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RESEARCH ARTICLE

Mycoheterotrophic plants living on arbuscular mycorrhizal fungi are generally enriched in ^{13}C, ^{15}N and ^{2}H isotopes

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Abstract

1. Fully mycoheterotrophic plants are thought to obtain carbon exclusively from their root-associated fungal partners. The general enrichment of these plants in the heavy isotopes ^{13}C and ^{15}N suggests that fungi are the main nutrient source for these plants. Yet, the majority of studies have targeted mycoheterotrophic plants associated with ectomycorrhizal, orchid mycorrhizal and saprotrophic fungi, while mycoheterotrophic plants living on arbuscular mycorrhizal fungi remain understudied.
2. Here, we sampled 13 species of arbuscular mycorrhizal fully mycoheterotrophic plants from five families and co-occurring autotrophic reference plants growing in forests of tropical South America, tropical South East Asia and temperate Australasia. We measured stable isotope natural abundances ($\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ and $\delta^{18}O$), determined total nitrogen concentrations and used high-throughput DNA sequencing to characterize the arbuscular mycorrhizal fungal communities associated with the sampled mycoheterotrophic plants.
3. We observed a general enrichment in ^{13}C and ^{15}N isotopes across mycoheterotrophic plant families and geographic regions. We confirm cases where no ^{15}N enrichment is present, but we show that in general arbuscular mycoheterotrophic plants are enriched in ^{15}N. Moreover, we demonstrate for the first time that these plants are significantly enriched in ^{2}H but not in ^{18}O in relation to their autotrophic references. The fungal communities targeted by the mycoheterotrophs mainly consist of Glomeraceae and show strong association with the isotopic signatures and geographic origin of the plants.
4. *Synthesis*. Our findings enlarge the limited knowledge on the multi-element stable isotopic signatures of mycoheterotrophic plants living on arbuscular mycorrhizal fungi. We show that these plants are enriched in ^{13}C and ^{2}H as expected due to their mycoheterotrophic nutrition, and that in general they are also enriched in ^{15}N, despite some exceptions. Variation in stable isotope signatures is likely influenced by plant taxonomy, geography and fungal community composition.

Sofia I. F. Gomes and Vincent S. F. T. Merckx contributed equally to this study.

KEYWORDS
arbuscular mycorrhizal fungi, carbon (C), hydrogen (H), mycoheterotrophy, mycorrhiza, nitrogen (N), oxygen (O), stable isotopes

1 | INTRODUCTION

Full mycoheterotrophy is an evolutionary widespread nutritional mode in which achlorophyllous plants obtain carbon (C) through their root-associated fungi (Leake, 1994; Merckx, 2013). Fully mycoheterotrophic plants can associate with different functional groups of fungi, including mycorrhizal and saprotrophic fungi (Merckx, 2013). Identification of the fungal partners by DNA sequencing of the root tips of these plants (e.g. Bidartondo et al., 2002; Cullings, Szaro, & Bruns, 1996; Ogura-Tsujita, Umata, & Yukawa, 2013; Taylor & Bruns, 1997), in combination with isotope natural abundances (e.g. Gebauer & Meyer, 2003; Merckx, Stöckel, Fleischmann, Bruns, & Gebauer, 2010; Ogura-Tsujita, Gebauer, Hashimoto, Umata, & Yukawa, 2009), has been instrumental to reveal the origin of the C obtained by mycoheterotrophic plants, and consequently that of their associated fungi (Leake & Cameron, 2010). Analysis of ^{13}C and ^{15}N natural abundances has been extensively used to provide insights into C and nitrogen (N) origin of mycoheterotrophic plants associated with ectomycorrhizal (Gebauer & Meyer, 2003; Hynson et al., 2013), and wood- and litter-decaying fungi (Lee, Yang, & Gebauer, 2015; Martos et al., 2009; Ogura-Tsujita et al., 2009). These studies show that these mycoheterotrophic plants are significantly enriched in ^{13}C and ^{15}N compared to neighbouring autotrophic understorey plants (Gebauer & Meyer, 2003; Zimmer, Meyer, & Gebauer, 2008). These isotopic enrichments indicate that mycoheterotrophic plants associated with ectomycorrhizal, wood- and litter-decaying fungi obtain C and N through distinct pathways compared to autotrophic plants, reflecting their fungal associates' isotopic signatures (Gebauer & Meyer, 2003).

In fact, when isotope data of fungal fruiting bodies are available for comparison, the isotope signatures of mycoheterotrophic plants resemble those of their associated fungi, providing further evidence that the fungi are the main C and N donors for these plants (Gebauer & Meyer, 2003; Schiebold, Bidartondo, Karasch, Gravendeel, & Gebauer, 2017; Trudell, Rygiewicz, & Edmonds, 2003).

Recently, Gebauer, Preiss, and Gebauer (2016) used hydrogen (H) isotope signatures in addition to the routinely employed C and N measurements to investigate nutrient flows in mycoheterotrophic interactions of orchids, reasoning that secondary heterotrophic organic compounds are enriched in ^{2}H compared to primary photosynthetic compounds (Yakir, 1992). Consequently, hydrogen stable isotope signatures could also be used as indicators for mycoheterotrophic nutrition (Cormier, Werner, Leuenberger, & Kahmen, 2019). Indeed, based on hydrogen stable isotope signatures, partial mycoheterotrophy in orchids that are associated with 'rhizoctonia' mycorrhizas was demonstrated by Gebauer, Preiss, and Gebauer (2016) and later by Schweiger, Bidartondo, and Gebauer (2018) and Schiebold, Bidartondo, Lenhard, Makiola, and Gebauer (2018), despite the lack of ^{13}C enrichment in this system.

Since most studies have targeted fully mycoheterotrophic plants in Ericaceae and Orchidaceae families, which associate with ectomycorrhizal, orchid mycorrhizal or saprotrophic fungi (Hynson et al., 2013), we know very little about the natural abundances of stable isotopes of full mycoheterotrophs associated with arbuscular mycorrhizal fungi. While these include over 250 species of angiosperms in eight families (Merckx, 2013), to date only two studies have reported on the stable isotope natural abundances of plants belonging to two of these families. Merckx et al. (2010) investigated the specimens of *Dictyostega orobanchoides* (Burmanniaceae) and *Voyria aphylla* (Gentianaceae) at a site in French Guiana and found that both species were significantly more enriched in ^{13}C than co-occurring autotrophic plants but lacked significant ^{15}N enrichment. Similar results were reported by Courty et al. (2011) for *Apteria aphylla* and *Gymnosiphon sphaerocarpus* (Burmanniaceae), and *Voyria tenella* and *V. aphylla* (Gentianaceae), collected at five sites on the Caribbean island Guadeloupe. Lack of differentiation in ^{15}N natural abundance between these mycoheterotrophic plants and reference autotrophic plants suggests that all of these plants use similar N sources, presumably inorganic N compounds obtained through their fungal partners (Hynson et al., 2013). However, these observations were limited in taxonomic and geographic scope, and additional information from other areas and other plant taxa are necessary to test the generality of this pattern (Hynson et al., 2013). In addition, in parallel to ectomycorrhizal systems, the use of hydrogen isotope signatures potentially offers a promising tool to shed more light on the flows of organic forms of C and N, and water in arbuscular mycorrhizal associations.

