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RESEARCH ARTICLE

Not only transitions in nutritional modes but also niche shifts facilitate mycorrhizal fungal specialization in *Burmannia*

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Abstract

1. Mycoheterotrophs are non-photosynthetic plants that obtain all of their carbon requirements through parasitizing mycorrhizal fungi. They originated from the autotrophic ancestors and usually have more specific relationships with fungi than that of green plants, for reasons that are largely unknown. Determining the factors that lead to specificity in mycoheterotrophs could provide insights on the constraints to mycoheterotrophic evolution.
2. Here we assess the fungal diversities in mycoheterotrophic plants and their co-occurring plants to determine the roles of ecological factors on the specific fungal associations in mycoheterotrophic plants. We investigated mycorrhizal fungal communities in 16 populations of seven *Burmannia* species with different trophic modes and their co-occurring plants using high-throughput sequencing to assess the tripartite relationships of fungi, mycoheterotrophs and co-occurring autotrophs.
3. We found that mycoheterotrophic species have similar fungal richness to their chlorophyllous relatives, indicating that they are not associated with a reduced set of fungal partners. The preference of mycoheterotrophic species toward specialized fungal assemblages is consistent with the pattern found in the green autotrophic plants within forest habitats, suggesting a coupling of the fungal phylogenetic constraints between mycoheterotrophs and their co-occurring autotrophs. We furthermore show that the turning to fungal communities having closer phylogenetic relationships during habitat shifts from open grasslands to shaded forests might provide the basis for the specialization of mycorrhizal associations in mycoheterotrophic species of *Burmannia*.
4. Our findings suggest that fungal niche shifts may have promoted fungal partner changes and specialization in mycoheterotrophic plants. Our results provide

new insights into the ecological factors leading to the specialized interactions between mycoheterotrophic plants and their fungal partners, expanding our understanding of the evolutionary trajectories followed by mycoheterotrophs.

KEYWORDS
evolutionary ecology, fungal diversity, mycoheterotroph, mycorrhizal symbiosis, niche shift

1 | INTRODUCTION

Most terrestrial plants have the potential to form symbiotic associations with arbuscular mycorrhizal fungi (AMF) that comprise Glomeromycotina (Mucoromycota; Wang & Qiu, 2006). In this symbiotic relationship the partners are usually mutualistic and benefit from reciprocal nutrient exchange: fungi provide soil nutrients such as nitrogen and phosphorus to plants, in return for photosynthetic carbohydrates and lipids from plants (Bago et al., 2000; Bonfante & Genre, 2010; Pfeffer et al., 1999; Smith et al., 2011). In contrast, mycoheterotrophic plants (MHPs) reverse the nutrient flux and can seize carbon from fungi (Leake, 1994). MHPs are completely achlorophyllous and hence non-photosynthetic, connecting to the autotrophic plants through mycorrhizal fungi on which they ultimately depend (Bidartondo, 2005). Although it is still unclear whether MHPs provide any benefit to their associated fungi, MHPs are usually treated as 'cheaters' of the mycorrhizal symbiosis.

MHPs evolved multiple times independently from autotrophic mutualistic ancestors, with at least 580 species reported across 10 plant families (Jacquemyn & Merckx, 2019). There are also many partially mycoheterotrophs that retain chlorophyll and are therefore photosynthetic (Jacquemyn & Merckx, 2019). Partially MHPs can also seize carbon nutrients from mycorrhizal fungi (Cameron & Bolin, 2010; Giesemann et al., 2021; Hynson et al., 2009; Johansson et al., 2015), although some of them have been proven to grow as complete autotrophs under high light levels (Merckx et al., 2010): they usually have fully green but much smaller leaves than their autotrophic relatives, and sometimes have subterranean organs quite similar to that of achlorophyllous mycoheterotrophic species (Imhof et al., 2013). Although they are not observed in some families with mycoheterotrophic species, including Triuridaceae and Thismiaceae, partially MHPs are thought to represent evolutionary intermediate stages from autotrophy to mycoheterotrophy (Jacquemyn & Merckx, 2019).

To better understand the evolution of mycoheterotrophic plants, there is increasing interest in uncovering the enigmatic relationships of MHPs and their fungal partners. Interestingly, previous studies have revealed that in many cases fungal partner shifts between plant species accompany either the transition from autotrophy to mycoheterotrophy or the split of mycoheterotrophic lineages (Jacquemyn & Merckx, 2019; Perez-Lamarque et al., 2020; Sykorova et al., 2007; Zhao et al., 2021), suggesting that the trophic mode switching may be a cause of the fungal partner shifts. Moreover, in comparison with either their autotrophic relatives or other autotrophic plants, MHPs tend to be associated with phylogenetically narrower fungal lineages (Gomes et al., 2017; Perez-Lamarque et al., 2020; Suetsugu et al., 2021; Yamato et al., 2014; Zhao et al., 2021), or in some extreme cases, only one or a few fungal species (Guo et al., 2019; Merckx et al., 2017; Suetsugu, Okada, et al., 2022). This specific relationship may vary among MHPs and be affected by biotic or abiotic factors, such as the composition of the local fungal community (Renny et al., 2017), but in general, mycoheterotrophic plants associate with specific and closely related fungi (Merckx et al., 2012; Perez-Lamarque et al., 2020). Until now, however, the mechanisms underlying these fungal partner changes and the specialization toward specific fungal lineages observed in the trajectory from autotrophy to partial mycoheterotrophy and mycoheterotrophy are still poorly understood. One explanation is that the fungal partner changes is helpful for avoiding or reducing inter-species competition between MHPs. However, considering the rarity of MHPs, with patchily distributed populations (Gomes et al., 2017, 2019; Leake, 1994), highly reduced vegetative growth, usually low population densities and self-pollination reproductive strategies (Zhang et al., 1999; Zhang & Saunders, 2000), it seems that their mycoheterotrophic habit could bring only limited pressure on the habitat, causing only less inter-species competition. Ecological factors other than the avoidance of inter-species competition might therefore have played more important roles in promoting fungal partner shifts in MHPs.

Most MHPs live in the understorey of deeply shaded forests (Leake, 1994), while their green-leaved relatives, including autotrophic and partially mycoheterotrophic species, usually grow in open habitats such as savannas, grassland and swamps. The habitat shifts and environmental vicariance may have influenced the diversification of MHPs from their autotrophic ancestors in some plant families, such as Burmanniaceae (Merckx et al., 2008). As the local habitats of autotrophic, partially mycoheterotrophic and fully mycoheterotrophic species differ significantly in vegetation structure and species composition, we thus hypothesize that the habitat-shifts would bring about differential AM fungal composition, providing a further basis for MHP partner shifts. However, it is unknown that whether the habitat shifts could also promote the fungal specialization during the evolution of mycoheterotrophs. Since the assemblage of fungal partners in MHPs is a subset of fungal communities in co-occurring autotrophic plants (Gomes et al., 2017, 2022; Perez-Lamarque et al., 2020), we would expect that the fungal specificity in MHPs may be framed by the associations between surrounding autotrophic plants and mycorrhizal fungi. As the survival of MHPs completely depend on mycorrhizal fungi which are obligately symbiotic with co-occurring autotrophic plants, we consequently propose two hypotheses for this fungal specificity: (1) MHPs tend to co-occur with particular groups of green plants, (1a)

either a phylogenetically constrained group, (1b) or dominant trees in particular types of vegetation, that provide a particular fungal assemblage; or (2) MHPs are associated with specific fungal communities assembled in particular habitats, independent of the composition of co-occurring plant species. In order to test these hypotheses, in this study we assayed the fungal and co-occurring plant communities of autotrophic, partially mycoheterotrophic, and fully mycoheterotrophic *Burmannia* species to address whether the habitat changes have affected the fungal partner shifts along the trophic switching. Furthermore, we assayed fungal communities of mycoheterotrophic species from different populations as well as their co-occurring plants to detect whether the mycorrhizal fungal specialization in MHPs was influenced by the co-occurring plants. This enabled us to identify the ecological factors influencing the evolutionary dynamics within the specialized interactions between MHPs and their fungal partners on the evolutionary trajectory from autotrophy to mycoheterotrophy.

2 | MATERIALS AND METHODS

2.1 | Plant sampling

Burmannia s.l. contains ~60 species composed of autotrophs, partial mycoheterotrophs, and full mycoheterotrophs, which represent multiple transitions from autotrophy to mycoheterotrophy in the genus. In this study, root samples from 16 populations (Table 1) of seven *Burmannia* species (Figure 1) with different habitat types (Table 2, Table S1) were collected following the method of a previous study by Zhao et al. (2021). Species sampled included a robust chlorophyllous, putatively autotrophic species (*B. disticha*), two candidate partially mycoheterotrophic species (*B. chinensis* and *B. filamentosa*) and four fully mycoheterotrophic species, representing two independent mycoheterotrophic lineages (a clade containing *B. decurrens*/*B. nepalensis*/*B. oblonga*/*B. chinensis*/*B. filamentosa* and the other clade containing *B. itoana* and *B. disticha*; Li et al., 2020; Merckx et al., 2008; Zhao et al., 2021). Hereafter, the terms autotrophic and partially mycoheterotrophic are used as convenient labels for species presumed based on morphological characteristics that require future verification.

