Mycorrhiza (2024) 34:283–292
https://doi.org/10.1007/s00572-024-01158-4

RESEARCH

Exploring mycorrhizal diversity in sympatric mycoheterotrophic plants: a comparative study of *Monotropastrum humile* var. *humile* and *M. humile* var. *glaberrimum*

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Received: 11 January 2024 / Accepted: 11 June 2024 / Published online: 25 June 2024

Abstract
Mycoheterotrophic plants (MHPs) rely on their mycorrhizal fungus for carbon and nutrient supply, thus a shift in mycobionts may play a crucial role in speciation. This study aims to explore the mycorrhizal diversity of two closely related and sympatric fully MHPs, *Monotropastrum humile* var. *humile* (Mhh) and *M. humile* var. *glaberrimum* (Mhg), and determine their mycorrhizal associations. A total of 1,108,710 and 1,119,071 ectomycorrhizal fungal reads were obtained from 31 Mhh and 31 Mhg, and these were finally assigned to 227 and 202 operational taxonomic units, respectively. Results show that sympatric Mhh and Mhg are predominantly associated with different fungal genera in Russulaceae. Mhh is consistently associated with members of *Russula*, whereas Mhg is associated with members of *Lactarius*. Associating with different mycobionts and limited sharing of fungal partners might reduce the competition and contribute to their coexistence. The ectomycorrhizal fungal communities are significantly different among the five forests in both Mhh and Mhg. The distinct mycorrhizal specificity between Mhh and Mhg suggests the possibility of different mycobionts triggered ecological speciation between sympatric species.

Keywords Coexisting mycoheterotrophic plants · *Monotropastrum humile* · Russulaceae · Mycoheterotrophy · Speciation

Introduction

Mycoheterotrophic plants (MHPs) lack chlorophyll and depend on mycorrhizal fungi for carbon sources and nutrients (Leake 1994). These plants are categorized into three trophic groups: initial, partial, and fully mycoheterotrophic, based on their reliance on mycorrhizal fungi in their life cycle. The fully MHPs, in particular, depend on fungal partners throughout their lifecycle and are considered the closest symbionts of mycorrhizal fungi (Merckx 2013). Approximately 580 achlorophyllous species have been identified, constituting a small subset of land plants (Jacquemyn and Merckx 2019). The transition from autotrophy to full mycoheterotrophy is estimated to have occurred independently at least 40 times during plant diversification (Jacquemyn and Merckx 2019).

The evolutionary trajectory of fully MHP is closely tied to their associated fungal partners (Ogura-Tsujita et al. 2012; Yamato et al. 2014). The adaptation and specialization of mycorrhizal fungi may play a role in the diversification or speciation of MHPs (Jacquemyn et al. 2023). Fully MHPs often form connections with phylogenetically restricted fungi, compared to their autotrophic relatives (Bidartondo and Bruns 2001; Grubisha et al. 2014; Dowie et al. 2017).

The subfamily Monotropoideae (family Ericaceae) is distributed throughout the Northern Hemisphere and is notable for its fully mycoheterotrophic characteristics (Bidartondo and Bruns 2001, 2002, 2005). Members within

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Monotropoideae, are associated with phylogenetically restricted fungi, such as Russulaceae, *Tricholoma*, and *Rhizopogon* (Bidartondo and Bruns 2001; Grubisha et al. 2014; Dowie et al. 2017). Phylogenetic congruence between Monotropoideae and ectomycorrhizal (ECM) fungi has been observed, indicating complex relationships between Monotropoideae plants and their fungal partners (Yang and Pfister 2006). Each of the five related plant lineages within the Monotropoideae is specialized to one of five distantly related ECM fungal lineages (Bidartondo and Bruns 2001, 2002, 2005), suggesting intricate and multifaceted interactions. Additionally, a shift in fungal partners may play a crucial role in the evolution and diversification of MHPs (Hynson and Bruns 2009; Ogura-Tsujita et al. 2012; Yamato et al. 2014; Suetsugu et al. 2020; Jacquemyn et al. 2023).

Monotropastrum humile often classified into two varieties, var. *humile* and var. *glaberrimum* (Hara 1965), exhibits distinct distribution patterns. *M. humile* var. *humile* (Mhh) is distributed in eastern Asia, from the Himalayas to Japan (Wallace 1975), while *M. humile* var. *glaberrimum* (Mhg) is only found in Taiwan and China. Chou and Zhou (1990) and Hsu et al. (1998) treated these varieties as different species. Mhg can be easily distinguished from Mhh by the absence of trichomes on the floral organs and differences in the shape and color of the floral disc (Tsukaya et al. 2008). Phylogenetic analysis based on ITS2 sequences shows that the Mhh formed a monophyletic group. However, Mhg was not the sister taxon of Mhh, but formed a monophyletic group with *Monotropa uniflora* (Tsukaya et al. 2008). Tsukaya et al. (2008) suggested that Mhg should be considered a distinct species.

