Letters

Stable isotope natural abundances of fungal hyphae extracted from the roots of arbuscular mycorrhizal mycoheterotrophs and rhizoctonia-associated orchids

Nutritional modes in fungi: contributions from stable isotope natural abundances

Since the first discovery of unique carbon (C) and nitrogen (N) isotope signatures in fungal fruiting bodies (Gebauer & Dietrich, 1993; Gleixner *et al.*, 1993), natural abundances of stable isotopes have been extensively used to identify the nutritional dynamics of fungi (Mayor *et al.*, 2009). Assigning ecological roles of fungi is essential to determine the role of individual taxa in nutrient cycling and forest ecology. The use of isotope natural abundances in forest ecosystems has been crucial in distinguishing fungi with two main modes of life: ectomycorrhizal and saprotrophic fungi (Henn & Chapela, 2001). Within saprotrophic fungi, isotope natural abundances further allow the identification of the substrates used (Kohzu *et al.*, 1999). Dual isotope analyses of the $\delta^{13}C$ and $\delta^{15}N$ values consistently indicate a differentiation in isotopic signatures between ectomycorrhizal and saprotrophic fungi within and among ecosystems (Henn & Chapela, 2001; Taylor *et al.*, 2003; Trudell *et al.*, 2004; Mayor *et al.*, 2009). These signatures have been shown to reflect the ecophysiology of fungi and demonstrate that fungi that can utilize organic nitrogen exhibit higher $\delta^{15}N$ than those fungi restricted to mineral nitrogen sources (Gebauer & Taylor, 1999; Lilleskov *et al.*, 2002). Still, the ability to distinguish fungal nutritional modes has been long restricted to fungi that produce macroscopic sporocarps, such as mushrooms, due to their large mass which allows for physical measurements. Thus, for many fungi, particularly those associated with plant roots that do not form evident fruiting bodies, isotope natural abundances of fungal hyphae are scarce.

Fungal groups with missing stable isotope natural abundance signatures

Besides ectomycorrhizal fungi, isotope natural abundances are known for sporocarp-forming ericoid (e.g. Hobbie & Hogberg, 2012) and orchid-associated nonrhizoctonia saprotrophic fungi (e.g. Ogura-Tsujita *et al.*, 2009). Yet, values of $\delta^{13}C$ and $\delta^{15}N$ are poorly known for arbuscular mycorrhizal fungi (but see e.g. Courty *et al.*, 2011; Suetsugu *et al.*, 2020, for isotope values of fungal spores), and the orchid-associated fungi known as 'rhizoctonia' in natural conditions. Recently, Klink *et al.* (2020) obtained the $\delta^{13}C$ and $\delta^{15}N$ of arbuscular mycorrhizal hyphae isolated from roots of a grass and a legume, inoculated in experimental conditions, thereby providing an efficient method to extract hyphae from roots. Using this method with a few modifications, here, we measured the isotope natural abundances $\delta^{13}C$ and $\delta^{15}N$ of naturally occurring arbuscular mycorrhizal (Fig. 1a–c) and orchid-associated hyphae (Fig. 1d–f) directly from roots (see Supporting Information Methods S1). To obtain hyphae of arbuscular mycorrhizal fungi, we selected two species of fully mycoheterotrophic plants: *Thismia megalongensis* C. A. Hunt, G. Steenbee. & V. Merckx and *Sciaphila megastyla* Fukuy. & T. Suzuki. Mycoheterotrophs are achlorophyllous plants that obtain carbon from their associated fungal partners (Leake, 1994; Merckx, 2013). Species in the plant genus *Thismia* have been demonstrated to be highly specialized on narrow lineages of Glomeromycotina fungi (Gomes *et al.*, 2017; Merckx *et al.*, 2017), while species of *Sciaphila* tend to associate with a wider phylogenetic diversity within the fungal subphylum (Merckx *et al.*, 2012; Suetsugu & Okada, 2021). For fungi associated with orchid roots, we selected two chlorophyllous partially mycoheterotrophic orchid species, known to associate with rhizoctonia symbionts, *Orchis militaris* L. and *Ophrys insectifera* L., for which both isotope natural abundances and Sanger sequencing of the root-associated fungi have been performed previously (Schweiger *et al.*, 2018). To be able to compare isotope values across sampling sites, the δ values of C and N stable isotope abundances were normalized by calculating enrichment factors (ε; see Methods S1).