To address these knowledge gaps, we investigated ^{13}C and ^{15}N natural abundance isotopic signatures of 13 species of arbuscular mycorrhizal fully mycoheterotrophic plants, sampled at 18 sites in tropical South America, tropical Asia and temperate Australasia. In addition, for representatives of three families of these mycoheterotrophic species, we report ^{2}H and ^{18}O natural abundances. We discuss the obtained isotope signatures of the mycoheterotrophic plants in relation to the identity of their associated fungi, and to previous published data of saprotrophic, arbuscular and ectomycorrhizal mycoheterotrophic interactions.

2 | MATERIALS AND METHODS

2.1 | Sampling

In total, 17 plots were sampled in French Guiana, Malaysia, Australia and New Zealand. Within these plots, a total of 13 arbuscular mycorrhizal fully mycoheterotrophic plant species were collected (see details in Table S1), from five different families: Burmanniaceae

(*D. orobanchoides*, *Hexapterella gentianoides*, *Gymnosiphon breviflorus*); Gentianaceae (*V. aphylla*, *Voyriella parviflora*); Polygalaceae (*Epirixanthes cylindrica*); Thismiaceae (*Thismia clavarioides*, *T. hillii*, *T. rodwayi* and an undescribed *Thismia* species); and Triuridaceae (*Sciaphila densiflora*, *Soridium spruceanum* and *Triuris hyalina*). Within each plot, samples of the target mycoheterotrophic plant species were taken in five replicates, if available, (resembling five 1-m^2 subplots). Within each of these subplots at least three reference autotrophic plant species were sampled as proposed by Gebauer and Meyer (2003). For sites in Tasmania, autotrophic reference plants were identified by local expert Mark Wapstra. For all other sites, leaf samples were identified to family level or below by sequencing *matK* or *trnL* following the methods described in Gomes, Aguirre-Gutiérrez, Bidartondo, and Merckx (2017). Reference plants that belong to Fabaceae or rely on C4 or CAM photosynthesis were excluded from the analyses, since these taxa have distinct stable isotope signatures of N and C respectively (Hynson et al., 2013). A total of 265 leaf samples obtained from autotrophic understorey species served as reference plants (Table S1). For DNA analysis of arbuscular mycorrhizal fungi, roots of the sampled mycoheterotrophs were rinsed with water and stored in CTAB buffer (cetyltrimethylammonium bromide) at −18°C until further processing.

2.2 | Analysis of stable isotope abundance

Above-ground samples of the 13 mycoheterotrophic species (n = 78) and autotrophic references (n = 265) were dried at 105°C and ground to a fine powder in a ball mill (Retsch Schwingmühle MM2). Relative C and N isotope natural abundances were measured in a dual element analysis mode with an elemental analyser (Carlo Erba Instruments 1108) coupled to a continuous flow isotope ratio mass spectrometer (delta S; Finnigan MAT) via a ConFlo III open-split interface (Thermo Fisher Scientific) as described in Bidartondo, Burghardt, Gebauer, Bruns, and Read (2004). Relative H and O isotope natural abundances of the samples were obtained only for a selection of representative species within each family because these measurements require the preparation of five samples in total, and for many species not enough material was available. Sufficient material was obtained for *E. cylindrica* (Polygalaceae), *G. breviflorus* (Burmanniaceae) and *V. parviflora* (Gentianaceae), and reference autotrophic plants (n = 31). Representatives of the families Thismiaceae and Triuridaceae were not measured for H and O because insufficient material was available. The H and O isotope natural abundances were measured with thermal conversion through pyrolysis (HTO; HEKAtech) coupled to a continuous flow isotope ratio mass spectrometer (delta V advantage; Thermo Fisher Scientific) via a ConFlo IV open-split interface (Thermo Fisher Scientific) as described in Gebauer et al. (2016).

Measured isotope relative abundances are denoted as δ values that were calculated according to the following equation: $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ or $\delta^{18}O = (R_{sample}/R_{standard} - 1) \times 1{,}000$ [‰], where R_{sample} and $R_{standard}$ are the ratios of heavy to light isotope of the samples and the respective standard. Standard gases were calibrated with respect to international standards (CO_2 vs. V-PDB, N_2 vs. N_2 in air, H_2 and CO vs. V-SMOW) with the reference substances ANU sucrose and NBS19 for C isotopes, N1 and N2 for N isotopes, CH7, V-SMOW and SLAP for hydrogen isotopes and IAEA601 and IAEA602 for O isotopes, all provided by the International Atomic Energy Agency, Vienna, Austria. Reproducibility and accuracy of the C and N isotope abundance measurements were routinely controlled by measuring the laboratory standard acetanilide (Gebauer & Schulze, 1991). For the relative C and N isotope natural abundance analyses, acetanilide was routinely analysed with variable sample weight at least six times within each batch of 50 samples. The maximum variation in $\delta^{13}C$ and $\delta^{15}N$ both within and between batches was always below 0.2‰. For relative H and O isotope natural abundance analyses, benzoic acid was routinely analysed with variable sample weight at least six times within each batch of 40 samples. The maximum variation in $\delta^{2}H$ and $\delta^{18}O$ both within and between batches was always below 4‰ and 0.6‰ respectively.

Total N concentrations in mycoheterotroph and reference plant above-ground samples were calculated from sample weights and peak areas using a six-point calibration curve per sample run based on measurements of the laboratory standard acetanilide with a known N concentration of 10.36% (Gebauer & Schulze, 1991).