Before collecting root samples, the composition of co-occurring species and dominant species were recorded. For the sampling of co-occurring autotrophic plants of MHPs, roots were collected from nine subplots of 0.1 × 0.1 m with 0.2 m in depth which were equally distributed in 10 × 10 m plots. Root tips were taken from each independent root and were collected as a single root sample. In addition, root tips of co-occurring autotrophs were also collected from the sites where the mycoheterotrophic specimens were removed. For the root sampling of plants co-occurring with *B. disticha* and *B. filamentosa*, a 2 × 2 m plot was set for each population and one individual was randomly selected for each species. Samples of plants co-occurring with *B. chinensis* were collected from a 1 × 2 m plot due to the limitation of the terrain. All root samples were rinsed, snap frozen using liquid nitrogen, and then stored in liquid nitrogen for the subsequent DNA extraction. In total, 468 root samples from 16 sampling plots were collected, including

TABLE 1 Replication statement.

Scale of inference	Scale at which factor of interest is applied	Number of replicates at the appropriate scale
Arbuscular mycorrhizal fungal communities	Sites	16 study sites (10 forest sites and 6 grassland sites)

FIGURE 1 *Burmannia* species. Autotrophic (*B. disticha*), putative partially mycoheterotrophic but green-leaved (*B. filamentosa* and *B. chinensis*), and fully mycoheterotrophic (*B. decurrens*, *B. oblonga*, *B. nepalensis* and *B. itoana*) species of *Burmannia*.

TABLE 2 Populations of each *Burmannia* species sampled in this study.

Burmannia species	Population	Niche type	Dominant species
B. disticha	WS	Grassy slope	*Cyperus rotundus*, *Echinochloa crus-galli*, *Hypolepis punctata*, *Paspalum scrobiculatum*, *Saccharum rufipilum*
	BPZ	Grassy slope	*Diplopterygium glaucum*, *Lepidosperma chinense*, *Hedyotis mellii*, *Dicranopteris pedata*, *Gahnia tristis*, *Cymbopogon* sp.
	DXG	Grassy slope	*Lepidosperma chinense*, *Isachne truncata*, *Ischaemum aristatum*, *Heteropogon contortus*
B. filamentosa	ZJ	Wet grassland	*Isachne globosa*, *Ischaemum barbatum*, *Stipa* sp., *Fimbristylis* sp., *Cyperus haspan*, *Pycreus flavidus*, *Sphaerocaryum malaccense*
	NX	Wet grassland	*Digitaria sanguinalis*, *Miscanthus floridulus*, *Paspalum scrobiculatum*, *Fimbristylis aestivalis*, *Eriocaulon australe*, *Sphaerocaryum malaccense*
B. chinensis	GT	Wet grassland	*Diplacrum caricinum*, *Lycopodium japonicum*, *Stenotaphrum helferi*, *Pogonatherum paniceum*, *Pycreus flavidus*, *Ischaemum barbatum*, *Sphaerocaryum malaccense*
B. decurrens	XRD	Bamboo broad-leaved mixed forest	*Phyllostachys edulis*, *Phyllostachys arcana*
	YKS	Bamboo broad-leaved mixed forest	*Phyllostachys edulis*, *Cunninghamia lanceolata*
B. oblonga	JFL	Broad-leaved forest	*Polyspora axillaris*, *Pseudostreblus indicus*, *Castanopsis carlesii*, *Lindera metcalfiana*
B. nepalnesis	XRD	Bamboo broad-leaved mixed forest	*Phyllostachys edulis*, *Cunninghamia lanceolata*
	CHD	Broad-leaved forest	*Machilus chinensis*, *Cinnamomum parthenoxylon*, *Castanopsis sieboldii*, *Cornus elliptica*
	NX	Bamboo broad-leaved mixed forest	*Phyllostachys heteroclada*, *Choerospondias axillaris*, *Mallotus repandus*, *Cornus elliptica*
	YKS	Broad-leaved forest	*Elaeocarpus japonicus*, *Callerya nitida*, *Callerya nitida*
B. itoana	NX	Bamboo broad-leaved mixed forest	*Cinnamomum parthenoxylon*, *Schima superba*, *Choerospondias axillaris*, *Mallotus repandus*, *Phyllostachys heteroclada*
	CHD	Broad-leaved forest	*Sloanea sinensis*, *Castanopsis concinna*, *Cinnamomum parthenoxylon*, *Castanopsis carlesii*, *Machilus duthiei*
	SM	Broad-leaved forest	*Machilus chinensis*, *Machilus chinensis*, *Viburnum odoratissimum*

65 samples from *Burmannia* species and 403 samples from co-occurring plants (Table S1).

2.2 | DNA extraction and plant identification

All the root samples were macerated in liquid nitrogen, and the total DNA of each sample were extracted using the Hi-DNASecure Plant Kit (TIANGEN, Beijing, China). These DNA samples were used for both plant identification and fungal community sequencing in the following analyses. To identify the plant species co-occurring with MHPs, fragments of matK, rbcL or ITS were selected for routine polymerase chain reaction (PCR) using respective primer pairs (Table S2). Finally, 385 samples were successfully amplified and sequenced using one of the three primer pairs, while 18 samples were failed. We assigned the scientific names to the obtained sequences using Blast searches with GenBank, and then checked the identification carefully with the recorded species names when establishing the plots. To assess the dissimilarities between plant communities, we calculated the Sørensen index for any pair of plant communities according to the equation $D_s=(B+C)/(2A+B+C)$, where A represents the number of species shared by two communities, B and C are the number of species unique to each of the two communities (Hao et al., 2019). Values of Sørensen index range from 0 to 1, with smaller values suggesting higher similarity.

2.3 | High-throughput sequencing of fungal communities and data processing

For the autotrophs co-occurring with *Burmannia* species, DNA samples from the same species within each sampling plot were mixed into one sample before fungal community sequencing, and species with less than three DNA samples were removed from the downstream

analyses. Finally, 151 DNA samples for co-occurring plants were obtained (Table S3). To assess the composition of fungal communities, the partial small subunit (SSU) regions of 18S rDNA were amplified by PCR using AMF-specific primers. Because MHPs in *Burmannia* associate with mycorrhizal fungi from the Glomeromycotina (Merckx et al., 2012; Ogura-Tsujita et al., 2013; Suetsugu et al., 2014; Suetsugu & Okada, 2021), to improve the AMF detection rate, in this study nested PCR method was used to establish the 18S rRNA gene amplicon libraries. The first PCR amplification was constructed with the primer set AML1 and AML2, and the second PCR amplification was constructed with the primer set AMV4.5NF and AMDGR (Table S2). Amplicon sequencing was performed using the Illumina novaseq 6000 PE250 platform (Illumina, USA).

Raw reads obtained from high-throughput sequencing were cleaned by removing barcode and primer sequences, and were further quality filtered by removing bad reads including those that were too short, of too low quality or with too many Ns using FastP 0.20.0 (Chen et al., 2018). Paired-end reads were assembled into tags using FLASH 1.2.11 (Magoc & Salzberg, 2011). We used the Vsearch 2.22.1 (Rognes et al., 2016) to remove chimeras and clustered sequences into operational taxonomic units (OTUs) at 97% similarity (Hijri & Sanders, 2005), and finally generated the OTU table (see Figure S1 for detailed flowchart of data processing). The most abundant sequence in each OTU cluster was selected as the representative sequence for the taxonomic annotation using Vsearch based on SILVA database (Quast et al., 2013), and only OTUs assigned to Glomeromycotina were used for downstream analyses.

2.4 | Diversity and statistical analysis of fungal communities

We first removed singleton OTUs and then standardized the abundance of OTUs by selecting the sample with the fewest reads. We calculated richness and Shannon index values using the package vegan (version 2.6–6.1) with default parameters in R 4.3.1 (all the R packages used in this study were run under the R 4.3.1 environment), and then constructed richness and Shannon rarefaction curves for all samples to examine whether sufficient sequencing had been achieved. We found that most samples reached a saturation plateau with far lower than 50,000 sequence reads (Figure S2a,b) which is lower than the number of tags generated by sequencing for each sample, indicating that our sequencing depths were sufficient to capture most fungal OTUs. Moreover, we constructed OTU accumulation for number of samples in each plot, and results showed that our sampling could capture most OTUs in plants within a community, particularly those of high abundance (relative abundance >0.01%; Figure S3a,b).