Arbuscular mycorrhizal fungi

The enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N$ were significantly different between the fungal hyphae, mycoheterotrophic and reference plants for both *T. megalongensis* and *S. megastyla* (Fig. 1g; Table 1). For both species, $\varepsilon^{13}C$ was not distinguishable between the mycoheterotrophs and respective fungal hyphae, while $\varepsilon^{15}N$ was significantly different between mycoheterotrophs and fungi for *S. megastyla*, and marginally significant for *T. megalongensis* (Fig. 1; Table 1). In relation to the reference plants, the fungi extracted from both mycoheterotrophic species were significantly enriched in $\varepsilon^{13}C$, and fungi from *S. megastyla* were marginally significantly depleted in $\varepsilon^{15}N$. Similarly, both mycoheterotrophic plants were enriched in $\varepsilon^{13}C$ although only significantly for *T. megalongensis*. This indicates that the $\varepsilon^{13}C$ of fungal hyphae drives the ^{13}C enrichment of arbuscular mycorrhizal fully mycoheterotrophic plants, and there seems to be a difference in nitrogen source between *T. megalongensis* and *S. megastyla*-associated fungi. Each

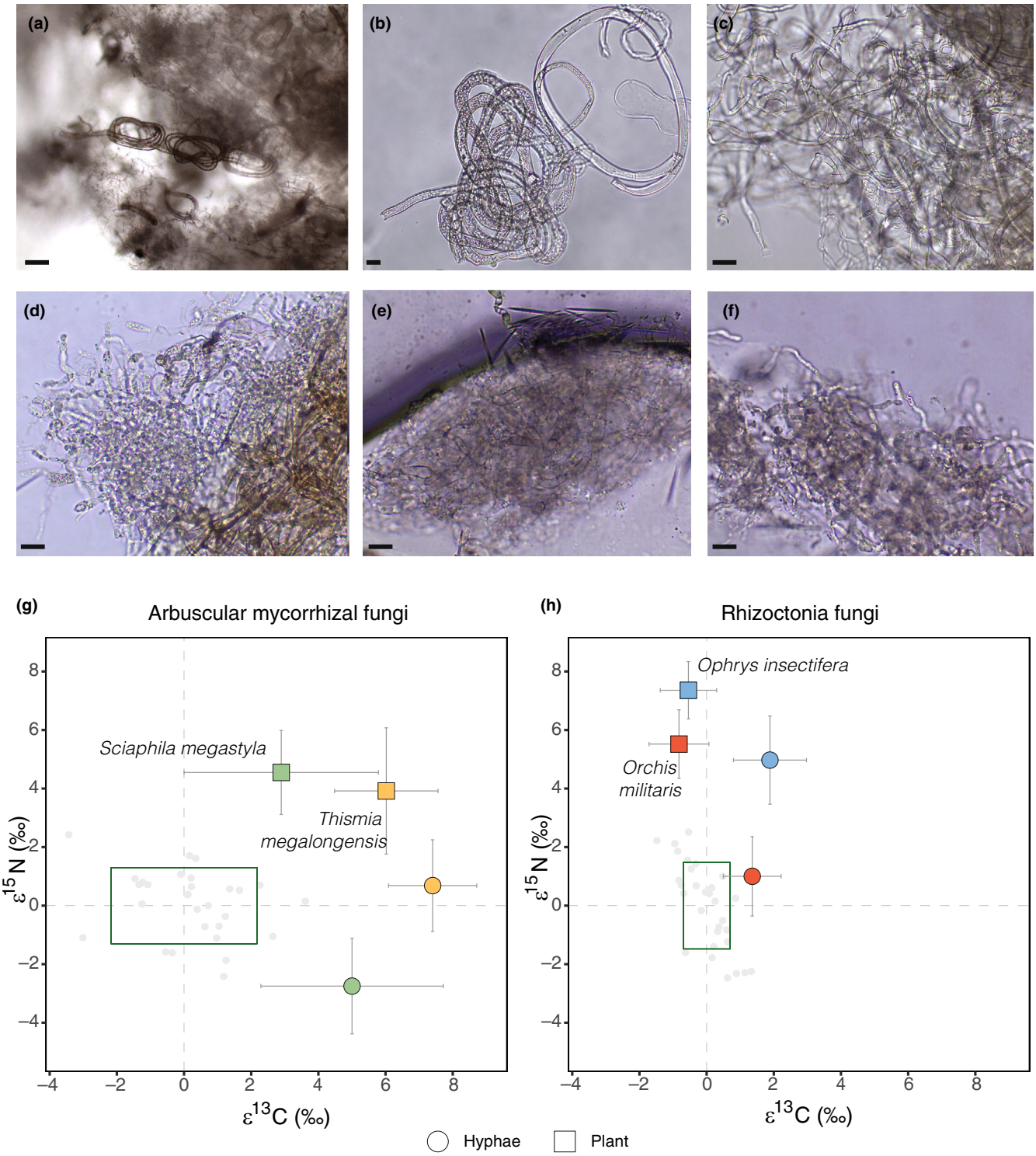

Fig. 1 Root-associated hyphae of *Thismia megalongensis* (a–c) and *Orchis militaris* (d–f). We observed coils (a, b) and pelotons (d, e) released from plant cells, and hyphae present in the pellets (c, f) recovered following the hyphal extraction protocol. Mean enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N \pm SD$ of the two fully mycoheterotrophic species associated with arbuscular mycorrhizal fungi – *Sciaphila megastyla* from Japan and *T. megalongensis* from Australia (g), and the two rhizoctonia-associated orchids – *Ophrys insectifera* and *O. militaris*, both collected in Germany (h) are depicted. Fungal hyphae (circles; $n = 5$ per plant species) and target plants (squares; $n = 5$ per plant species) are represented. The green rectangle represents the SD of the reference plants ($n = 3$ per plant species, except for *S. megastyla* where only two plant species were available). Bars, 20 μm.

Table 1 ^{13}C and ^{15}N enrichment factor differences between types of material: target plants (plant), fungal hyphae (hyphae) and reference plants (Ref), calculated using the Dunn's test.

			Ref-plant		Ref-hyphae		Plant-hyphae	
Fungal type	Plant species	Isotope	*Z*	*P*	*Z*	*P*	*Z*	*P*
AMF	*Thismia megalongensis*	$\varepsilon^{13}C$	2.68	**0.007**	3.89	**<0.001**	0.99	0.162
		$\varepsilon^{15}N$	2.91	**0.005**	0.86	0.195	−1.68	0.094
	Sciaphila megastyla	$\varepsilon^{13}C$	1.08	0.140	2.53	**0.017**	1.22	0.223
		$\varepsilon^{15}N$	2.64	**0.008**	−1.58	0.057	−3.55	**<0.001**
Rhizoctonia	*Orchis militaris*	$\varepsilon^{13}C$	−1.55	0.060	2.21	**0.027**	3.07	**0.003**
		$\varepsilon^{15}N$	3.53	**<0.001**	0.95	0.172	−2.11	**0.035**
	Ophrys insectifera	$\varepsilon^{13}C$	−1.18	0.118	2.63	**0.001**	3.12	**0.003**
		$\varepsilon^{15}N$	3.93	**<0.001**	2.56	**0.010**	−1.12	0.132