2.3 | Identification of arbuscular mycorrhizal fungi

Fungal DNA was extracted from the CTAB preserved roots with the KingFisher Flex Magnetic Particle Processors (Thermo Fisher Scientific) using the NucleoMag 96 Plant Kit (Machery-Nagel Gmbh & Co.). Because morphological observations suggest that the mycoheterotrophic plants of this study are associated with fungi that belong to arbuscular mycorrhizal fungi (Merckx, 2013 and references therein), we targeted Glomeromycotina fungi. The ITS2 region (approximately 250 bp) was sequenced using the primers fITS7 (Ihrmark et al., 2012) and ITS4 (White, Bruns, Lee, & Taylor, 1990) with a Personal Genome Machine (Ion Torrent; Life Technologies) with 850 flows. These primers potentially exclude mycorrhizal fungi within the Mucoromycotina (Ihrmark et al., 2012), yet there is no prior indication that these fungi associate with mycoheterotrophic plants. Raw reads were processed using the UPARSE algorithm (Edgar, 2013) incorporated in USearch v.7. Briefly, raw reads were screened for quality control and trimmed at the first base with a Phred score of Q < 20. Then, reads were dereplicated with singletons and sequences with length <100 bp removed. The resulting reads were clustered into operational taxonomic untis (OTUs) at 97% similarity (Blaalid et al., 2013), and taxonomy was assigned by querying against the UNITE + INSD database (released on 10 September 2014). We only retained fungal OTUs classified as Glomeromycotina in the subsequent analysis. The OTUs that were represented by <5 reads in each sample were excluded to avoid spurious OTUs (Lindahl et al., 2013). The fungal OTUs were aligned together with sequences from 40 reference

Glomeromycotina taxa from Krüger, Krüger, Walker, Stockinger, and Schüßler (2012), and the MaarjAM database (Opik et al., 2010) using the MUSCLE algorithm (Edgar, 2004). The phylogenetic relationships were inferred with RAxML v.8.2.12 using the GTRCAT model of evolution (Stamatakis, 2014). Paraglomeraceae and Archaeosporaceae reference sequences were selected as out-groups and the phylogeny was transformed into an ultrametric tree with TreePL (Smith & O'Meara, 2012) fixing the root at 505 million years ago (Davison et al., 2015). Since the ITS2 region is likely variable below species level in Glomeromycotina, we used the phylogeny to estimate evolutionarily independent clusters of DNA sequences by differentiating branches within clusters, associated with neutral coalescent (merging of lineages within a population) processes, from branches between clusters, associated with speciation events (Powell, Monaghan, Öpik, & Rillig, 2011), using a single-threshold Generalized Mixed Yule Coalescent (GMYC) approach (Pons et al., 2006) implemented in the SPLITS R package (Ezard, Fujisawa, & Barraclough, 2009). The GMYC table is available in Table S2.

2.4 | Statistical analyses

We normalized the δ values of C, N, H and O stable isotope abundances by calculating the enrichment factors (ε) for each sample as $\varepsilon = \delta_S - \delta_{REF}$, where δ_S is a single $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ or $\delta^{18}O$ value of a mycoheterotroph or an autotrophic reference plant and δ_{REF} is the mean value of all autotrophic reference plants within a plot (Preiss & Gebauer, 2008). The mean δ and ε values ± standard deviations of all studied species are available in Tables S3 and S4. Overall differences in isotopic enrichment factors ($\varepsilon^{13}C$, $\varepsilon^{15}N$, $\varepsilon^{2}H$, $\varepsilon^{18}O$) and N concentrations between the mycoheterotrophic species and their corresponding autotrophic reference plants were assessed. The normality test *shapiro.test* in the STATS R package (R Core Team, 2016) revealed non-normality in $\varepsilon^{13}C$ ($W = 0.873$, $p < 0.001$), $\varepsilon^{15}N$ ($W = 0.944$, $p < 0.001$) and $\varepsilon^{2}H$ ($W = 0.909$, $p < 0.001$), and normality in $\varepsilon^{18}O$ ($W = 0.977$, $p = 0.247$). Because most of the variables showed non-normality, differences in enrichment factors were assessed with a non-parametrical Kruskal–Wallis rank sum test using the STATS R package. The Dunn's test with Bonferroni–Holm corrections using the *dunn.test* R package (Dinno, 2017) was performed to assess pairwise differences in enrichment factors among the mycoheterotrophic plants, and between those and their corresponding autotrophic references. To test whether enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N$ of mycoheterotrophic plants were conserved among taxonomic group or geographic origin, we performed two one-way PERMANOVA with 999 permutations using the function *adonis2* in the VEGAN R package (Oksanen et al., 2015).

To test if the fungal communities associated with the mycoheterotrophic plants were conserved among taxonomic group or geographic origin, we performed two one-way PERMANOVA with 999 permutations. We estimated the proportions of the total variation in fungal community composition that were explained by plant enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N$, geographic origin (French Guiana, Malaysia, Australia, New Zealand) and taxonomic group (plant family; Legendre, Borcard, & Peres-Neto, 2005). Geographic origin was correlated with taxonomic group (Pearson's χ^2 test = 22, $df = 12$, $p = 0.038$), thus only one of the variables was included in the analysis. To investigate whether the fungal communities of each mycoheterotrophic plant species were significantly associated with the isotope enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N$, we performed a distance-based redundancy analysis (dbRDA), using the VEGAN R package, with fungal distances between the mycoheterotrophic species calculated as the phylogenetic community dissimilarity metric (pcd), using the PICANTE R package (Kembel et al., 2010). The dbRDA model was assessed for significance using *anova.cca* in the VEGAN R package. To further understand the relationships between fungal community composition and the isotope signatures, we tested for correlations between the enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N$, and two measures of associated fungal phylogenetic diversity, using *cor.test* in R. We used the net relatedness index (NRI) and the nearest taxon index (NTI) to evaluate phylogenetic clustering or overdispersion (Webb, 2000). Positive values indicate that fungal communities are phylogenetically more clustered than expected by chance, while negative values indicate they are overdispersed. Numerically, NTI and NRI are the inverse of the standardized effect size of the mean phylogenetic distance and mean nearest phylogenetic taxon distance, respectively, and were calculated using PICANTE.

3 | RESULTS

3.1 | Stable isotope natural abundances

Mean enrichment in ^{13}C of all mycoheterotrophic species varied between 1.59 ± 1.15‰ (*T. rodwayi*) and 8.57‰ (*D. orobanchoides*) and in ^{15}N between −0.23 ± 2.21‰ (*V. parviflora*) and 8.47 ± 4.17‰ (*Thismia* sp.; Figure 1). Mean enrichment in ^{2}H varied between 19.24 ± 9.47‰ (*E. cylindrica*) and 42.24 ± 14.25‰ (*V. parviflora*), and in ^{18}O between −0.66 ± 1.19‰ (*G. breviflorus*) and 0.38 ± 1.05‰ (*V. parviflora*; Figure 2). On average, the individual mycoheterotrophic plants in this study were significantly enriched in ^{13}C (Kruskal–Wallis $\chi^2 = 143.19$, $df = 1$, $p < 0.001$), in ^{15}N ($\chi^2 = 135.66$, $df = 1$, $p < 0.001$), in ^{2}H ($\chi^2 = 27.02$, $df = 1$, $p < 0.001$), but they were not distinguishable in ^{18}O ($\chi^2 = 0.74$, $df = 1$, $p = 0.391$) in relation to their autotrophic references.