To assess differences among plants with regard to AMF communities, we calculated values of several α-diversity indices including Chao1, ACE (abundance-based coverage estimator), Shannon and Simpson indices using the package vegan. Two distance indices, Bray–Curtis dissimilarity (Bray & Curtis, 1957) and UniFrac dissimilarity (Lozupone et al., 2011; Lozupone & Knight, 2005), are widely used to assess pairwise dissimilarity between samples or sample groups (β-diversity). The former describes microbial dissimilarities using the abundance (counts) of fungal OTUs, while the latter takes into account the phylogenetic relatedness of OTUs. In this study, we used UniFrac distances to assess the dissimilarities of fungal communities among plants. UniFrac distance matrices of fungal communities among different plant species or groups were constructed using the function UniFrac in R package phyloseq (version 1.48.0; McMurdie & Holmes, 2013) with default parameters, and the weighted UniFrac (Lozupone et al., 2007) distance matrix was constructed based on OTU abundances. To reveal relationships among samples and/or groups based on UniFrac distances, we performed nonmetric multidimensional scaling (NMDS) analysis using function metaMDS in R/vegan 2.6–6.1 package and hierarchical clustering analysis of fungal communities using the unweighted pair-group method with arithmetic mean (UPGMA) with the R/phyloseq package.

Wilcoxon tests for the indices of fungal α-diversity (Chao1, ACE, Shannon and Simpson) were performed to assess the significance ($p < 0.05$) of differences between different plant groups using the function stat_compare_means in R/ggpubr (version 0.6.0) package with default parameters. We performed a one-way PERMANOVA test (Anderson, 2001) to determine the differences of β-diversities between plant groups using the function Adonis2 from vegan package with 999 permutations.

2.5 | Correlation analysis of fungal assemblages in MHPs and co-occurring plants

To detect the mycorrhizal fungal associations between MHPs and co-occurring plants, we related the abundances of fungal OTUs in MHPs to those in co-occurring plants based on Spearman correlation coefficients. Spearman rank correlation coefficients (Sedgwick, 2014) between fungal OTUs of any pair of samples were calculated and visualized using R/corrplot 0.92 package (Wei & Simko, 2021), and *p* values were calculated at 95% confidence level. The coexistence network based on Spearman rank correlation coefficients was presented by Cytoscape (Shannon et al., 2003).

2.6 | Evolutionary analysis of arbuscular mycorrhizal fungi

Multiple alignments of 18S rRNA sequences were constructed using Muscle program (Edgar, 2022) with Super5 algorithm. Maximum-likelihood (ML) phylogenetic trees were constructed using FastTree program (Price et al., 2010) with generalized time-reversible (gtr) model and -spr 4+-gamma settings. For each phylogenetic tree, two 18S rRNA sequences of Mucoromycotina fungi (GenBank ID: LC431087.1 from *Vinositunica radiata*; LR997510.1 from *Endogone lactiflua*) were used as outgroup. To assess the phylogenetic clustering for fungal species associated with plants investigated in this study, the

standardized effect size (SES) of the mean pairwise distance (SES_{MPD}) (Webb et al., 2002) for fungal partners of each *Burmannia* species and co-occurring plant group were calculated using the R package picante (Kembel et al., 2010) with 1000 runs based on the phylogenetic tree. SES is used to measure the mean pairwise phylogenetic distance of taxa in a sample by quantifying overall clustering of taxa on a tree, in which increased clustering of taxa will result in the decrease of SES, and negative SES values indicate phylogenetic clustering, or small phylogenetic distances among co-occurring species than expected. Generalized linear mixed models (GLMMs) were used to evaluate the effect of phylogenetic constraints (SES) of fungal species associated with plants in the sampling plots on that of *Burmannia* species. We performed GLMMs analysis using R package glmmTMB (Brooks et al., 2017) with Gaussian distribution for our data. For the GLMM analysis, SES of each *Burmannia* population were used as the dependent variable (DV), SES of co-occurring plants and nutritional modes of *Burmannia* species were used as an independent variable (fixed factor), and habitat types as a random factor.

3 | RESULTS

3.1 | Plant–plant co-occurrence

In total, 109 species were identified from the root samples of plants co-occurring with seven *Burmannia* species (Table S3). Among these, 41 species from the grassland plots are herbs or shrubs, and 68 from the forest plots are mostly trees or shrubs. The plants co-occurring with green-leaved *Burmannia* species (*B. disticha*, *B. filamentosa* and *B. chinensis*) are distributed in 20 families, among which the most prevalent family is Poaceae, followed by Cyperaceae and Lycopodiaceae. The composition of plants co-occurring with *B. disticha* differs from that of partially mycoheterotrophic *B. filamentosa* and *B. chinensis* at both the family and species levels ($D_s > 0.9$ for all comparisons; Figure S4a; Table S4). *B. filamentosa* had similar composition of co-occurring plants to that of *B. chinensis* at the family level but differed significantly at the species levels ($D_s > 0.7$ for all comparisons; Figure S4a; Table S4). For fully MHPs, the composition of co-occurring plants was more distinct at the species than family levels (Figure S4b) and even differed among populations within one species ($D_s > 0.6$ for all comparisons; Figure S4c,d): for example, the two *B. nepalensis* populations YKS and NX had quite different co-occurring plant composition ($D_s = 0.89$) and shared only one autotrophic species (*Callerya nitida*, Fabaceae). For *B. itoana*, the most frequently sampled co-occurring plants are from Lauraceae followed by Fagaceae; in contrast, the most frequently sampled co-occurring plants for *B. nepalensis* are from Fabaceae (Table S3).

The dominant tree species as well as vegetation type in different populations of the same *Burmannia* MHPs differed significantly: for example, the dominant plant species co-occurring with *B. itoana* are different in each population (Table 2); similarly, populations of *B. nepalensis* had quite different dominant tree species and vegetation type, sharing only one tree species, *Cornus elliptica*.

3.2 | The distribution of fungal OTUs

We successfully sequenced the fungal communities from 65 samples of *Burmannia* species and 151 samples from their co-occurring plants. In total, 2804 Glomeromycotina OTUs were identified after aggregating effective sequences at 97% sequence similarity. Most of these OTUs were assigned to Glomeraceae (accounting for 1588 or 56.6% of taxa), followed by Gigasporaceae (5.2%) and Acaulosporaceae (4.2%). The relative abundance at family level of AMF differed among the co-occurring plants of *Burmannia*, for example, the co-occurring plants of MHPs showed a significant higher preference for Glomeraceae but less preference for Acaulosporaceae and Gigasporaceae than that of autotrophic and partially mycoheterotrophic *Burmannia* species (Figure S5). Within Glomeraceae, OTUs were dominated by *Glomus*, which accounted for 483 (30.4%) of the taxa, and then *Rhizophagus* (12.3%). In terms of the abundance of sequences, *Glomus* OTU986 accounted for 5.7% of total sequences, followed by *Glomus* OTU319 (3.7%) and *Rhizophagus* OTU1135 (3.1%).

In total, 1952 fungal OTUs were found to be associated with *Burmannia* plants, and around a half of them were associated with more than one species of *Burmannia*; however, none were shared by all *Burmannia* species (Figure S6). The autotrophic *B. disticha* and the partially mycoheterotrophic *B. filamentosa* had the highest number of shared OTUs, and both shared many OTUs with the full mycoheterotroph *B. itoana*. Moreover, *B. filamentosa* also shared a significant number of OTUs with fully mycoheterotrophic species *B. nepalensis*, *B. decurrens* and *B. oblonga*. *B. itoana* notably shared only a few OTUs with any of *B. nepalensis*, *B. decurrens* or *B. oblonga* (Figure S6), which were from the other lineage we sampled.

Among the 2615 OTUs identified from plants co-occurring with *Burmannia* species, 16 were commonly shared by all the plant hosts, while most OTUs were associated exclusively with co-occurring plants of individual *Burmannia* species (Figure 2a). There were also many OTUs shared by co-occurring plants from different vegetation types: for example, 121 were uniquely shared by plants co-occurring with *B. disticha* from grasslands and *B. itoana* under dense forests, of which the former are mainly herbs and the later mainly trees.

The majority (58.7%) of fungal OTUs were shared by root samples from both grassland and forests, although the sequence abundance varied considerably. When taking into account the fungal abundance, most OTUs showed significantly different habitat preference, and only 10 OTUs were evenly distributed across all habitats (Figure 2b). Moreover, there were 481 grassland-specific OTUs which were never observed from the root samples from forests, while 674 OTUs were only identified in the forest samples.

