to *P. rotundifolia* linked by shared mycorrhizal fungi, preliminary reports of a ^{13}C -labelling pot experiment suggest C transfer from *Larix kaempferi* to *Pyrola incarnata* (Kunishi et al. 2004). More studies using in situ C labelling are needed to directly demonstrate the C transfer from autotrophic trees via fungi to pyroloids in environmental conditions.

Pyroloids harbour a wide spectrum of EcM fungi, including *Suillus variegatus* that is considered as *Pinus*-specific. Pyroloids have less specific mycorrhizal associations than MH species, although a preference was observed for *Tricholoma* species (Fig. 4), which also specifically associate with several MH monotropoids (Bidartondo 2005). Similarly, mixotrophic orchids display mycorrhizal preferences, but no strict specificity (Bidartondo et al. 2004, Selosse et al. 2004; Julou et al. 2005). Full abandonment of photosynthesis may require higher specificity, perhaps because elevated functional compatibility is achievable with only a limited number of partners (Bruns et al. 2002).

Ecological implications of mixotrophy in pyroloids

Mixotrophy likely allows pyroloids to colonize shaded forest environments since they display low photosynthetic activities in natural shady conditions (Isogai et al. 2003 and references therein) and do not increase photosynthesis after shading (Hunt and Hope-Simpson 1990). Hunt and Hope-Simpson (1990) suggested that the vegetative vigour of *P. rotundifolia* in these conditions might be explained by exploitation of fungal C. Thus, pyroloids have two adaptations to understorey niches: (1) vernal photosynthesis allowed by their evergreenness (Isogai et al. 2003), and (2) exploitation of fungal C, probably later in the year after tree photosynthesis has started. Thus, we can speculate that $\delta^{13}C$ may fluctuate over the year in pyroloids. Gebauer (2005) proposed that heterotrophy level is inversely correlated with light availability, as reported for mixotrophic orchids (Julou et al. 2005) and mixotrophic planktonic algae (Jakobsen et al. 2000). For example, *P. rotundifolia* from a luminous forest edge had a photosynthetic rate comparable to that of neighbouring green plants (M.-A. Selosse and C. Damesin, unpublished data); in our data, both *O. secunda* and *C. umbellata* had higher C gain from fungi at Värska versus Kärla, that is less shaded, but *P. chlorantha* had lower C gain at Värska. Obviously, analysis of other sites is required to further support Gebauer's appealing hypothesis. Despite comparable perennial growth, mixotrophic evergreen pyroloids contrast with mixotrophic forest orchids that survive belowground in winter and early spring, and do not access the light available in spring.

Mixotrophs exert considerable grazing pressure on primary producers and affect competitive balance in marine food webs (Havskum and Riemann 1996; Jeong et al. 2005). Similarly, parasitic and hemiparasitic terrestrial plants shape both the diversity and productivity of vegetation and related microflora in grasslands (Press and Phoenix 2005; Bardgett et al. 2006). Is a similar effect also exerted by mycorrhiza-dependent mixotrophs? Pyroloids, in contrast to mixotrophic orchids, cover large surfaces by vegetative spread, can be dominant (as at our study sites) and respire C around the year due to evergreenness. Thus, they may drain significant amounts of C from their fungal and plant associates. Analogously, high abundance of pyroloids and other mixotrophs may decrease reproduction and competitiveness of both EcM autotrophic plants and even associated fungi. Boreal forests, with low primary productivity, may be especially vulnerable. Moreover, species of *Moneses, Orthilia* and *Pyrola* are alternate hosts for the pathogenic rust *Chrysomyxa*

pirolata, which substantially reduces the production and germination ability of *Picea* spp. seeds (Singh and Carew 1990). Altogether, this highlights the intriguing possibility that forest mixotrophs are important drivers of dynamics, competitive interactions and biodiversity, both in the plant and fungal communities. More generally, there is a need to reconsider ecological roles of forest understorey plants, which are often underestimated owing to their low biomass.

Evolution of mixo- and mycoheterotrophy

In this study, the phylogenetic position of pyroloids (Fig. 1) successfully predicted their mixotrophy, supporting that a parallel evolution occurred in forest orchids and Monotropoideae. In particular, the phylogenetic position of Pyroleae as a sister tribe to MH tribes (Kron et al. 2002, Fig. 1) suggests two scenarios, either: (1) mixotrophy was acquired by the ancestor of all Monotropoideae and predisposed to the rise of MH clades, or (2) mixotrophy and MH evolved independently and repeatedly in the Monotropoideae from a common ancestor presenting some (ecological?) predisposition to exploiting fungal C. The latter scenario is less parsimonious. Unpublished phylogenies on nuclear ribosomal genes suggest that pyroloids are paraphyletic with respect to the MH clades (K. Kron, personal communication) and strongly support the first scenario. Trophic analysis of *Moneses uniflora*, that is likely mixotrophic under the most parsimonious evolutionary scenario, is highly relevant. The association with EcM fungi, which are also mycorrhizal on Arbutoideae (Richard et al. 2005), likely evolved in the ancestor common to all Ericaceae, except Enkianthoideae (Fig. 1). If mixotrophic traits were already established in that ancestor, then species from the Ericoideae and Vaccinioideae tribes (Fig. 1) that can associate with some EcM partners of surrounding trees (Villarreal-Ruiz et al. 2004) could be mixotrophic. The abundance and ecological importance of these Ericaceae renders such studies highly relevant. Similarly, other forest taxa related to MHs (e.g. in Gentianaceae and Polygalaceae; Leake 2004) may include mixotrophic species.

We showed that pyroloids use a C source similar to that of MH plants in boreal forests, substantiating and quantifying the use of fungal C. The fungal associates most likely create mycelial links to surrounding green plants and allow a net C flow. Additional studies are needed to demonstrate this flow in situ and to establish its importance spatially and seasonally. Investigations on other Pyroleae and Ericaceae in general will reveal the evolution of mixotrophy in this family. Study of other understorey plants may unravel additional mixotrophs, whose ecological role in C cycling and dynamics of forest plant communities awaits further testing.

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