Some studies reveal the existence of two closely related varieties of *M. humile* that establish symbiotic relationships with distinct ECM fungal families. Mhh forms associations with fungi from the Russulaceae family (Bidartondo and Bruns 2001), whereas Mhg is connected with the Thelephoraceae family (Yokoyama et al. 2005). Notably, the symbiosis between Mhg and its affiliated ECM fungi marks a significant deviation in the pattern of mycorrhizal associations observed within the subfamily Monotropoideae (Yokoyama et al. 2005). However, these differences have been observed in limited samples (2 Mhg and 1 Mhh) within a single site (Yokoyama et al. 2005), without considering the spatial structure in the associated fungal community.

Microscopic characteristics of the mycorrhizal fungal sheath of Mhh were examined by Yamada et al. (2008). They categorized 78 samples of adult *M. humile* var. *humile* individuals into 37 root mycorrhizal morphotypes, with 24 types identified as *Russula* or *Lactarius* fungal taxa within the Russulaceae family. However, the remaining 13 types were left unidentified, suggesting the potential association of non-Russulaceae fungi with Mhh. While microscopic evidence is valuable for assessing these ECM fungal mycorrhizal formations, the limited characteristics make it challenging to comprehensively understand their ECM fungal diversity. This limitation may result in underestimating ECM fungi that deviate from the classic mycorrhizal structure and exhibit non-dominant colonization patterns.

Over the past decades, advancements in culture-independent approaches, particularly high-throughput sequencing, have significantly expanded our comprehension of the global diversity of root mycobionts. These cutting-edge techniques have provided a more comprehensive understanding of the intricate relationships between plants and mycorrhizal fungi. This progress underscores the need to revisit and reevaluate previous findings, especially in the context of mycorrhizal associations. In Taiwan, Mhh and Mhg often coexist in the same geographic area, providing a valuable model to investigate the mycorrhizal fungal community of these closely related MHPs in sympatry.

This study aims to assess the diversity of mycorrhizal fungi associated with Mhh and Mhg in Taiwan. Five sites were selected to identify variations in fungal preferences of these co-occurring MHPs by addressing the following research questions: (1) What is the identity and structure of the fungal communities associated with two closely related MHPs? (2) Do the co-occurring Mhh and Mhg overlap in their respective ECM fungi within their roots? (3) Do populations of Mhh or Mhg from different areas host distinct ECM fungal communities?

Materials and methods

Study site and sampling procedure

Throughout the flowering periods of MHP from 2017 to 2020, we collected samples of Mhh and Mhg from five locations across Taiwan, including Jailishan (JL), Henglingshan (HL), Hehuanshan (HH), Sun Link Sea (SLS), and Jinshuiying (JS). These sites are situated at elevations ranging from 1,430 to 3,030 m above sea level. The diverse habitats sampled include broadleaved forests with ectomycorrhizal (ECM) trees from the Fagaceae family, arbuscular mycorrhizal (AM) trees from the Symplocaceae family, and ericoid mycorrhizal (ERM) trees from the Ericaceae family; mixed conifer-broadleaved forests with ECM trees (Fagaceae and Pinaceae), AM trees (Lauraceae and Cupressaceae), and ERM trees (Ericaceae); and conifer forests dominated by *Abies kawakamii* and *Tsuga chinensis*, as detailed in Table 1. In the course of our fieldwork, individual plants were identified morphologically as one of the two MHP varieties. Five to nine individuals per MHP variety were sampled in each site, with a distance of more

Table 1 Collection sites, forest type, elevation, and sample sizes for the Mhh and Mhg specimens used in this study

Site	Forest type (Dominant tree species or families)	Elevation (m)	Sample size		Sampling time (month-year)	
			Mhh	Mhg	Mhh	Mhg
Hehuanshan (HH)	*Abies-Tsuga* (*Abies kawakamii* and *Tsuga chinensis*)	3,030	6	9	Jun-19	May-17, May-18
Jailishan (JL)	Conifer-broadleaf (Cupressaceae, Ericaceae, Fagaceae and Lauraceae)	2,200	7	5	Mar-20	Mar-20
Henglingshan (HL)	Conifer-broadleaf (Cupressaceae, Ericaceae, Fagaceae and Lauraceae)	1,900	5	6	Jun-20	Mar-20
Sun Link Sea (SLS)	Conifer-broadleaf (Cupressaceae, Ericaceae and Fagaceae)	1,600	6	6	Jun-20	Mar-20
Jinshuiying (JS)	Broadleaved (Ericaceae, Fagaceae and Symplocaceae)	1,430	7	5	Mar-20 – Apr-20	Feb-20 –Apr-20

Note The mycorrhizal type of dominant tree species or families: ectomycorrhizal type: *Abies kawakamii*, *Tsuga chinensis*, Fagaceae; arbuscular mycorrhizal type: Cupressaceae, Lauraceae, and Symplocaceae; ericoid mycorrhizal type: Ericaceae

than one meter between specimens to prevent sampling the same plant population (Matsuda et al. 2011). The distance between MHPs at the HH site ranged from 30.9 to 234.1 m; at the HL site, the range was from 7.8 to 1,241.6 m; the distance at the JL site spanned from 8.5 to 62.9 m; at the JS site, it varied from 17.3 to 419.5 m; and, finally, at the SLS site, the MHPs were separated by distances ranging from 36.8 to 239.8 m.