3.3 | Diversity of AMF in plant groups

Each *Burmannia* species varied in fungal α-diversity (richness and evenness), but in general, mycoheterotrophic species had similar α-diversities with green-leaved species (Wilcoxon tests, $p > 0.05$), except that *B. itoana* had significantly lower α-diversity than others

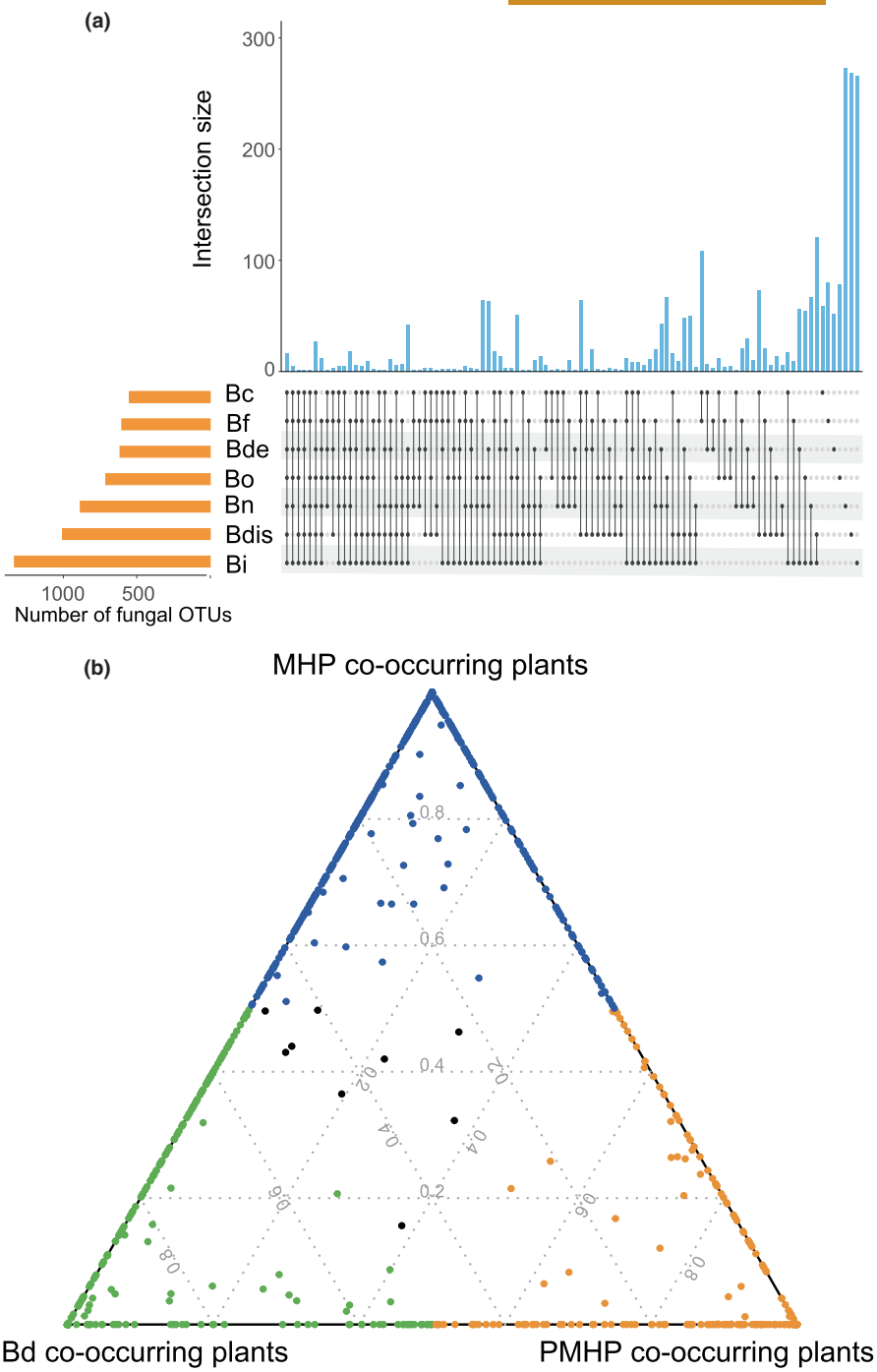

FIGURE 2 The distribution of arbuscular mycorrhizal fungal (AMF) OTUs. (a) Shared and unique AMF OTUs in sampled co-occurring plants of *Burmannia* species. Bc: *B. chinensis*, Bd: *B. disticha*, Bf: *B. filamentosa*, Bi: *B. itoana*, Bde: *B. decurrens*, Bo: *B. oblonga*, Bn: *B. nepalensis*. (b) Distribution of fungal OTUs in different habitats. Green, orange and blue dots indicate the distribution of fungal OTUs in co-occurring plant groups of *B. disticha* (grassland), partially MHPs (grassland), and MHPs (forest) with total relative abundance >50%, respectively, and black dots indicate fungal OTUs of which the total relative abundance lower than 50% in each of the three plant groups. Bd: *B. disticha*, PMHP: partially mycoheterotrophic plant, MHP: mycoheterotrophic plant.

(Figure S7). In contrast, MHPs had lower within-species β-diversity (dissimilarity of fungal composition, quantified by UniFrac distances) than green-leaved species based on either weighted UniFrac distances or unweighted UniFrac distances (Figure S7, Table S5 for PERMANOVA tests). The green-leaved species *B. chinensis* had lower β-diversity than some achlorophyllous species (e.g. *B. oblonga*), although this exception was likely caused by the limited plot and/or sampling size of *B. chinensis*.

To determine whether the variation of fungal diversities in *Burmannia* species were caused by the local fungal communities, we investigated the fungal diversity in the co-occurring plants of each *Burmannia* population. We found that the overall α-diversities were higher in plants co-occurring with MHPs than in those of autotrophic and partially MHP species (Wilcoxon tests, $p<0.05$; Figure 3a,b). All the paired comparisons of differences on the α-diversities between the plants co-occurring with green-leaved *Burmannia* were not significant (Wilcoxon tests, $p>0.05$; Figure 3a,b). Similarly, most comparisons between plant groups co-occurring with fully mycoheterotrophic species were not significant either. In contrast, plants co-occurring with fully mycoheterotrophic species had significantly lower β-diversities than those of green-leaved *Burmannia* species (PERMANOVA tests, $p<0.05$; Table S6) based on either unweighted or weighted UniFrac distances (Figure 3c,d), except that of the plants co-occurring with *B. chinensis*, which had lower unweighted UniFrac distances than those

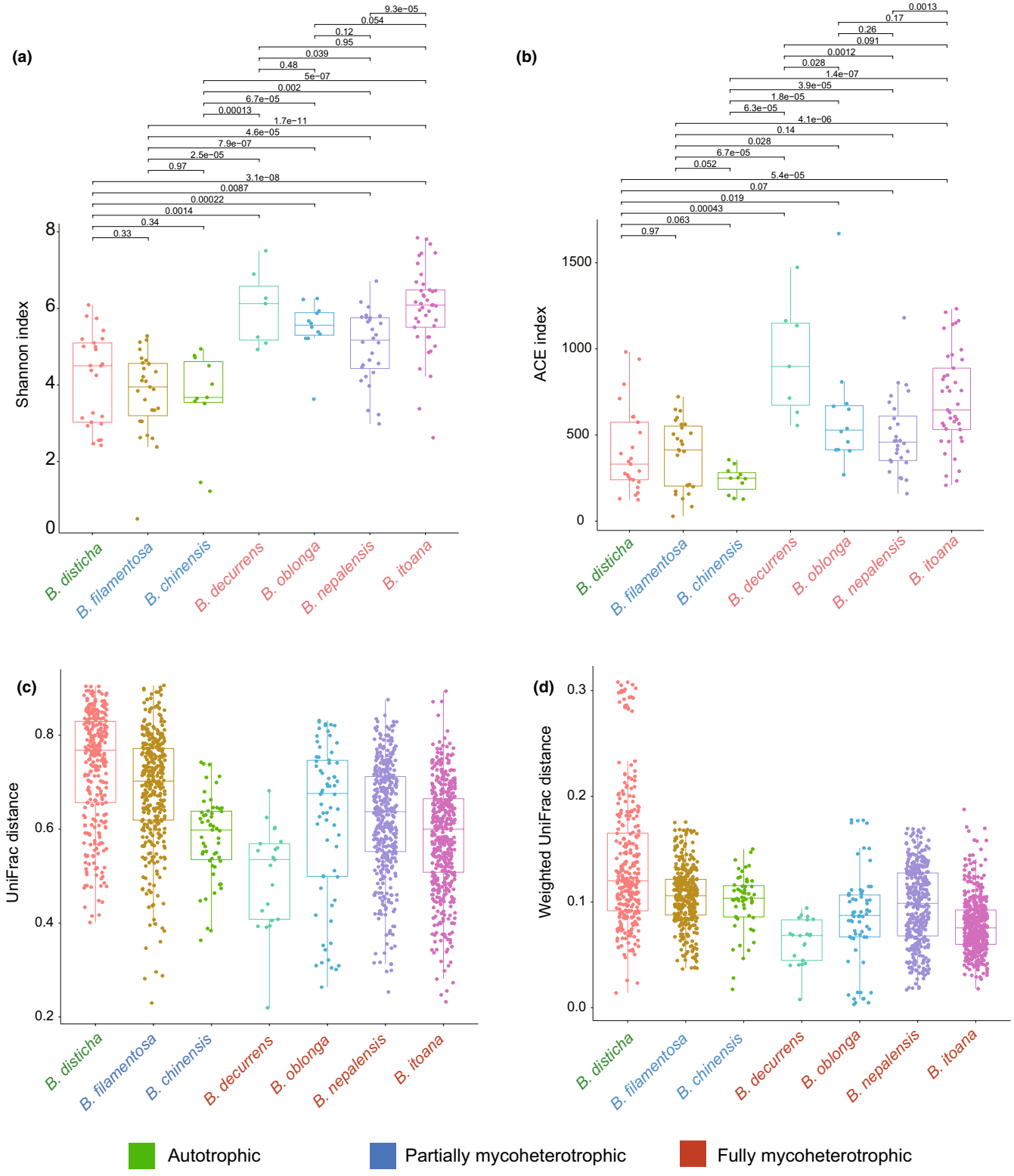

FIGURE 3 Diversities of fungal communities in co-occurring plants of each *Burmannia* species. (a) Shannon index. (b) ACE index. *p* values for each paired comparison were obtained from Wilcoxon tests. (c) Unweighted UniFrac distances. (d) Weighted UniFrac distances using fungal abundances.