At the family level, all the families of mycoheterotrophic plants were significantly enriched in ^{13}C and ^{15}N relative to their references, with the exception of Gentianaceae, which was not distinguished in ^{15}N (Figure 1; Table 1). Thismiaceae presented on average the lowest enrichment in ^{13}C ($\varepsilon_{MH\text{-}R} = 3.48‰$), followed by Polygalaceae ($\varepsilon_{MH\text{-}R} = 5.41‰$), Triuridaceae ($\varepsilon_{MH\text{-}R} = 6.85‰$), Gentianaceae ($\varepsilon_{MH\text{-}R} = 7.24‰$) and Burmanniaceae ($\varepsilon_{MH\text{-}R} = 7.86‰$). In relation to the ^{15}N isotope, Gentianaceae ($\varepsilon_{MH\text{-}R} = 0.50‰$) was not significantly enriched in comparison to the reference autotrophic plants and neither to the other mycoheterotrophic plant families, which did not present

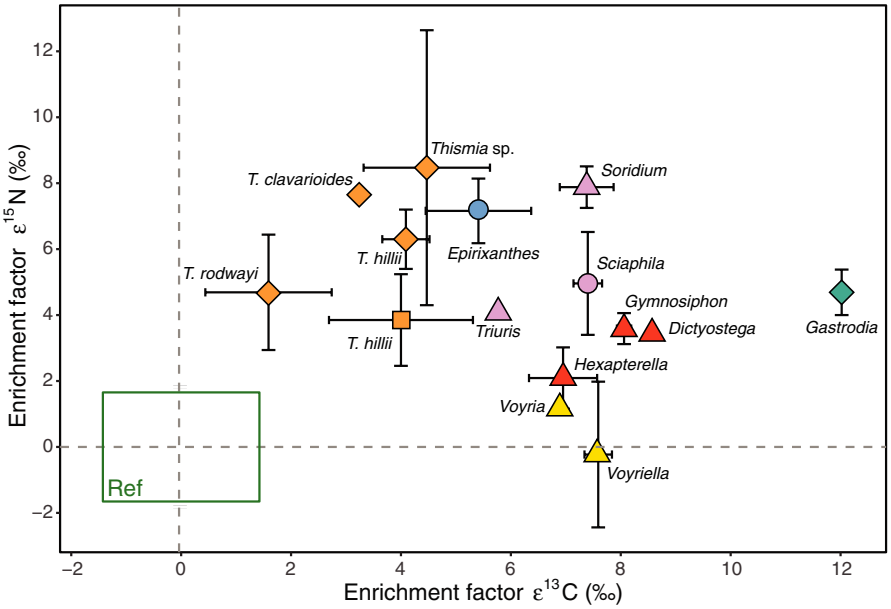

FIGURE 1 Mean enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N$ ± *SD* of 13 species of mycoheterotrophic plants associated with arbuscular mycorrhizal fungi. Colours correspond to the different families of mycoheterotrophic plants: Burmanniaceae (red; *Dictyostega* n = 1, *Gymnosiphon* n = 4, *Hexapterella* n = 5), Gentianaceae (yellow; *Voyria* n = 1, *Voyriella* n = 5), Polygalaceae (blue; *Epirixanthes* n = 12), Thismiaceae (orange; *Thismia* sp. n = 4, *T. clavarioides* n = 2, *T. hillii* from Australia n = 3 and from New Zealand n = 11, *T. rodwayi* n = 20), Triuridaceae (pink; *Sciaphila* n = 5, *Soridium* n = 3, *Triuris* n = 2). The Orchidaceae *Gastrodia sesamoides* (green diamond; n = 2) was included in the graph as reference of a fully mycoheterotrophic plant that associates with saprotrophic non-rhizoctonia fungi also collected in this study. Symbols correspond to geographic locations where samples were collected: Australia and Tasmania (diamonds), New Zealand (square), Malaysia (circles) and South America (triangles). The green box represents the mean enrichment factors ± *SD* for the autotrophic reference plants, which are zero by definition, that were sampled together with the target plants (Ref, n = 265)

a significant different ^{15}N enrichment among each other. On average, Burmanniaceae ($\varepsilon_{MH\text{-}R}$ = 3.04‰) had the lowest enrichment, followed by Triuridaceae ($\varepsilon_{MH\text{-}R}$ = 5.66‰), Thismiaceae ($\varepsilon_{MH\text{-}R}$ = 6.19‰) and Polygalaceae ($\varepsilon_{MH\text{-}R}$ = 7.16‰). The isotope signatures of mycoheterotrophic plants in this study were significantly structured by taxonomic group (R^2 = 0.59, df = 12, F = 25.81, p = 0.001) and geographic origin (R^2 = 0.67, df = 12, F = 37.64, p = 0.001; see Figure S1).

3.2 | Nitrogen concentration

Total N concentrations of mycoheterotrophic plants were significantly higher than the reference plants (total N mean = 1.18 ± 0.54 mmol/g dw) for the families Polygalaceae (total N mean = 1.73 ± 0.27 mmol/g dw; Dunn's test: Z = 3.940, p < 0.001), Thismiaceae (total N mean = 1.91 ± 0.44 mmol/g dw; Z = 7.614, p < 0.001) and Triuridaceae (total N mean = 2.13 ± 0.72 mmol/g dw; Z = 4.143, p < 0.001), while there were no significant differences in Burmanniaceae (total N mean = 1.35 ± 0.37 mmol/g dw; Z = 1.307, p = 1) and Gentianaceae (total N mean = 1.20 ± 0.15 mmol/g dw; Z = 0.607, p = 1).

3.3 | Fungal identification

The roots of the 58 mycoheterotrophic plant individuals yielded 1,227,231 quality-filtered reads, of which 398,206 belonged to the subphylum Glomeromycotina, representing 228 OTUs retained for subsequent analysis (1 Gigasporaceae, 10 Acaulosporaceae, 6 Claroideoglomeraceae and 211 Glomeraceae). These were reduced to 29 GMYC groups (Figure S2), of which 23 belonged to Glomeraceae, three to Claroideoglomeraceae, one to Gigasporaceae and two to Acaulosporaceae. The associations of these GMYC groups range from a single (GMYC 15) to six plant species (GMYC 17 and 10). Plant associations range from one (*D. orobanchoides*) to eight (*T. hyalina*) GMYC groups.