A total of 31 Mhh and 31 Mhg individuals were collected. All samples were stored at 4 °C and processed within 48 h for DNA extraction. To confirm the taxonomic identity of these two MHPs, we meticulously selected multiple samples from both Mhh and Mhg. These samples were subjected to ITS sequence amplification and subsequent alignment with ITS sequences of other monotropoids, which are available in the GenBank international DNA database (Bidartondo and Bruns 2001).

Root Processing

The root structure within the rootball of two varieties of *Monotropastrum humile* differs significantly from that of autotrophic plants (Fig. S1). This distinction allows us to differentiate them and avoid mistakenly taking them based on their root morphology. The rootball was carefully rinsed in tap water using forceps to eliminate soil particles, tree roots, and debris. For each MHP specimen, we randomly selected at least 15 root tips (approximately 1 cm in length, totaling around 1 gram), spanning from the inner to the outer sections of the rootball. This approach was adopted to guarantee a thorough and representative sample.

To eliminate any surface contaminants or non-mycorrhizal fungi present, the mycorrhizal roots were subjected to a surface sterilization procedure. This involved the use of a 1% sodium hypochlorite (NaClO) solution, followed by thorough rinses in sterile deionized water (Suetsugu et al. 2021). This step is crucial for ensuring that the observed mycorrhizal associations are not influenced by external microbial or fungal populations.

DNA extraction, PCR, and high-throughput sequencing

Each root sample was pooled with 15 root tips and extracted DNA using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1987). DNA purity and concentration were determined with a NanoDrop spectrophotometer. Fungal-specific primers ITS1F (Gardes and Bruns 1993) and ITS2 (White et al. 1990) were used to amplify the nuclear internal transcribed spacer region (Boeraeve et al. 2018; Truong et al. 2019). PCR was performed using FastStart polymerase buffer and *Taq* DNA Polymerase (Roche, Germany) with the following PCR conditions, an initial denaturation for 5 min at 95°C, followed by 35 cycles of 30 sec at 95°C, 55°C and 72°C, and a final extension for 5 min at 72°C. Secondary PCR was performed using the following forward (5'-[8-mer NS]-[ITS1F]-3') and reverse (5'-[8-mer NS]-[ITS2]-3') sequencing primers fused with an 8-mer barcode as follows: an initial denaturation for 3 min at 95 °C, followed by 5 cycles of 20 s at 98 °C, 57.5 °C and 72 °C; and a final extension for 3 min at 72 °C. The Illumina MiSeq platform processed the high-throughput sequencing libraries (Tri-I Biotech Inc, Taiwan).

Bioinformatics

Raw data underwent quality control analysis using CLC Genomics Workbench v10. Mothur v. 1.35.1 was employed to screen reads with a Phred score > 20, aligning quality-filtered sequences against the UNITE database. Bayesian classifier with the UNITE training database was utilized to classify sequences, and USEARCH 7.0 clustered these sequences into OTUs at 97% similarity. OTU sequences comprising less than 0.05% of the total reads in any sample were removed (Mujic et al., 2023). Taxonomy assignment

at the generic level (identity > 95%), family level (identity > 90%), and order level (identity > 80%) was performed (Tedersoo et al. 2015; Nilsson et al. 2019). The functional group of fungal OTUs was recognized using FUNGuild (Nguyen et al. 2016) and research articles (Rinaldi et al. 2008; Tedersoo and Smith 2013; Tedersoo et al. 2014). The ECM guild taxa were used for further analysis.

Statistical analysis

Sequencing depth adequacy was measured by rarefaction analysis using Past 4 software version 4.03 (Hammer et al. 2001). R software version 4.1.2 was used for statistical procedures. T-tests and ANOVA compared alpha diversity between MHPs and among sites, respectively. The GUniFrac package normalized reads for each sample (Chen and Zhang 2021). Multidimensional scaling (MDS) plot was used to visualize the ECM fungal community pattern based on Hellinger-based distances (Pérez-Izquierdo et al. 2020) by Primer 6 software (Clarke et al. 2014). PERMANOVA tested the difference in ECM fungal communities between two MHPs or among sites. Venn diagram was generated using InteractiVenn (http://www.interactivenn.net) (Heberle et al. 2015).

Results

Overall fungal community composition

A total of 62 DNA samples from plant roots were amplified and sequenced, with the effective tags generated by high-throughput sequencing aggregated at 97% sequence similarity, yielding 1,942 fungal OTUs (2,861,545 sequencing reads). After analysis, 77.9% of the sequences (2,227,781 sequencing reads, 408 OTUs) were assigned to putative ECM fungi (Table S1). Notably, the ECM guild represented the predominant fungal group associated with the MHPs (Table S2, Table S3). The rarefaction curves (Fig. S2) constructed for these samples indicated that the OTU diversity reached near-saturation, suggesting that our sequencing depth captured the majority of the fungal diversity present in each sample.