of *B. oblonga*, *B. itoana* and *B. nepalensis*—a result that may have been caused by the extremely small size of the local plant community associated with *B. chinensis*. Moreover, NMDS analysis supported this observation and the results showed that the fungal communities can be well separated based on either unweighted (stress = 0.21) or weighted UniFrac distances (stress = 0.05; Figure S8).

To assess whether the transitions of nutritional modes affected the change of fungal specificity, we compared β-diversities between

conspecific mycoheterotrophic samples of *Burmannia* species and their co-occurring autotrophic samples from all the sampled sites (Figure 4a). Results showed that the autotrophic *B. distich* has similar β-diversity to its co-occurring plants ($R^2=0.021$, $p=0.47$), while the partially mycoheterotrophic *B. filamentosa* has lower β-diversity than its co-occurring plants ($R^2=0.068$, $p<0.05$; Figure 4b), and all the fully mycoheterotrophic species showed much lower β-diversities than their co-occurring plants ($R^2>0.2$, $p<0.01$; Figure 4b), suggesting that the transition of nutritional modes may bring in the elevation of fungal specificity.

3.4 | Tripartite relationships of AMF, MHPs and co-occurring plants

Spearman correlation coefficient analysis showed that all the *Burmannia* species had significantly positive relationships with most of their own co-occurring plants, and none showed more significant correlations with specific co-occurring plants (Figure 5a), for example, dominant or tree species (listed in Figure 1b) within the same community (Figure S9).

In general, the relationships among autotrophic plants were well organized by local communities, sharing fungal assemblage with their co-occurring *Burmannia* species, and most had weak or negative relationships with plants from other communities (Figure 5b). However, it is noteworthy that there were also positive relationships between different communities: for example, many plants co-occurring with *B. disticha* had weak positive relationships with those of *B. itoana*, and similarly between the plants co-occurring with *B. filamentosa* and *B. nepalensis* (Figure 5b,c), indicating that they shared some highly abundant fungal OTUs.

3.5 | Fungal phylogenetic community structure of vegetation types

Within *Burmannia*, AMF associated with MHPs showed closer phylogenetic relationships than those associated with green-leaved species

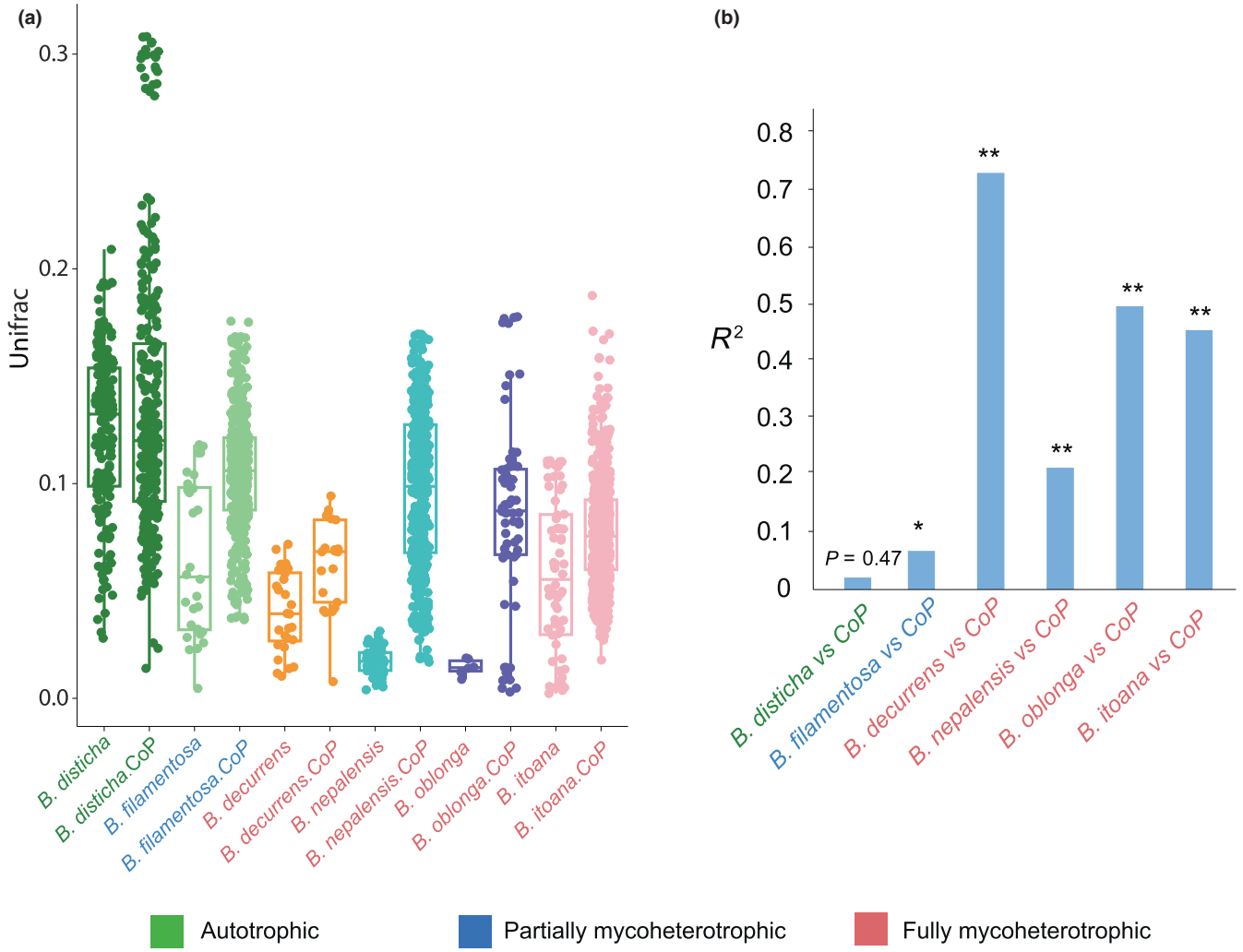

FIGURE 4 Fungal specificities based on beta-diversities in *Burmannia* and their autotrophic co-occurring plants. (a) Weighted UniFrac distances in *Burmannia* and their autotrophic co-occurring plants. CoP: Co-occurring plants. (b) PERMANOVA tests for the degree of fungal compositional difference between pairs of *Burmannia* species and their co-occurring plants. $^*p<0.05$, $^{**}p<0.01$.