The fungal communities of mycoheterotrophic plants were not significantly structured by plant family (p = 0.555) but they were significantly structured by geographic origin (df = 3, R^2 = 0.45, F = 2.765, p = 0.002). Variation partitioning analyses indicated that the isotope enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N$, and the geographic origin of the plants explained 21.5% of the total variation observed in the fungal community composition of mycoheterotrophic plants. The effect of $\varepsilon^{13}C$ and $\varepsilon^{15}N$ explained 10% of the variation, but only 1% after accounting for the variation explained by the geographic origin, which alone explained 20.6% of the total variation, and only 11% after accounting for the effect of isotope enrichment factors. Only geographic origin was included in the variation partitioning analysis because fungal communities were not structured by taxonomic group (see PERMANOVA above). The dbRDA model including the variables $\varepsilon^{13}C$ and $\varepsilon^{15}N$ was statistically significant (df = 2, F = 2.160, p = 0.005), despite only $\varepsilon^{13}C$ being statistically significant ($\varepsilon^{13}C$: df = 1, F = 2.936, p = 0.001). Moreover, the first

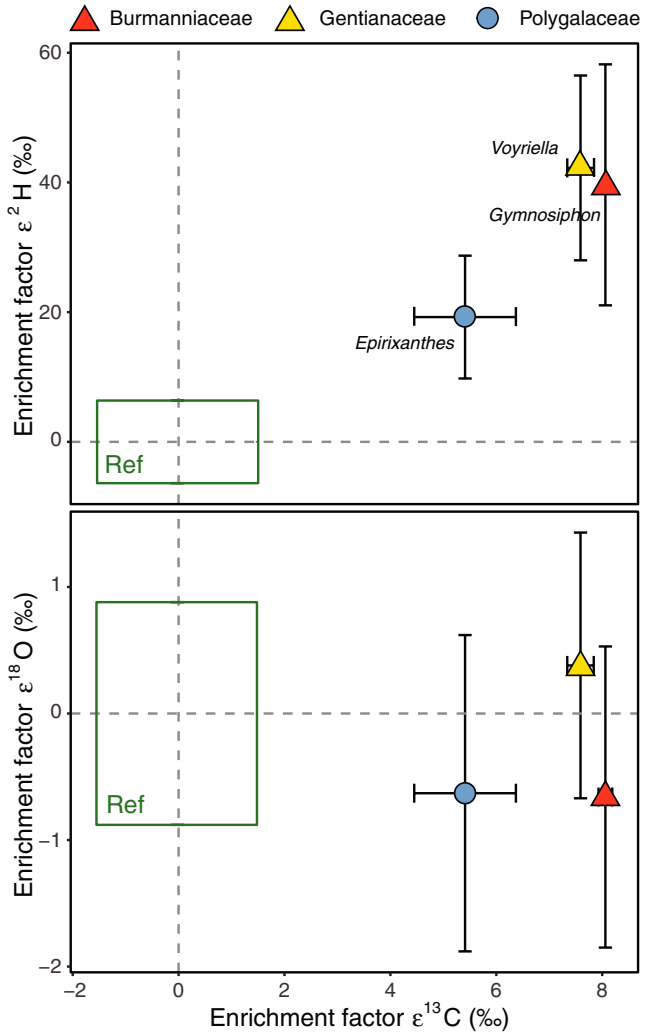

FIGURE 2 Mean enrichment factors ε^{2}H and ε^{18}O ± *SD* of three species of mycoheterotrophic plants representative of three families in this study. Symbols correspond to geographic locations where samples were collected: Malaysia (circle) and South America (triangles). The green box represents the mean enrichment factors ± *SD* for the autotrophic reference plants, which are zero by definition, that were sampled together with the target plants (Ref, *n* = 31)

axis (*df* = 1, *F* = 3.060, *p* = 0.005; Figure 3) of the dbRDA was statistically significant explaining 20% of the total variance (see details in Table S5).

The NRI values were not associated with the enrichment factors of either ε^{13}C or ε^{15}N, while the NTI was negatively correlated with ε^{13}C (R^2 = 0.38, *p* = 0.025; Figure 4). The species *D. orobanchoides* was associated with only one GMYC group, and thus was removed from the analysis.

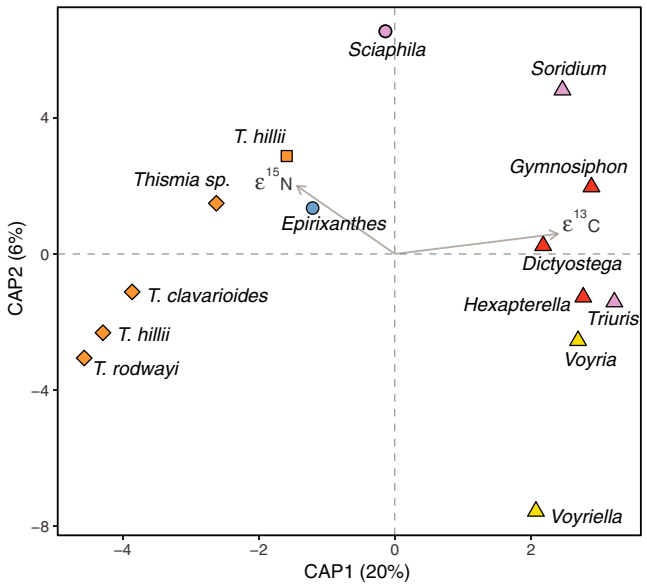

FIGURE 3 Distance-based redundancy analysis model of the fungal communities found in the roots of the mycoheterotrophic plants according to the isotopic enrichment factors. Arrows represent the ε^{13}C and ε^{15}N measured from the above-ground parts of the mycoheterotrophic plants. Only the ε^{13}C is statistically significant, but both arrows are depicted to represent the full model. Colours correspond to the different families of mycoheterotrophic plants: Burmanniaceae (red), Gentianaceae (yellow), Polygalaceae (blue), Thismiaceae (orange), Triuridaceae (pink). Symbols correspond to geographic locations where samples were collected: Australia (diamonds), New Zealand (square), Malaysia (circles) and South America (triangles)

TABLE 1 Results from pairwise comparisons for enrichment factors ε^{13}C, ε^{15}N, ε^{2}H and ε^{18}O between arbuscular mycoheterotrophic plant families and their autotrophic references using the Dunn's test. *p*-values were adjusted with Bonferroni–Holm corrections

	ε^{13}C		ε^{15}N		ε^{2}H		ε^{18}O	
Family	***Z***	***p***	***Z***	***p***	***Z***	***p***	***Z***	***p***
Burmanniaceae	6.11	**<0.001**	3.90	**<0.001**	3.38	**0.002**	1.16	0.732
Gentianaceae	4.73	**<0.001**	0.07	1	3.92	**<0.001**	0.86	1
Polygalaceae	5.74	**<0.001**	6.50	**<0.001**	2.82	**0.015**	1.44	0.447
Thismiaceae	7.30	**<0.001**	9.48	**<0.001**				
Triuridaceae	5.89	**<0.001**	5.37	**<0.001**				

Note: Kruskal–Wallis tests indicated significant differences between all groups of investigated families and the respective autotrophic reference plants (ε^{13}C, χ^2 = 143.19, ε^{15}N, χ^2 = 136.66, ε^{2}H, χ^2 = 27.02; in all cases *df* = 1, *p* < 0.001), except one (ε^{18}O, χ^2 = 0.74, *df* = 1, *p* = 0.391). Significant *p* values (α < 0.05) are highlighted in bold.