Kruskal–Wallis tests indicated significant differences between tissue of target plant, fungal hyphae and reference plants (*T. megalongensis*: $\varepsilon^{13}C$: $\chi^2 = 18.28$, df = 2, $P < 0.001$; $\varepsilon^{15}N$: $\chi^2 = 8.50$, $P = 0.010$; *S. megastyla*: $\varepsilon^{13}C$: $\chi^2 = 6.51$, $P = 0.040$; $\varepsilon^{15}N$: $\chi^2 = 13.07$, $P < 0.001$; *O. militaris*: $\varepsilon^{13}C$: $\chi^2 = 9.61$, $P = 0.010$; $\varepsilon^{15}N$: $\chi^2 = 12.44$, $P < 0.001$; *O. insectifera*: $\varepsilon^{13}C$: $\chi^2 = 10.55$, $P = 0.010$; $\varepsilon^{15}N$: $\chi^2 = 18.10$, $P < 0.001$). Significant *P*-values ($\alpha < 0.05$) are highlighted in bold. Target plants and fungal hyphae are represented by five individual samples. Reference plants are represented by three plant species per target plant, except for *S. megastyla* where only two plant species were available on the sampling location (Supporting Information Table S1 for details). *P*-values were adjusted using Bonferroni–Holm corrections for multiple comparisons. AMF, arbuscular mycorrhizal fungi.

mycoheterotrophic plant species is associated with nonoverlapping fungal clades within the Glomeromycotina (Fig. 2a). *Sciaphila megastyla* harboured fungi belonging to the genera *Dominikia*, *Kamienskia* and two unidentified amplicon sequence variants, while the fungi in the roots of *T. megalongensis* belonged exclusively to the genus *Rhizophagus*, supporting a specialization on fungal interactions of different degrees between these plant lineages (Gomes *et al.*, 2020; Suetsugu & Okada, 2021).

Rhizoctonia fungi associated with orchids

The enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N$ were generally significantly different between fungal hyphae, orchids and reference plants for both *O. militaris* and *O. insectifera* (Fig. 1h; Table 1). Both $\varepsilon^{13}C$ and $\varepsilon^{15}N$ were significantly different between orchid leaves and hyphae for *O. militaris*, while for *O. insectifera*, only $\varepsilon^{13}C$ was significantly higher in the hyphae in comparison with the plant tissue (Fig. 1; Table 1). In both orchid species, fungal hyphae were significantly enriched in $\varepsilon^{13}C$, and in *O. insectifera* fungi were also enriched in $\varepsilon^{15}N$ in relation to the reference plants. The fungal hyphae extracted from the two orchid species were only weakly enriched in $\varepsilon^{13}C$ in comparison with reference plants and far less enriched in ^{13}C than tissues of ectomycorrhizal fungi reported previously (Mayor *et al.*, 2009). This observation is consistent with previous findings of absence of ^{13}C enrichment in fully mycoheterotrophic protocorms of *O. militaris*, which were also associated with rhizoctonia fungi by Schweiger *et al.* (2018). Interestingly, in that study, protocorms of *O. insectifera* were somewhat enriched in ^{13}C.

We detected most sequenced reads obtained from root pieces to belong to the fungal order Helotiales. Fungi in the genus *Ilyonectria* were also detected, concordant with previous observations of these orchid species collected at the same site (Schweiger *et al.*, 2018). Both Helotiales and *Ilyonectria* were present in the roots of both orchid species and, as far as we know, have an unknown ecological function. Helotiales have also been detected in the species studied in Zahn *et al.* (2023). In addition, we detected rhizoctonia fungi belonging to the families Ceratobasidiaceae, Serendipitaceae and Thelephoraceae in the roots of *O. insectifera*, and to the families Ceratobasidiaceae and Thelephoraceae in the roots of *O. militaris* (Fig. 2b). One orchid individual of *O. militaris* presented a high relative abundance of Ceratobasidiaceae, and another of Thelephoraceae in their roots. We cannot exclude that Tulasnellaceae are underrepresented in our data influenced by the primers used (Vogt-Schilb *et al.*, 2020), as these taxa have been shown to be present in *O. insectifera* roots (Schweiger *et al.*, 2019). Besides rhizoctonia fungi, we also found fungi known to form ectomycorrhizas (according to FungalTraits; Põlme *et al.*, 2020), such as *Sebacina* (Sebacinaceae), *Amphinema* (Atheliaceae), *Hebeloma* and *Hymenogaster* (Hymenogastraceae) in two *O. insectifera* individuals. In terms of isotope signatures, no apparent differences were observed between individual samples where rhizoctonia fungi are present and those where Helotiales are predominant, and neither in relation to the plant material between specimens. Yet, a larger sample size would be needed to properly evaluate this.