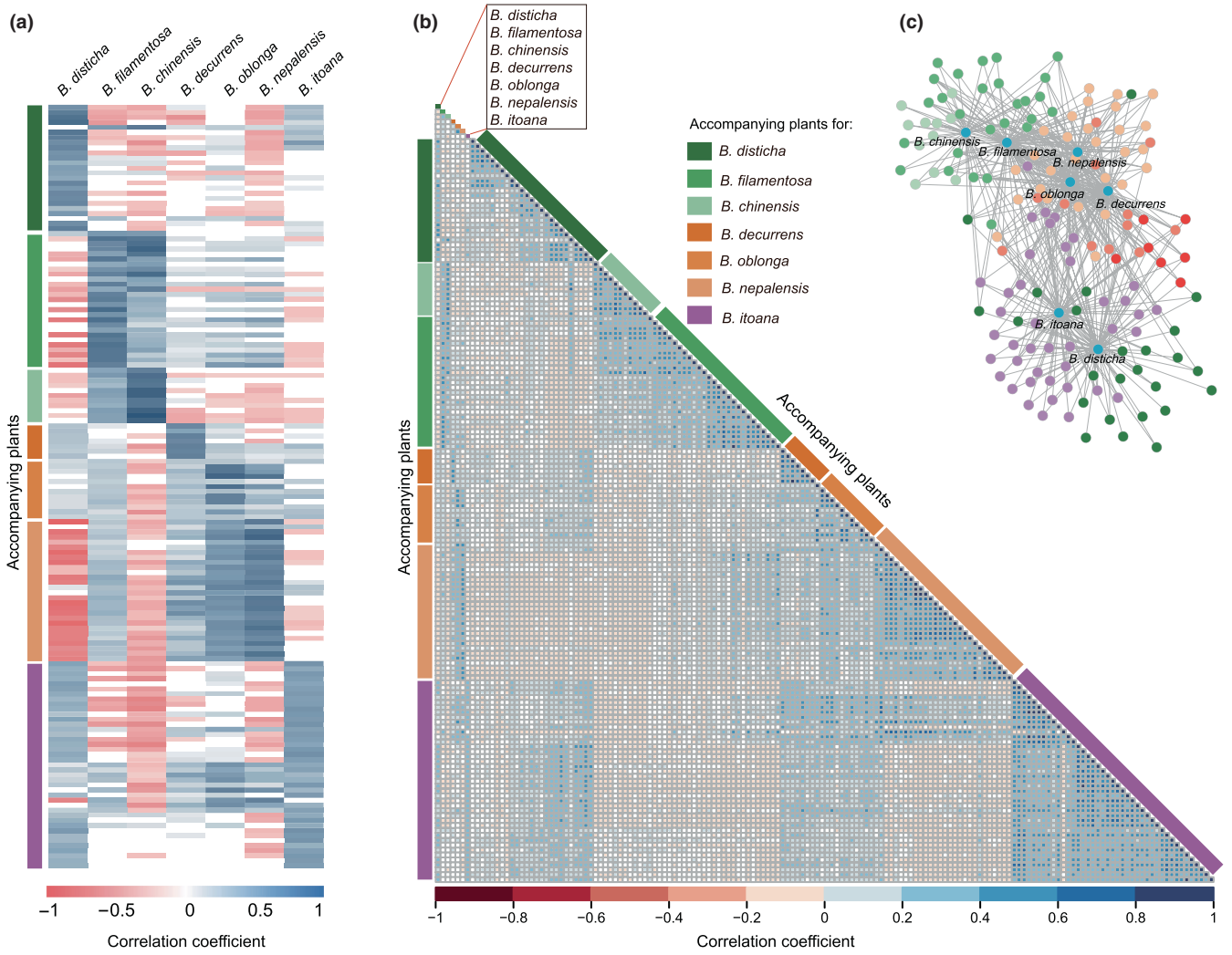

FIGURE 5 Correlations between *Burmannia* species and autotrophic co-occurring plants. (a) Spearman correlation coefficients between *Burmannia* species and co-occurring plants. The correlation strength is indicated by the scale colour of the filled circles. (b) Putative relationships between *Burmannia* species and co-occurring plants based on Spearman correlation coefficients at 95% confidence level. The box was left null if the correlation is not significant. (c) Network of all significant co-occurrences for *Burmannia* species and their co-occurring plants.

(Figure 6). Consistent with that observation, all the MHPs had negative SES_{MPD} values, which were generally lower than those of green-leaved species (Figure 7a), suggesting increased fungal phylogenetic clustering in MHPs than in green-leaved species. The AMF in plants co-occurring with MHPs also showed concentrated phylogenetic distributions; in contrast, AMF in plants co-occurring with green-leaved *Burmannia* species were highly dispersed across the phylogenetic tree (Figure 6). The plants co-occurring with MHPs, most of which are trees from subtropical forests, generally had much lower SES_{MPD} values than those from grasslands (Figure 7b), indicating a significant phylogenetic clustering of fungal communities. Moreover, we found that the SES_{MPD} values of *Burmannia* species showed positive correlation coefficient with that of co-occurring autotrophic plants (Figure 7c) and were positively affected by that of co-occurring plants (GLMM coefficient estimates: estimate = 0.68, Std. Error = 0.16, z value = 4.2, $p < 0.01$) but not nutritional mode (estimate = 0.52, Std. Error = 0.42, z value = 1.24, $p > 0.05$), however, the insignificant effect of nutritional mode may be misled by the low SES_{MPD} values caused by the too-small sampling size of a partially mycoheterotrophic species, *B. chinensis* and its accompanying green species.

To further investigate the overall interaction patterns between co-occurring plants and AMF, we performed UPGMA clustering analysis based on UniFrac distances. The results showed that most samples from co-occurring plants of one *Burmannia* species were specifically clustered in the UPGMA tree, although some samples were discrete (Figure 7d). Samples from autotrophic plants (trees) accompanied with MHPs are prone to be more aggregated than those (most of which are herbs) accompanied with green-leaved *Burmannia* species. Furthermore, samples of one species were clustered with those collected from the same sampling site rather than those of the same species: for example, three samples (14, 190 and 234) of the species *Cinnamomum parthenoxylon* (Lauraceae), were respectively collected from three sites, *B. itoana* CHD, *B. itoana* NX and *B. nepalensis* CHD, and each of them was clustered with the samples from

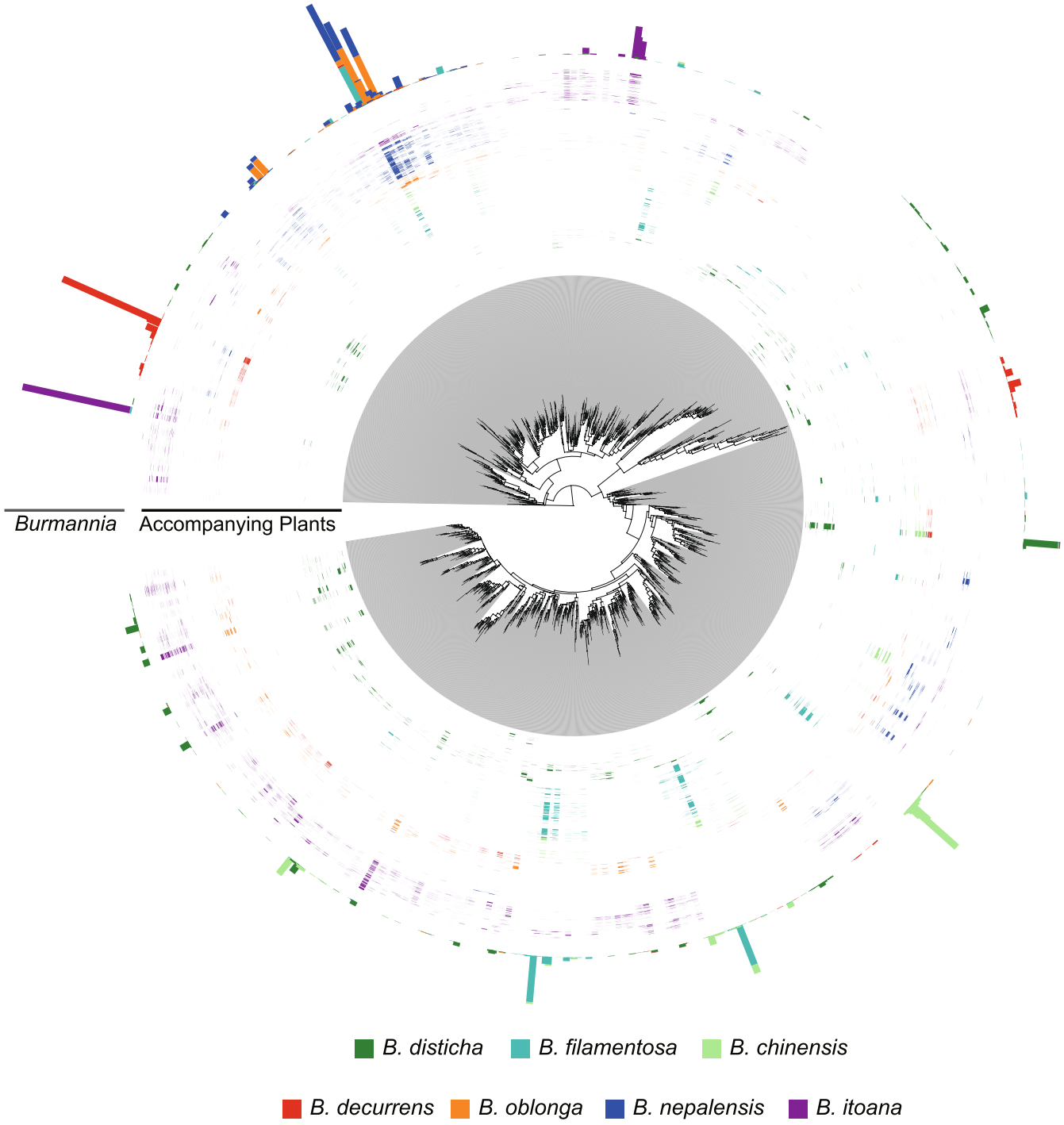

FIGURE 6 Phylogenetic distributions of arbuscular mycorrhizal fungi in *Burmannia* and their co-occurring plants. The phylogenetic tree was constructed based on fungal OTUs using Maximum-likelihood approach. Bars in each circle out of the phylogenetic tree indicate the relative abundance of fungal OTUs.

their respective sampling site, rather than aggregated in the UPGMA tree (Figure S10).