ECM fungal diversity associated with *M. humile* var. *humile* and *M. humile* var. *glaberrimum*

The totals of ECM fungal OTUs in 31 samples of *Monotropastrum humile* var. *humile* (Mhh) and 31 samples of *M. humile* var. *glaberrimum* (Mhg) were 227 (with 1,108,710 sequencing reads) and 202 (with 1,119,071 reads), respectively. Among these, only 21 ECM OTUs were communal between the two MHPs. A comparison of OTU richness showed that Mhh and Mhg had a similar number of ECM fungal OTUs, with no significant difference (t = -0.67057, $p = 0.5051$) in the number of ECM fungal OTU between Mhh (13.1 ± 7.9; mean ± SD) and Mhg (11.7 ± 7.7; mean ± SD). On average, there were 35,764.8 ± 9,881.6 reads and 36,099.1 ± 10,735.1 reads per individual associated with Mhh and Mhg, respectively (t = 0.12754, $p = 0.8989$). Table S2 compared ECM fungal OTU numbers detected in Mhh and Mhg from the five study sites. The number of ECM fungal OTUs of Mhh ranged from 8.2 ± 3.6 to 18.4 ± 14, which was not significantly different among the five sites (ANOVA, $F = 1.42$, $p = 0.255$). In contrast, the number of ECM fungal OTUs of Mhg ranged from 6.8 ± 4.4 to 21.6 ± 4.9, significantly different among the five sites (ANOVA, F = 4.529, $p = 0.007$). The highest ECM fungal OTUs were detected in the HL site (Table S2).

ECM fungal composition among MHP varieties and sites

To examine whether the ECM fungal OTU composition varied between two MHP varieties, ECM fungal OTU matrices were selected for MDS and PERMANOVA. The OTU-level MDS plot illustrated distinct ECM fungal communities between Mhh and Mhg (Fig. 1), confirmed by PERMANOVA (pseudo-$F = 5.2018$, $p = 0.001$). There were thirteen ECM fungal genera and one Boletaceae unclassified genus associated with Mhh. Members of *Russula* were most abundant accounting for 92.3% of total ECM guild reads, followed by *Lactarius* (3.4%), *Sebacina* (2.0%), and *Chloridium* (1.6%). Members of *Amanita*, *Cortinarius*, *Elaphomyces*, *Entoloma*, *Inocybe*, *Phylloporus*, *Piloderma*, *Tomentella, Tuber*, and one Boletaceae unclassified genus represented < 1% relative read abundance (Fig. 2). Fifteen ECM fungal genera were associated with Mhg. Members of *Lactarius* were most abundant accounting for 80.2%, followed by *Russula* (14.0%), *Sebacina* (2.2%), *Elaphomyces* (1.3%), and *Chloridium* (1.1%). Members of *Amanita*, *Amaurodon*, *Cortinarius*, *Entoloma*, *Hydnum*, *Inocybe*, *Lactifluus*, *Piloderma*, *Thelephora* and *Tomentella* represented < 1% relative read abundance (Fig. 2).

The top 100 ECM fungal OTUs were used to compare the dominant ECM fungal composition and relative read abundance between two MHP varieties (Fig. 3). Each MHP was predominantly associated with one to four ECM fungal OTUs at each site. Two MHP varieties did not share dominant ECM fungal OTUs in three sites (HL, JL, and SLS; Fig. 3b, c and e). In contrast, five ECM fungal OTUs were shared between two MHP varieties in the HH site (Fig. 3a), and one ECM fungal OTU was detected in both MHP

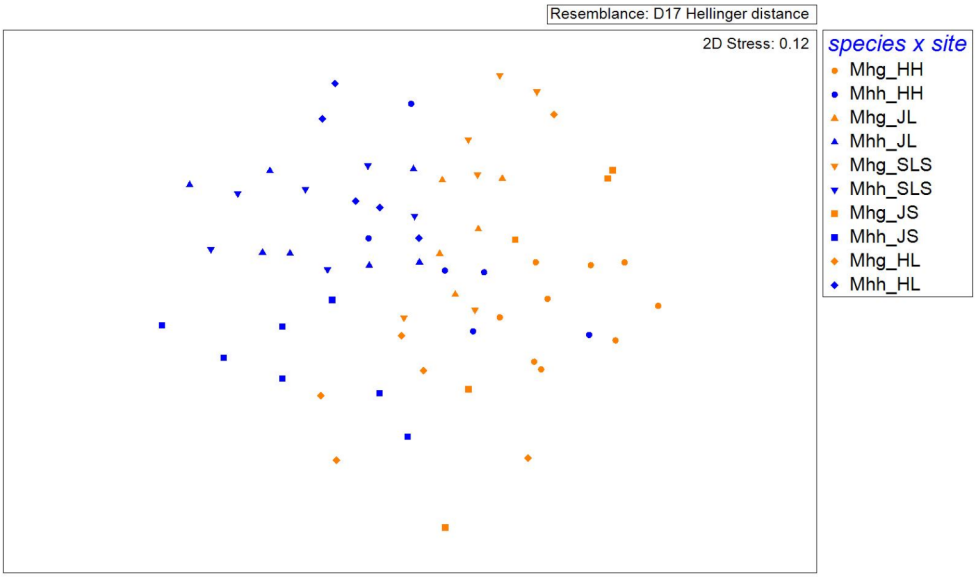

Fig. 1 Variation in ECM fungal communities (OTU-level) associating with two MHPs. MDS graph displays variation in ECM fungal community composition between individuals of *M. humile* var. *humile* (Mhh) and *M. humile* var. *glaberrimum* (Mhg) sampled at Hehuanshan (HH), Jailishan (JL), Henglingshan (HL) Sun Link Sea (SLS) and Jinshuiying (JS)