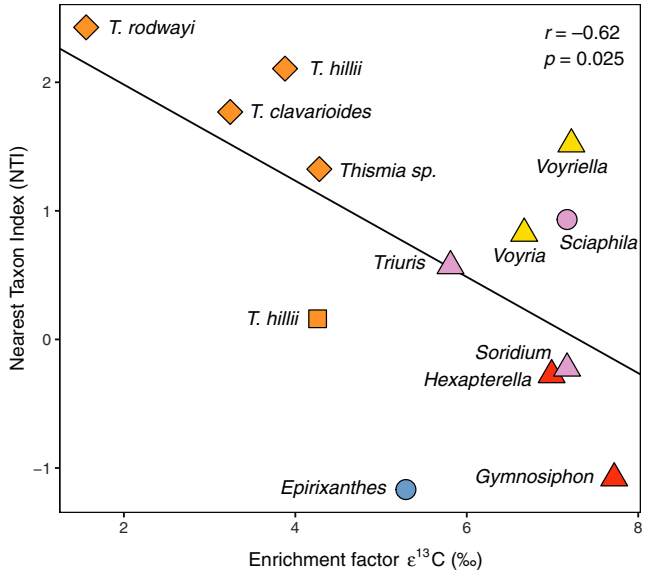

FIGURE 4 Pearson correlation coefficient (*r*) and *p*-value (*p*) between the enrichment factor $\varepsilon^{13}C$ and the nearest taxon index (NTI). Positive NTI values indicate that fungal communities are more clustered than expected by chance; negative NTI values indicate overdispersion. Symbols correspond to geographic locations where samples were collected: Australia and Tasmania (diamonds), New Zealand (square), Malaysia (circles) and South America (triangles)

4 | DISCUSSION

4.1 | Emerging patterns of ^{13}C, ^{15}N, ^{2}H and ^{18}O stable isotope natural abundances of arbuscular mycorrhizal mycoheterotrophic plants

Our study reveals a general pattern of enrichment in ^{13}C and ^{15}N for the targeted fully mycoheterotrophic plants associated with arbuscular mycorrhizal fungi. Consistent only with the two previous studies that reported C and N signatures of these plants (Courty et al., 2011; Merckx et al., 2010), we confirm an overall pattern of enrichment in ^{13}C at the family level (Table 1). In contrast to previous knowledge, we also detect a general enrichment of ^{15}N of arbuscular mycorrhizal mycoheterotrophic plants compared to photosynthetic references. Enrichment in ^{15}N, however, is not universal and as shown by previously published results species of Gentianaceae and some Burmanniaceae lack ^{15}N enrichment.

The observed enrichment in ^{13}C and ^{15}N is consistent with the pattern observed for mycoheterotrophic plants associated with ectomycorrhizal fungi (Hynson, Schiebold, & Gebauer, 2016), for which the fungi are the most likely alternative C and N source of fully mycoheterotrophic plants. In contrast to the latter system, however, we still lack information on the isotope natural abundances of the arbuscular mycorrhizal fungi themselves, due to the absence of macroscopic fruiting bodies of these fungi, which hinders their measurement. Therefore, we cannot directly compare the isotope signatures of the mycoheterotrophs and their arbuscular mycorrhizal fungi to definitely prove that the fungi are the principal C and N donors for the plants. Yet, our study suggests that mycoheterotrophs associated with arbuscular mycorrhizal fungi may be able to utilize N from different origins, such as from their associated fungal partners (in the cases where we observed N enrichment), or by taking up N from the substrate themselves (no N enrichment). However, evidence to support this hypothesis is lacking. Alternatively, local conditions may determine the origin of the N that is available to both arbuscular mycorrhizal fungi and mycoheterotrophic plants, and thus the signatures of the mycoheterotrophs reflect the local availability of different N sources (Robinson, 2001). Understanding whether the isotope natural abundance of arbuscular mycorrhizal fungi vary according to local conditions, leading these fungi to obtain N from different origins, requires further study.

Our study provides the first stable isotope abundance data for ^{2}H and ^{18}O for arbuscular mycorrhizal mycoheterotrophs. The three species assessed for H and O isotope abundances exhibit a significant enrichment in ^{2}H compared to the references, while no differences in ^{18}O abundances were observed. Physiological features can potentially explain the observed ^{2}H enrichment. The simultaneous enrichment in ^{2}H and ^{13}C is often influenced by the photosynthetic pathway type of a plant (Ziegler, 1995). Yet, fully mycoheterotrophic plants lack a functional photosynthesis apparatus, and thus cannot perform photosynthesis (Graham, Lam, & Merckx, 2017). Consequently, the potential effect of transpiration which is known to simultaneously affect C (Farquhar, Ehleringer, & Hubick, 1989), H (Ziegler, 1989) and O (Barbour, 2007) isotope abundances is unlikely to have an impact, further supported by the reduced number of stomata in these leafless plants (Leake, 1994). High transpiration rates have been observed in hemiparasites, which in turn were depleted in ^{13}C and ^{18}O (Cernusak, Pate, & Farquhar, 2004) and should potentially be depleted in ^{2}H (but see Ziegler, 1996). In our study, we found an enrichment in ^{13}C and ^{2}H and no differences in ^{18}O isotope abundance, thus a transpiration effect can also be ruled out. Alternatively, an enrichment in ^{2}H can be elucidative of the C and energy metabolism of plants (Cormier et al., 2018). Because of a consistently higher ^{2}H enrichment in carbohydrates than in lipids observed in heterotrophic plants in comparison to autotrophs, Cormier et al. (2019) suggested a similar ^{2}H enrichment observed across different families of mycoheterotrophic plants associated with ectomycorrhizal fungi distinguished from autotrophic plants. As possible mechanisms, Cormier et al. (2019) proposed either a characteristic level of ^{2}H fractionation associated with the heterotrophic production of NADPH, or to the potential of these plants to take up lipids, which would lead to a less-enriched C signature when compared to the uptake of carbohydrates (Gleixner, Danier, Werner, & Schmidt, 1993). Recent independent studies demonstrated with different approaches that plants transfer significant amounts of lipids to their arbuscular mycorrhizal fungal partners as C sources to sustain the mycorrhizal colonization, which lack genes encoding fatty acid synthase I subunits (Jiang et al., 2017; Keymer et al., 2017; Luginbuehl et al., 2017). This supports the hypothesis that the ^{2}H enrichment in arbuscular mycoheterotrophic plants is due to the uptake of lipids, in this case from their associated arbuscular mycorrhizal fungi, either through the digestion of the arbuscules inside their root cells, as commonly observed in mycoheterotrophic plants (Imhof, Massicotte, Melville, & Peterson, 2013), or through active fungus–plant membrane transport.