Fungal hyphae of arbuscular mycorrhizal and orchid-associated fungi are depleted in ^{15}N

While $\varepsilon^{13}C$ values of hyphae are within the same range as found for the respective plant tissues of the AM mycoheterotrophic plants, as expected, $\varepsilon^{15}N$ values of the hyphae were considerably lower than $\varepsilon^{15}N$ of the respective plant tissues. This relative ^{15}N depletion of hyphae in comparison with plant tissue was also observed for the two orchid species. One could wonder whether this depletion is either due to potential loss of hyphal content during extraction considering that nitrogen in chitin is depleted in ^{15}N by *c.* 10‰ in comparison with fungal protein (Taylor *et al.*, 1997; Hobbie & Hogberg, 2012) or due to a selective transport of ^{15}N-enriched protein-derived compounds from fungal to plant tissues. Similarly, Zahn *et al.* (2023) show an equal depletion in ^{15}N of hyphae extracted from two rhizoctonia-associated orchid species and for identically extracted hyphae from ectomycorrhiza-associated orchid roots an even larger depletion in ^{15}N in relation to orchid leaves.

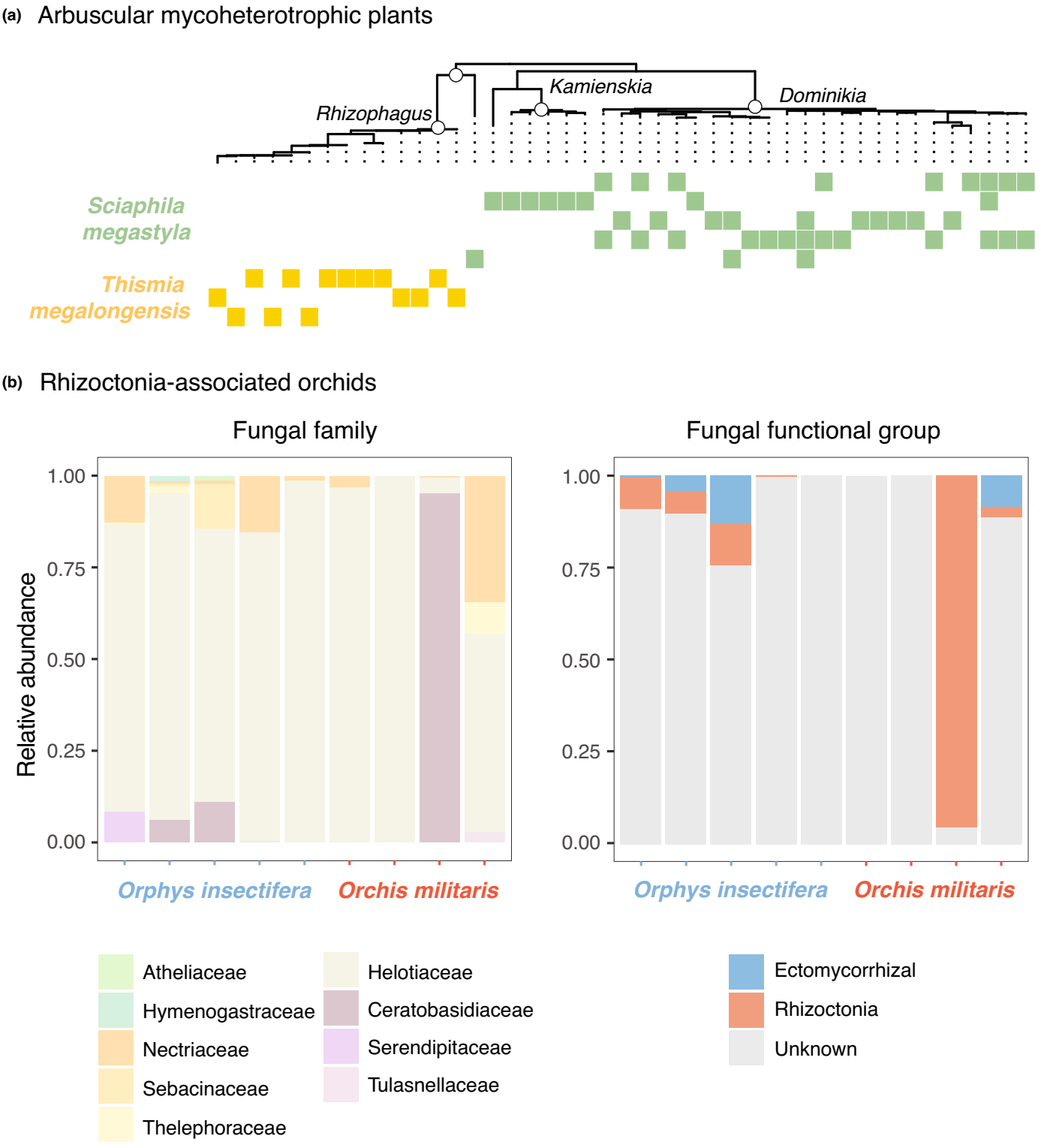

Fig. 2 Fungal diversity detected in the roots of individual plants of (a) the arbuscular mycorrhizal mycoheterotrophic plants: *Sciaphila megastyla* ($n = 5$) and *Thismia megalongensis* ($n = 3$), represented with the phylogenetic relationship between the fungi, where open circles indicate bootstrap support values > 90% for major clades; and (b) the orchid species: *Ophrys insectifera* ($n = 5$) and *O. militaris* ($n = 4$), represented per fungal family (left panel) and fungal functional group (right panel).