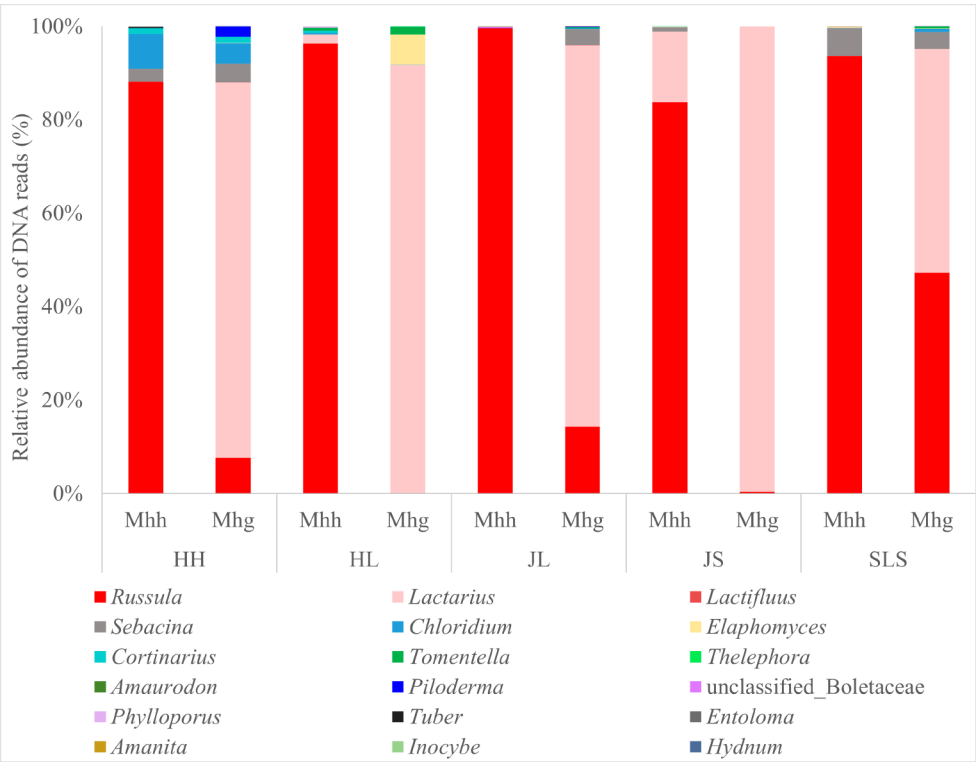

Fig. 2 Generic level of ECM fungal communities observed in the coexisting populations of *M. humile* var. *humile* (Mhh) and *M. humile* var. *glaberrimum* (Mhg). Bar charts representing the cumulative proportions of sequences belonging to different ectomycorrhizal fungal genera observed in the sampled Mhh and Mhg populations from Hehuanshan (HH), Jailishan (JL), Henglingshan (HL) Sun Link Sea (SLS) and Jinshuiying (JS)

varieties in JS (Fig. 3d). These limited ECM fungal OTUs sharing between two MHPs had lower relative read abundance. In the HH site, *Lactarius* OTU00007 was detected in both roots of MHPs but was only predominantly associated with Mhg (Fig. 3a). At the OTU level, we also found two MHPs had differences in ECM fungal associations in the co-occurring site.

PERMANOVA test revealed that sampling sites significantly influenced the ECM fungal communities associated with MHPs (pseudo-$F = 3.526$, $p = 0.001$). MDS plot showed that ECM fungal communities associated with Mhh were grouped according to sampling sites (Fig. 4a) and the ANOSIM test show that the ECM fungal communities were significantly different among sites ($p = 0.001$). Only ECM fungal communities associated with Mhh from SLS and HL were not significantly different. The Venn diagram revealed that 11, 10, 5, 1, and 1 OTUs were shared between Mhh populations from SLS-JL, SLS-JS, SLS-HL, HH-JL, and HH-JS, respectively (Fig. 4c). Similar site effects were also found in ECM fungal communities associated with Mhg