4.2 | Isotope signatures of arbuscular mycorrhizal mycoheterotrophs compared to other mycoheterotrophic plants

We compiled ^{13}C, ^{15}N, ^{2}H and ^{18}O stable isotope natural abundance data of fully mycoheterotrophic species living on multiple fungal functional groups from all available publications (Hynson et al., 2016; Merckx et al., 2010; Schweiger, 2018) and calculated mean values and standard deviations of their enrichment factors (Figure 5). Raw data from Courty et al. (2011) were not available. We observe that mycoheterotrophic plants associated with arbuscular mycorrhizal fungi are on average the least enriched in ^{13}C, followed by the plants associated with ectomycorrhizal and litter-decaying fungi, and finally wood saprotrophs. In terms of ^{15}N, ectomycorrhizal mycoheterotrophs are the most enriched of the four groups. For ^{2}H, the data available are still limited, but it seems that mycoheterotrophic plants associated with ectomycorrhizal fungi are more enriched than those associated with arbuscular mycorrhizal fungi. One possible explanation lies in the recent discovery that arbuscular mycorrhizal fungi receive lipids as C source, pivotal to sustain the mycorrhizal colonization (Jiang et al., 2017; Keymer et al., 2017; Luginbuehl et al., 2017), while ectomycorrhizal fungi preferentially receive carbohydrates, which are more enriched in ^{13}C and ^{2}H than lipids (Cormier et al., 2019; Gleixner et al., 1993; Nehls, Göhringer, Wittulsky, & Dietz, 2010). Finally, while Gebauer et al. (2016) and Schiebold et al. (2018) separately found no significant distinction in ^{18}O of ectomycorrhizal mycoheterotrophs in comparison with their reference autotrophic plants, these plants seem to display a slight depletion in ^{18}O in comparison to the overall signal of autotrophic plants (Figure 5). The mycoheterotrophs associated with arbuscular mycorrhizal fungi had no distinguished ^{18}O isotope signature when compared to the reference autotrophic plants. Interestingly, arbuscular mycorrhizal mycoheterotrophs show a higher variability in ^{13}C and ^{2}H signatures, and a similar variability in ^{15}N signatures compared to ectomycorrhizal mycoheterotrophs. Although the origin of this high variability is unknown, possible explanations include the higher diversity of arbuscular mycorrhizal mycoheterotrophic plant lineages, that is ectomycorrhizal mycoheterotrophs are represented by one eudicot and one monocot family, while arbuscular mycorrhizal mycoheterotrophs are represented by two eudicot and three monocot families. Also, the evolutionary diversity of the associated fungi is likely higher for arbuscular mycorrhizal mycoheterotrophs than ectomycorrhizal mycoheterotrophs: the Glomerales–Diversisporales clade, which includes the arbuscular mycorrhizal fungi detected here, has an inferred crown node age of *c*. 331 million years (Sun et al., 2019), while Agaricomycetes—the main associates of ectomycorrhizal orchids—have a crown node of *c*. 300 million years, but the origin of the ectomycorrhizal symbiosis within this clade is likely considerably younger (Lutzoni et al., 2018). Finally, isotopic data on ectomycorrhizal mycoheterotrophs are predominantly from temperate climates, while in this study we include arbuscular mycoheterotrophs that occur in tropical regions as well as in temperate climates (Thismiaceae).

4.3 | Variation of isotope signatures within arbuscular mycorrhizal mycoheterotrophs

We found that the enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N$ of the mycoheterotrophic plants were significantly associated with plant family and geographic origin (Figure 1). However, in our dataset, most plant