In addition, the N concentrations of the extracted fungal hyphae of both *T. megalongensis* (2.25 ± 0.53 mmol g_{dw}^{-1}) and *S. megastyla* (2.18 ± 0.42 mmol g_{dw}^{-1}) were not distinguishable from those of the mycoheterotrophic plant tissues (1.95 ± 0.28 and 1.88 ± 0.45 mmol g_{dw}^{-1} respectively), in congruence with Klink *et al.* (2020), while reference plants presented lower N

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concentrations (1.36 ± 0.44 and 1.17 ± 0.19 mmol g_{dw}^{-1} for each set respectively) in relation to both fungal hyphae (*T. megalongensis*: $Z = 2.772$, $P = 0.008$, and *S. megastyla:* $Z = 3.231$, $P = 0.002$) and mycoheterotrophic plants (*T. megalongensis*: $Z = 2.140$, $P = 0.032$ and *S. megastyla:* $Z = 2.710$, $P = 0.007$). The N concentrations between fungal hyphae (1.19 ± 0.23 mmol g_{dw}^{-1} for *O. militaris* and 1.64 ± 0.17 mmol g_{dw}^{-1} for *O. insectifera*), orchids (1.74 ± 0.07 and 2.19 ± 0.17 mmol g_{dw}^{-1} respectively) and reference plants (1.62 ± 0.76 and 1.79 ± 0.79 mmol g_{dw}^{-1} for each set respectively) were not statistically different for both orchid species. In Zahn *et al.* (2023), the fungal hyphae extracted from rhizoctonia-associated orchids were also nondistinguishable from reference plants, while for one species (*Anoectochilus sandvicensis*), fungal hyphae had significantly lower N concentration than the orchid leaves.

Isotopic signatures and fungal identification

The arbuscular mycorrhizal diversity in the roots of the mycoheterotrophic plant species did not overlap between *T. megalongensis* and *S. megastyla*, and the fungal enrichment in $\varepsilon^{15}N$ was variable between plant species. The association with different fungal genera, in addition to local soil nitrogen availability, could have contributed to the differences in $\varepsilon^{15}N$ between species. Further studies are required to assess the source of variation and generality of isotope values among arbuscular mycorrhizal fungi.