4 | DISCUSSION

Most mycoheterotrophs have scattered distributions in small populations. This may be determined by various factors such as soil pH and nitrate levels (Gomes et al., 2019), but a different hypothesis is that they are tightly associated with certain co-occurring autotrophs or dominant tree species in the local environment, which may result in the limited distribution of mycoheterotrophs. However, our results show that different populations of the same mycoheterotrophic species in *Burmannia* could occur in very different habitats with different co-occurring plants, although sometimes they indeed have some common co-occurring plants: for example, both *B. itoana*

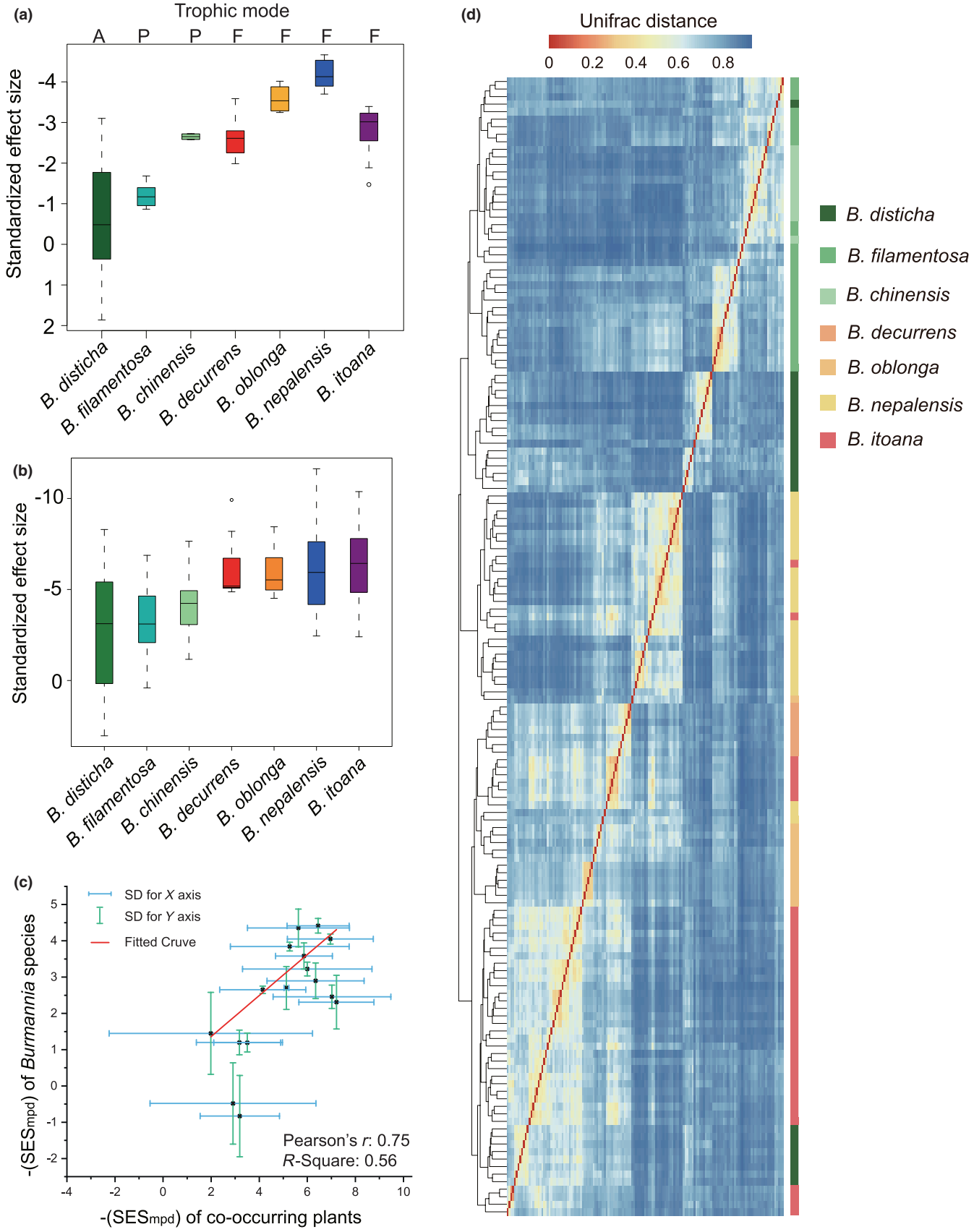

FIGURE 7 Phylogenetic community structure of arbuscular mycorrhizal fungi in *Burmannia* and their co-occurring plants. (a) SES_{MPD} (Standardized effect size for the mean pairwise distance) in *Burmannia* plants. A: Autotrophic; F: Fully mycoheterotrophic; P: Partially mycoheterotrophic. (b) SES_{MPD} in co-occurring plants. (c) Correlationship of SES_{MPD} values between *Burmannia* and co-occurring plants. (d) UPGMA analysis for fungal communities in co-occurring plants of *Burmannia*. Colour strips indicate the UniFrac distances between each paired plant samples and the higher values indicate higher dissimilarities between paired samples.

and *B. nepalensis* grow in either broad-leaved forests or bamboo thickets with distinct co-occurring plants. Moreover, we found that individuals of mycoheterotrophic species can be linked with most of the autotrophic plants in the community through shared AMF. This pattern is due to the fact that most autotrophic plants within a community have similar fungal composition despite variation in the abundance of fungal OTUs. These results suggest that mycoheterotrophic species have no preference on specialized sets of autotrophic plants and can rely on different carbon sources within or between populations. This pattern may be prevalent since several MHP species from different families have been also observed to be indirectly linked to most autotrophic plants through fungi highly connected to autotrophic plants (Gomes et al., 2022). Moreover, a high preference toward specialized fungal assemblage in mycoheterotrophic species has been revealed (Zhao et al., 2021), suggesting that the preference toward similar fungal assemblages in mycoheterotrophs does not require similar composition of co-occurring plants.

The transition in nutritional mode from autotrophy to mycoheterotrophy may prompt the diversification and evolution of mycoheterotrophic plant families, resulting in the adaption of shaded forest for MHPs (Merckx et al., 2008). In several such families, fungal partner changes/specialization accompanying nutritional mode transition in MHPs have been shown, even though some MHPs retain fungal partners similar to those of their autotrophic relatives (Perez-Lamarque et al., 2020; Zhao et al., 2021): fungal partner shifts might therefore be caused by the turnover of symbiotic relationships between MHPs and mycorrhizal fungi. As a consequence, we would expect to observe evolutionary co-diversification between MHPs and fungi; indeed, the delayed co-speciation between MHPs and mycorrhizal fungi has been suggested in *Afrothismia* (Merckx & Bidartondo, 2008). However, the co-evolutionary trajectory between MHPs and fungi along the transition of nutritional mode has never been reported, and whether the shift of fungal partners occurred before or after diversification of nutritional modes in plants remains unknown. In *Burmannia*, apparent co-phylogenetic relationship between fungal partners and *Burmannia* species including autotrophs, partial mycoheterotrophs and full mycoheterotrophs was not detected (Zhao et al., 2021). Our results show that grassland is either barren or lacks most mycorrhizal fungi required by MHPs, implying the absence of an adequate selective pressure or evolutionary advantage for the fungal partner shifts in the process of mycoheterotrophy. This supports the previous conjecture that shifts in fungal partners probably occurred after species divergence in *Burmannia* (Zhao et al., 2021).

Although the fungal composition was significantly different among the vegetation types, we found that there were still dozens of fungal OTUs shared by different habitats. More interestingly, some of mycorrhizal fungi shared by grassland and forest habitats were associated with both chlorophyllous and mycoheterotrophic *Burmannia* species: for example, OTU986 was shared by chlorophyllous *B. filamentosa* and *B. nepalensis* with high abundance, and was also identified from the roots of achlorophyllous *B. oblonga* and *B. decurrens*. This pattern was also observed in other plant lineages such as *Pyrola* (Suetsugu et al., 2021), Polygalaceae (Perez-Lamarque et al., 2020) and orchid lineages *Cremastra* and *Cymbidium* (Ogura-Tsujita et al., 2012; Suetsugu, Haraguchi, et al., 2022). We therefore hypothesize that while the distinct fungal compositions between grassland and forest habitats enforced the fungal shifts in MHPs, the common mycorrhizal fungi shared across different habitats may provide the basis for the primary colonization of MHPs to shaded forests, whereupon MHPs recruited their own fungal partners locally to achieve intense partner shifts.