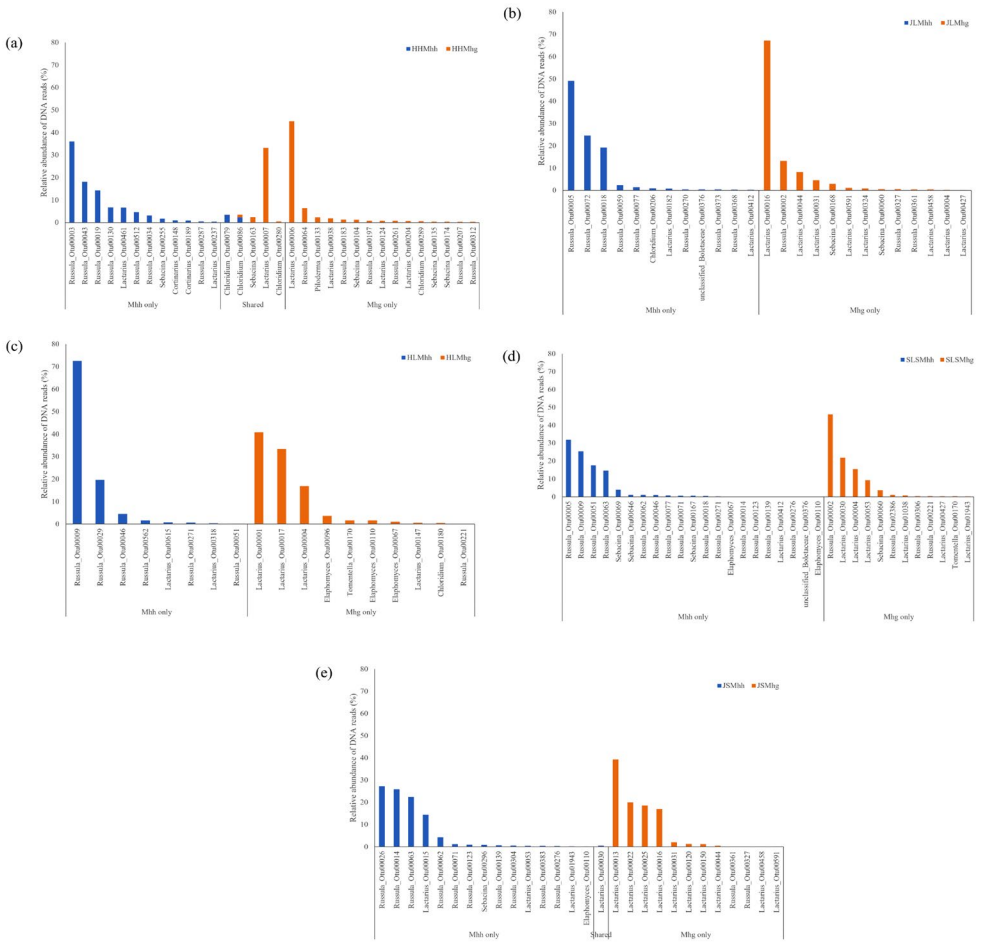

Fig. 3 Relative read abundance of the top 100 dominant ECM fungal OTUs in the coexisting populations of *M. humile* var. *humile* (Mhh) and *M. humile* var. *glaberrimum* (Mhg) from Hehuanshan (**a**), Jailishan (**b**), Henglingshan (**c**), Sun Link Sea (**d**) and Jinshuiying (**e**)

(Fig. 4b, $p = 0.001$). The Venn diagram revealed that 1 OTU was shared among Mhg populations from JL, SLS, and HL. Eleven, seven, four, and one OTUs were shared between Mhg populations from JS-JL, JL-SLS, HL-SLS, and SLS-JS, respectively (Fig. 4d).

Discussion

This study investigated the ECM fungal community of monotropoid roots through Illumina sequencing of the ITS1 region. Our findings reveal a high ECM fungal diversity associated with *Monotropastrum humile* var. *humile* (Mhh) and *M. humile* var. *glaberrimum* (Mhg). The dominant association pattern emerged, with Mhh having a consistent link to Russulaceae fungi, particularly members of *Russula*, while Mhg preferred members of *Lactarius*. Additionally, our study also identified a few undominant ECM taxa, such as *Sebacina*, *Chloridium*, *Elaphomyces* and *Thelephora* associated with Mhh and Mhg. Roots of *M. humile* were found to have a highly specialized association with Russulaceae (Yokoyama et al. 2005; Yamada et al. 2008; Matsuda et al. 2011). However, Thelephoraceae (Yokoyama et al. 2005; Matsuda et al. 2011), *Phellodon* sp. and *Gymnopilus* aff. *penetrans* (Shen et al. 2012) were found in the root-ball of *M. humile*. Contrary to prior research (Bidartondo and Bruns 2001; Yokoyama et al. 2005; Yamada et al. 2008; Matsuda et al. 2011; Shen et al. 2012), our findings suggest that MHPs can host a diverse array of ECM fungal species, with certain fungi potentially outcompeting others or being preferentially selected by the host, leading to the dominance of specific ECM fungi while others remain less abundant.