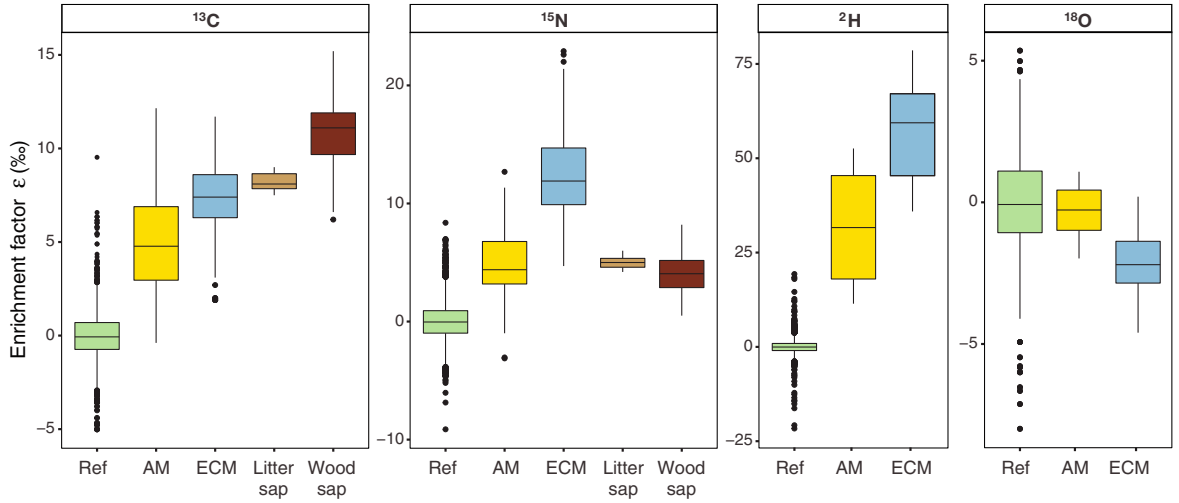

FIGURE 5 Mean enrichment factors $\varepsilon^{13}C$, $\varepsilon^{15}N$, $\varepsilon^{2}H$ and $\varepsilon^{18}O$ of fully mycoheterotrophic plants associated with different fungal functional groups (AM, arbuscular mycorrhizal fungi; ECM, ectomycorrhizal fungi; Litter sap, saprotrophs on litter; Wood sap, wood decaying fungi). Ref represents the mean enrichment factors of the respective autotrophic reference plants. The data for the arbuscular mycorrhizal mycoheterotrophs ($\varepsilon^{13}C_{mean}$ = 4.74 ± 2.43‰, $\varepsilon^{15}N_{mean}$ = 4.99 ± 2.89‰, $\varepsilon^{2}H_{mean}$ = 33.28 ± 16.99‰, $\varepsilon^{18}O_{mean}$ = −0.28 ± 1.19‰) were obtained in this study and combined with Merckx et al. (2010). Raw data from Courty et al. (2011) were not available. The data from the other fungal functional groups (ECM: $\varepsilon^{13}C_{mean}$ = 7.35 ± 1.78‰, $\varepsilon^{15}N_{mean}$ = 12.23 ± 3.53‰, $\varepsilon^{2}H_{mean}$ = 57.28 ± 13.85‰, $\varepsilon^{18}O_{mean}$ = −2.18 ± 1.34‰; Litter sap: $\varepsilon^{13}C_{mean}$ = 8.69 ± 1.37‰, $\varepsilon^{15}N_{mean}$ = 4.99 ± 0.53‰; and Wood sap: $\varepsilon^{13}C_{mean}$ = 10.61 ± 1.98‰, $\varepsilon^{15}N_{mean}$ = 3.96 ± 1.83‰) are a compilation from all the available published works since 2003 (Hynson et al., 2016; Schweiger, 2018)

families were collected at a single geographic location, hampering the separation of the effects of plant family and geographic origin. In addition, we found that the fungal community composition associated with mycoheterotrophic plant species was correlated with the plant's enrichment factor $\varepsilon^{13}C$. At the same time, the fungal community structure was not associated with plant taxonomic group, but it was structured by geographic origin (Figure 3). This shows a non-random association of fungal communities reflecting their isotope signature, and potentially indicates different environmental and geographical niches occupied by the different fungal communities. However, since most of the plant species investigated here do not associate with a monophyletic clade of Glomeromycotina fungi, and since plant taxonomy and sampling location are correlated, it is challenging to attribute isotope signatures to particular Glomeromycotina clades, or to clearly disentangle effects of fungal community and geographic origin. Within South America, the species within the families Burmanniaceae, Gentianaceae and Triuridaceae are clustered by plant family based on the isotopic signatures of the plants, yet this structure is not mirrored in their fungal communities. In this case, we suggest that the evolutionary history of the mycoheterotrophs probably shapes their family-level isotope natural abundance signature, particularly of N, rather than their associated fungal communities.

Previous studies have shown that the ^{15}N isotope abundance in mycoheterotrophic plants associated with ectomycorrhizal and saprotrophic non-rhizoctonia fungi exhibit particular distinguishable N signatures, which allow to identify the respective substrate available for the fungus (Hynson et al., 2013; Lee et al., 2015). While it is more straightforward for Basidiomycota and Ascomycota to delimitate taxonomic and functional groups, species delimitation and functionality are poorly understood for arbuscular mycorrhizal fungi (subphylum Glomeromycotina). Yet, there is some evidence that life-history strategies are maintained at the family level in arbuscular mycorrhizal fungi (Chagnon, Bradley, Maherali, & Klironomos, 2013), which could reflect different N acquisition strategies among these groups. The mycoheterotrophic plants in this study were found to be associated with four families of arbuscular mycorrhizal fungi (Figure S2). The majority of fungi (86%) were found in only one or two plant species. These may represent facultative associations (Renny et al., 2017). The most commonly detected fungi include GMYC group 17, which was found in the five *Thismia* species and *Sciaphila* from Australasia and Malaysia, respectively, and GMYC group 10 which was found in the roots of *Dictyostega*, *Gymnosiphon*, *Hexapterella*, *Soridium*, *Triuris* and *Voyria*, all from South America. Both Glomeraceae fungi associate with mycoheterotrophic plants from different families but are also restricted to different geographic regions. Therefore, it is not possible to disentangle whether these associations are driven by the geographic distribution of the fungi or by plant family preferences. While we recognize the difficulty to unravel the functions provided by the different arbuscular mycorrhizal fungi individually to the mycoheterotrophic plants, we observed that the plant's enrichment factor $\varepsilon^{13}C$ increases with the association with phylogenetically more diverse fungal communities. It has been previously demonstrated that mycoheterotrophic plants associate with fungal communities that range from highly clustered to overdispersed (Gomes, Aguirre-Gutiérrez, et al., 2017; Gomes, Merckx, & Saavedra, 2017), potentially to increase the chances of finding the best suitable partners from the available fungal pool. If phylogenetic dispersion can be linked with functional complementary of arbuscular mycorrhizal fungi, then the species with clustered fungal communities may be limited in fungal functional diversity for C uptake (Maherali & Klironomos, 2007). Whether the association with more diverse fungal communities reflects functional redundancy or offers mycoheterotrophic plants the ability to choose fungi that obtain C from different hosts, for example by associating simultaneously with C3 and C4 plants (Hobbie & Werner, 2004), which are characterized by distinct C signatures, is unknown.

5 | CONCLUSIONS

This study broadens our knowledge on multielement isotope natural abundances of mycoheterotrophic plants associated with arbuscular mycorrhizal fungi from different independent evolutionary lineages. We confirm that mycoheterotrophic plants associated with arbuscular mycorrhizal fungi are generally enriched in ^{13}C, yet we demonstrate that previous knowledge on the lack of ^{15}N enrichment does not hold for the majority of species, and we show that these mycoheterotrophs are enriched in ^{15}N. Moreover, we present the first data on ^{2}H and ^{18}O isotope abundances for arbuscular mycoheterotrophic plants, which appear to be enriched in ^{2}H and not different from the reference plants in ^{18}O. Finally, their enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N$ appear to group the mycoheterotrophic plants by taxonomic group and geographic origin, while their fungal community composition is not structured by plant taxonomic group but is better explained by their geographic origin. This suggests that different arbuscular mycorrhizal fungal communities may lead to a similar isotopic signature, but inherent phylogenetic conserved mechanisms in the plant metabolism of C and particularly of N further determine their higher similarity in isotopic pattern according to taxonomic group.

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AUTHORS' CONTRIBUTIONS

V.S.F.T.M. and G.G. designed the study; S.I.F.G. and V.S.F.T.M. collected the samples and led the writing of the manuscript; J.K. and G.G. generated and processed the isotope data, and S.I.F.G. the molecular data; S.I.F.G. and J.K. analysed the data. All the authors contributed critically to the drafts and gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.95x69p8g2 (Gomes, Merckx, Kehl, & Gebauer, 2020).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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