MHPs are thought to be more specific toward fungi than autotrophic plants, hosting only subset of the fungi in autotrophic plants (Bidartondo et al., 2002; Gomes et al., 2017), shaped by the plant trophic mode (Gomes et al., 2017). We found that most MHPs had similar fungal α-diversities to their autotrophic relatives, with *B. itoana* as the only exception, as it had quite low fungal α-diversity. This trend is not only observed in *Burmannia* but also in *Pyrola aphylla* (Ericaceae) which associate with a range of root-inhabiting mycorrhizal fungi (Hynson & Bruns, 2009), implying that the transition from autotrophy to full mycoheterotrophy does not always result in a reduction of fungal partners at fungal species (OTU) level. Since that MHPs may have lost more partners out of Glomeraceae than their autotrophic relatives (Zhao et al., 2021), this conclusion might be biased due to the different genetic variability level among Glomeromycotina lineages. However, MHPs in *Burmannia* did have lower fungal beta-diversity than their autotrophic relatives, which is in consistent with the previous study of Zhao et al. (2021). We found that, in contrast with their autotrophic relatives, MHPs tend to associate with fungal taxa with closer phylogenetic relationships when fungal shifts occurred, supporting the phylogenetic constraints of fungal partners (Merckx et al., 2012; Perez-Lamarque et al., 2020). Our results thus support that the transition in nutritional modes may contribute to fungal specialization in *Burmannia*. However, the fungal specificity varies dramatically among different mycoheterotrophic species, suggesting that other factors may also contribute to the fungal specialization in MHPs. The ecological and evolutionary mechanisms underlying this pattern of phylogenetic constraints in fungal partners targeted by MHPs, however, have been largely unknown. It has been shown that in some MHPs of *Burmannia*, the increased fungal specificity may result from the gradual loss of non-Glomeraceae fungal partners (Zhao et al., 2021). Here we show that woody plants from subtropical forests investigated in this study also showed an increased preference toward Glomeraceae, which may make the associated mycorrhizal fungi of higher fungal richness but lower beta-diversity than those green plants (most of which are herbs) from grasslands. Our studies thus support the previous observations that vegetation of forests is usually richer in fungi than grasslands (Öpik et al., 2006; Rodriguez-Echeverria et al., 2017). We further reveal that within vegetation of forests both *Burmannia* species and their co-occurring autotrophic species were associated with fungal partners with lower beta-diversities and SES_{MPD} values, suggesting their preference for more closely related fungi with narrower phylogenetic distances, resulting in the specificity toward fungi in host plants. Although the comprehensive comparison of beta-diversity between

vegetation types of grasslands and forests have been poorly studied, some earlier reports indicated that grassland plants tend to have distinct fungal communities (Davison et al., 2020; Reinhart & Anacker, 2014; Valyi et al., 2015; Vandenkoornhuyse et al., 2003), and lower beta-diversities were observed during ecosystem development from open turf vegetation and shrub-land to the tall forest communities (Martinez-Garcia et al., 2015).

UPGMA analysis showed that most plant hosts formed site-specific groups, and samples from the same plant species but different communities did not cluster together (Figure S10), suggesting that plant hosts have low fungal specificity at the species level, supporting previous studies that host phylogeny at low taxonomic level has only a weak effect on the community composition of AMF (Wang et al., 2019). Our results also show that most plant hosts within a community tend to associate with the same or similar (with low UniFrac distances) fungi, which in turn result in the homogenization of local fungal communities in each plant host. This tendency suggests that plant hosts within a community may have localized fungal partner specificity or preference, which in turn structures the localized fungal composition. Indeed, some studies have suggested that plant communities as whole likely dominate the composition and diversity of AMF in the local environment (Martinez-Garcia et al., 2015; Yang et al., 2012), albeit the composition and diversity of AMF could also be influenced by various biotic and abiotic environmental factors such as soil properties (Guo & Gong, 2014; Hazard et al., 2013; Valyi et al., 2016; Yang et al., 2012). Considering that the root samples for the forest trees may represent only a subset of their fungal associations, and some tree species at the study sites were missed in our sampling, cautions are needed to conclude the extent of the fungal partner specificity in autotrophic plants of subtropical forests due to the constraints of plant sampling in this study. However, the large proportion of overlapped fungal OTUs between MHPs and their co-occurring plants and also among co-occurring autotrophic plants within sites suggested that the sampled root fungal communities likely well characterized the fungal preference of forests and grasslands in spots where *Burmannia* occur. Taken together, our results suggest that the autotrophic plants within a community co-occurring with mycoheterotrophic *Burmannia* species likely have localized fungal partner preference, forming the AMF network to provide the basis for the fungal specialization in fully mycoheterotrophic plants. It is worthy to note that the fungal partners of MHPs are a subset of fungal communities in co-occurring plants, which may be selected by plant hosts because of their possible effectiveness for nutrient uptake of MHPs (Zhao et al., 2021). However, the mechanistic mechanisms on how the fungal partner changes and/or specialization prompt the nutrient uptake of MHPs are still unclear, and further studies are needed to address the functions of fungal partners involved in the nutritional mode changes of MHPs.

Some MHPs interact with only one or a few fungal species (Guo et al., 2019; Merckx et al., 2017; Suetsugu, Okada, et al., 2022), showing high specificity toward mycorrhizal fungi. Moreover, some MHPs, for example, *Pyrola subaphylla* (Suetsugu et al., 2021), have higher specificity toward mycorrhizal fungi than their autotrophic relatives even within a sympatric population. In these MHP lineages, not only habitat shifts but also changes of nutritional mode have prompted the specialization of fungal associations. It may suggest that, distinct evolutionary scenarios of plant-fungi interactions have occurred in different MHP lineages. However, how the nutritional mode changes affect the fungal specialization is still poorly studied. In *Burmannia* mycoheterotrophs, the specificity toward fungal assemblage is stable and more likely defined by interspecific genetic differentiation of plants (Zhao et al., 2021), suggesting that there are genetic traits governing the association patterns with fungal partners. It has been known that plants percept molecular signals from mycorrhizal fungi and then establish symbiotic relationships with fungi through a series of gene cascades, for example, LRR and LysM receptor-like kinases (Oldroyd, 2013; Parniske, 2008), and transfer of some of these genes could alter the recognition of microbial partners in plant hosts (Radutoiu et al., 2007). Therefore, we suppose that the genomic changes (e.g. evolutionary diversifications of genes, gene losses) related to the fungal perception accompanying the evolution of mycoheterotrophy may have substantially determined the changes/specialization of fungal associations in MHPs, however, further works such as genome sequencing and comparative genomic analysis are needed to provide valuable information for understanding the molecular mechanisms underlying the rise to fungal specificity in MHPs.

AUTHOR CONTRIBUTIONS

Zhongtao Zhao and Dianxiang Zhang conceived and designed the study, Vincent S. F. T. Merckx and Richard M. K. Saunders contribute to the generation of scientific questions driving this study. Zhongtao Zhao, Tieyao Tu and Yu Zhang performed plant sampling, data collection and bioinformatic analyses. Ruifan Meng, Li Fan and Vincent S. F. T. Merckx participated in the data analysis. Zhongtao Zhao, Dianxiang Zhang and Richard M. K. Saunders led the writing of this manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The raw data underlying this article are available in the Short Read Archive (SRA) at https://www.ncbi.nlm.nih.gov/ and can be accessed with the project no. PRJNA1100211. The processed data are available from the figshare repository: https://doi.org/10.6084/m9.figshare.27625965.v1 (Zhao et al., 2024).

STATEMENT ON INCLUSION

Our study brings together authors from a number of different countries, including scientists based in the country where the study was carried out. All authors were engaged early on with the research and study design to ensure that the diverse sets of perspectives they represent was considered from the onset. Whenever relevant, literature published by scientists from the region was cited; efforts were made to consider relevant work published in the local language.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. Flowchart of data processing in this study.

Figure S2. Accumulation curves for the sequencing of fungal communities in plant root samples.

Figure S3. OTU accumulation for number of samples in each population of *Burmannia* species and their co-occurring plants.

Figure S4. Numbers of shared and unique co-occurring plants among *Burmannia* species.

Figure S5. Fungal composition at family level for *Burmannia* co-occurring plants.

Figure S6. Shared and unique arbuscular mycorrhizal fungal OTUs among sampled *Burmannia* species.

Figure S7. Diversities of fungal communities within *Burmannia* species.

Figure S8. Nonmetric multidimensional scaling (NMDS) analysis for fungal communities.

Figure S9. Correlations between *Burmannia* species and autotrophic co-occurring plants.

Figure S10. UPGMA analysis for fungal communities in co-occurring plants of *Burmannia*.

Table S1. Summary of sample collection for *Burmannia* species and the co-occurring plants (CoPs).

Table S2. Primers used in PCR.

Table S3. Co-occurring autotrophic plants of *Burmannia* species in each population.

Table S4. Sørensen dissimilarity indices between plant communities co-occurring with each *Burmannia* population.

Table S5. PERMANOVA tests for the degree of fungal compositional difference between pairs of *Burmannia* species.

Table S6. PERMANOVA tests for the degree of fungal compositional difference between pairs of co-occurring plant groups of *Burmannia* species.

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