Our results align with prior research suggesting that plant phylogenetic constraints play a crucial role in shaping mycorrhizal communities in MHP species. Jacquemyn et al. (2011) and Xing et al. (2020) proposed that phylogenetic constraints influence the specificity levels of dominant mycorrhizal partners. Our study observed diversified mycobionts in the roots of Mhh and Mhg, with the changes in specificity levels (i.e., phylogenetic breadth) of dominant mycobionts influenced by phylogenetic constraints. Previous studies showed that plant lineages are specifically dependent on different lineages of fungi in the monotropoid mycorrhizal symbiosis and that MHP plants in the clades of *Monotropastrum* and *Monotropa* are constraint associated with Russulaceae fungi (Bidartondo and Bruns 2001).

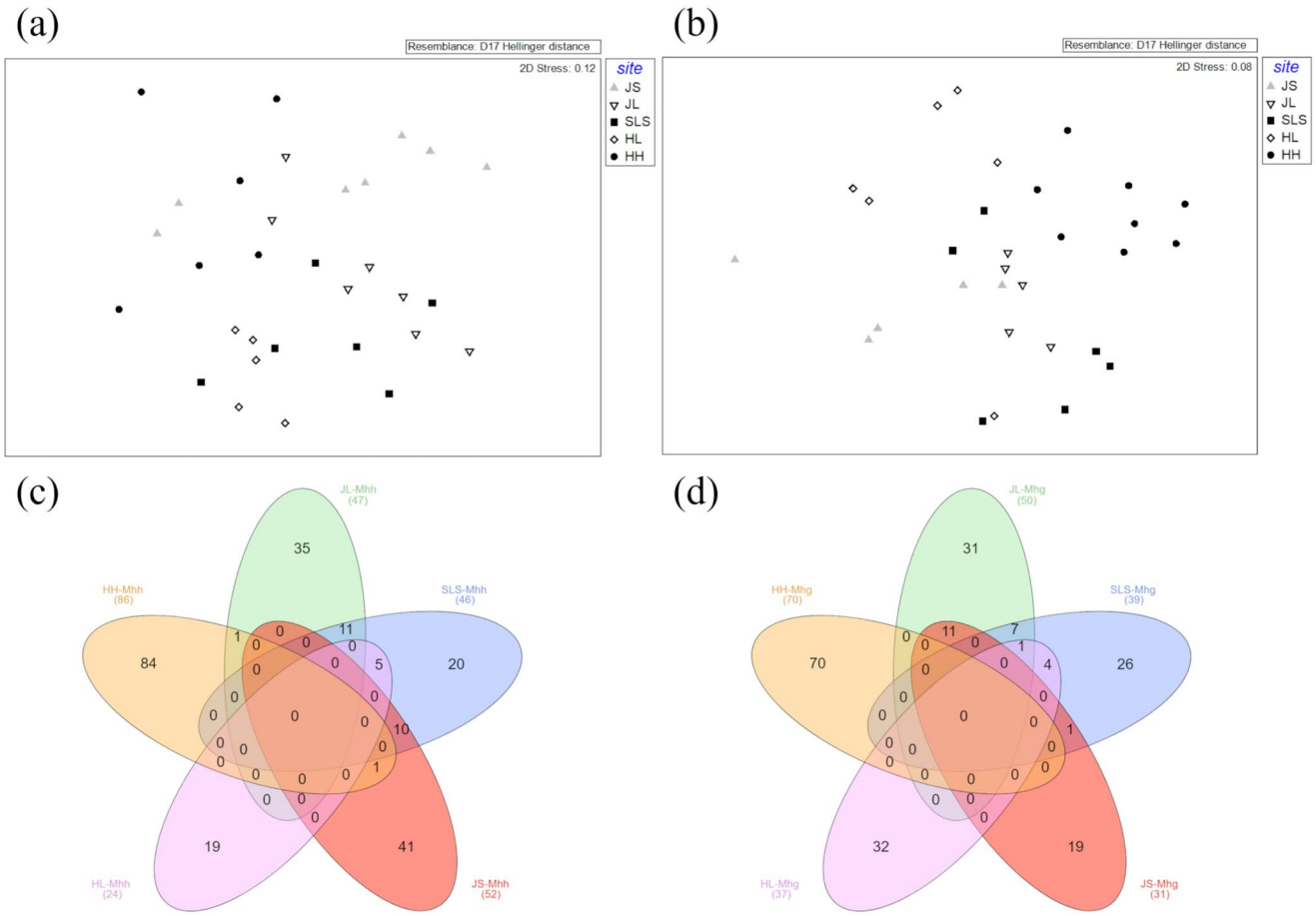

Fig. 4 Variation in ECM fungal communities (OTU-level) associating with Mhh (**a**) and Mhg (**b**) among the five sites. The MDS graph displayed variation in ECM fungal community composition between individuals of Mhh (**a**) and Mhg (**b**) sampled at five sites. A Venn diagram showing the number of ECM fungal OTUs shared among Mhh (**c**) or Mhg (**d**) populations from Hehuanshan (HH), Jailishan (JL), Henglingshan (HL) Sun Link Sea (SLS) and Jinshuiying (JS)

However, the identity of the fungal symbiotic partners of the *Monotropastrum* used limited samples. The present study has the largest sample, numerically, taxonomically and geographically.