In the orchid-associated fungi, the fungal composition was variable between individual specimens, yet without reflection on the isotopic values of the extracted hyphae. The absence of differences in fungal isotopic values may indicate an artefact on the integration of both techniques. The apparent dominance of specific fungal groups in the roots could reflect spatial segregation of fungi, as a small piece of root was used for sequencing, while for the hyphal extraction, the remainder of the root system was used. Furthermore, ectomycorrhizal fungi were detected in two individuals of *O. insectifera*, while rhizoctonia fungi were detected in three individuals. The presence of ectomycorrhizal fungi in the roots of some rhizoctonia-associated orchids is commonly reported in the literature (e.g. Jacquemyn *et al.*, 2021), yet it remains to be demonstrated whether these fungi indeed establish a mycorrhizal symbiosis with the orchid, in a sporadic or constant way during the orchid development, or represent endophytic fungi as it has been shown in typical nonmycorrhizal hosts (Schneider-Maunoury *et al.*, 2020). Nevertheless, we cannot exclude those ectomycorrhizal fungi found in the roots of *O. insectifera* to be responsible for the slight enrichment in ^{13}C and ^{15}N of the hyphae extracted from *O. insectifera* in comparison with *O. militaris*. However, these isotopic differences are rather small and are not seen in the leaves of these two species, that is there appears to be no major plant matter gain from these ectomycorrhizal fungi. In addition, our results reveal that sporadic appearance of ectomycorrhizal fungi in orchids hitherto classified as rhizoctonia-associated does obviously not affect their isotope signature. Zahn *et al.* (2023) present further isotope signatures and diversity of root-associated fungi of orchids associated with ectomycorrhizal fungi.

The assessment of fungal diversity often comprises a qualitative snapshot of a fraction of the root system, and although different fungal species or guilds may contribute differently to nutrient uptake at multiple occasions, we still lack a solid framework to quantify the contribution of each of these fungi to fungal–plant matter exchange. By contrast, isotopic abundance data are a temporal and spatial integrator (Dawson *et al.*, 2002) over all fungal–plant matter exchange processes without providing direct information about the role of the individual potential fungal players, which is less sensitive to occasional changes in carbon or nitrogen supply.

Conclusion

To the best of our knowledge, we reveal for the first-time isotope signatures of hyphae of arbuscular mycorrhizal fungi in mycoheterotrophic plants and, together with Zahn *et al.* (2023), of fungal pelotons present in chlorophyllous orchids in relation to the plant tissues from their roots. Arbuscular mycorrhizal hyphae have isotope signatures that allow a significant distinction in $\varepsilon^{13}C$ abundance in relation to reference plants. Subsequently, hyphae resemble the $\varepsilon^{13}C$ of the mycoheterotrophic plants, suggesting that these mycoheterotrophs gain carbon from the detected fungi. For the orchid-associated fungi, hyphae are only slightly enriched in ^{13}C in relation to both reference and orchid plants, remaining unclear whether these orchids gain C from the associated fungi based on these results. However, the significant enrichment in ^{15}N of hyphae or orchid leaves indirectly indicates a partial mycoheterotrophic matter gain by these orchids.

Our study appeals to a careful interpretation when integrating root-associated fungal diversity and isotope natural abundances considering their inherent ecological significance as each method contains fundamentally different categories of information. Still, the combination of both approaches is greatly valuable and contributes to understand complex patterns in plant–fungal interactions, for example considering spatial and temporal fungal colonization in roots, and both advantages and caveats of each technique should be considered in the subsequent interpretation of ecological patterns. Finally, including the isotopic signatures of root-associated fungi in the context of mycorrhizal symbiosis contributes to a direct observation of fungal participation to organic matter gain of the plant.

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Competing interests

None declared.

Author contributions

SIFG and GG designed the research and collected the orchid material. PG and SK guided the fungal hyphal extraction by SIFG. CH and KS collected the arbuscular mycorrhizal plant material. GG supervised the isotope abundance analyses. SIFG performed the molecular analysis, analysed the data, and together with GG wrote the manuscript. All authors commented and approved the final version of the manuscript.

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Data availability

Isotope abundance data are available in the Supporting Information. Raw sequencing data are available in GenBank/SRA under project number PRJNA966927.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Methods S1 Methods used in this paper.

Table S1 Isotope raw data and identity of reference plants at genus level.

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