The phylogenetic tree for the subfamily Monotropoideae was constructed and presented in Fig. S3. This phylogenetic tree indicates that *M. humile* var. *humile* (Mhh) samples form a monophyletic group. In contrast, *M. humil*e var. *glaberrimum* (Mhg) samples form a monophyletic group closely related to *Monotropa uniflora*. This finding corroborated the study by Tsukaya et al. (2008), disclosing that these two *M. humile* varieties and *Monotropa uniflora*, despite belonging to different genera, form a monophyletic clade. Therefore, our results support the reclassification of *Monotropastrum humile* var. *glaberrimum* as a separate species.

Additionally, our research uncovers that Mhh and Mhg are associated with distinct dominant ECM fungi. *Monotropastrum humile* var. *humile* (Bidartondo and Bruns 2001; Suetsugu et al. 2023), *Monotropastrum kirishimense* (Suetsugu et al. 2023), and *Monotropa uniflora* (Kong et al. 2015) are predominantly associated with *Russula* fungi. *Monotropastrum humil*e var. *glaberrimum* (Mhg) samples form a monophyletic group closely related to *Monotropa uniflora* (Fig. S3), and they preferred to be associated with members of *Lactarius*. *Monotropa brittonii* is closely related to *Monotropa uniflora* (Fig. S3) and Keesling et al. (2021) found that *Monotropa brittonii* is only associated with *Lactifluus* which is a member of the Russulaceae family. This demonstrates that the symbiotic associations between the MHPs and fungi are dictated by the plants' phylogenetic relationships, with a predilection for specific ECM genera in the Russulaceae family. A thorough examination of the ECM community and taxonomies of these MHPs in the *Monotropastrum-Monotropa* clade is necessary to uncover their ECM association and shed light on this complicated evolutionary process.

Our results revealed that coexisting Mhh and Mhg are associated with distinct sets of fungal partners. This observation aligns with existing studies suggesting that co-occurring MHP species prefer distinct mycorrhizal fungi

(Waterman et al. 2011; Jacquemyn et al. 2012, 2014). In sympatric habitats, MHP species tend to engage with different fungal partners, minimizing overlap, and reducing competition (Gomes et al. 2017). The limited sharing of fungal partners between Mhh and Mhg might reduce competition and allow coexistence.

The significant role of habitat in shaping ECM fungal communities associated with both Mhh and Mhg was observed in this study, thereby demonstrating the profound influence of site effects on such communities. Bidartondo and Bruns (2001) conducted an expansive investigation into the phylogenetic patterns of Ericaceae from North America and Eurasia, thereby inferring the existence of geographical patterns of specificity. Our research provides further substantiating findings, suggesting that *Monotropastrum* plant species have affiliations with local fungal partners that are area-specific. Although approximately 200 ECM fungal OTUs may establish associations with MHPs, typically only 12–13 OTUs emerge as dominant. Across different sites, 2–4 ECM fungal OTUs tend to dominate, with some regions hosting three dominant ECM fungi. For instance, HH site harbors unique autotrophic host plant species, resulting in a distinct ECM fungal pool. The Venn diagram illustrates that ECM fungi associated with Mhh/Mhg from the HH site do not overlap with those from the other four sites. The presence of varied ECM fungi in different habitats provides MHPs with ample colonization opportunities across diverse elevations and forest types. The composition of the ECM fungal pool is influenced by the forest type, thus impacting the associations between MHPs and ECM fungi.

Additionally, the selective symbiotic relationship between Mhh/Mhg and their fungal symbionts likely evolved over time. For example, Johansson et al. (2017) demonstrated that *Hypopitys monotropa*, during its seed germination stage, can form associations with various genera of ECM fungi, but as it progresses to later stages, it exhibits heightened mycorrhizal specificity. This specialization could potentially enhance the efficiency of carbon acquisition (Leake and Cameron 2010). While dominant ECM fungi may contribute to carbon acquisition efficiency, further research is required to fully elucidate this relationship.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00572-024-01158-4.

Acknowledgements The work was supported by funding from the Ministry of Science and Technology, Taiwan (MOST 108-2621-B-080-001-MY2). The authors would like to express their gratitude to several organizations for granting permission to collect samples of two Monotropastrum taxa in Taiwan. The acknowledgments go to Taroko National Park Headquarters, Endemic Species Research Institute, Dongshih Forest District Office, Pingtung Forest District Office, and Sun-Link-Sea Resort. We also thank Dr. Yu-Ting Wu, Dr. Ko-Hsuan Chen for their critical reading, insightful discussions, and constructive comments greatly enhanced the quality of the research. This research was conducted by Ren-Cheng Liu as part of his doctoral dissertation, under the guidance and supervision of Dr. Wan-Rou Lin and Dr. Pi-Han Wang.

Author contributions R.C.L. conducted research, analyzed the data and contributed to writing the manuscript; P.H.W. designed the experiments, contributed to writing the manuscript and reviewed the manuscript; W.R.L. obtained funding, planned the experiments, analyzed the data, and wrote the manuscript.

Funding The work was supported by funding from the Ministry of Science and Technology, Taiwan (MOST 108-2621-B-080-001-MY2).

Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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