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ORIGINAL PAPER

Specificity and preference of mycorrhizal associations in two species of the genus *Dendrobium* (Orchidaceae)

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Abstract *Dendrobium* is a large genus of tropical epiphytic orchids. Some members of this genus are in danger of extinction across China. To investigate orchid mycorrhizal associations of the genus *Dendrobium*, plants from two *Dendrobium* species (*Dendrobium officinale* and *Dendrobium fimbriatum*) were collected from two habitats in Guangxi Province, China, and clone libraries were constructed to identify the mycorrhizal fungi of individual plants. A low and high degree of specificity was observed in *D. officinale* and *D. fimbriatum*, respectively. Phylogenetic analysis revealed that the majority of *Dendrobium* mycorrhizal fungi are members of the Tulasnellaceae, but, in some plants, members of the Ceratobasidiaceae and Pluteaceae were also found. In *D. officinale*, individual plants associated with more than three fungi simultaneously, and, in some cases, associations with five fungi at the same time. One fungus was shared by individual plants of *D. officinale* collected from the two habitats. In *D. fimbriatum*, only one fungal partner was found in each population, and this fungus differed between populations. The two species of *Dendrobium* sampled from the same habitat did not share any fungal taxa. These results provide valuable information for conservation of these orchid species.

Keywords Orchid mycorrhizae · *Dendrobium* · Specificity · Preference

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Introduction

Most orchid species are dependent on mycorrhizal fungi for completion of their life cycle, at least during the early stages of their development (Smith and Read 2008; Rasmussen and Rasmussen 2009). In adult photosynthetic orchids, mycorrhizal associations are often maintained (Cameron et al. 2007). The fungi that form orchid mycorrhizas are usually basidiomycetes (Rasmussen 2002; Dearnaley 2007) and largely belong to the *Rhizoctonia* form genus (Bougoure et al. 2005; Waterman and Bidartondo 2008). This is an artificial grouping of unrelated fungi based on anamorphic life stage and usually includes members of the *Ceratobasidium*, *Sebacina*, and *Tulasnella* genera (Smith and Read 2008).

In general, in order to identify orchid fungal symbionts, fungal strains had to be isolated and described morphologically. However, some mycorrhizal fungi are difficult to culture in vitro, and identification using morphological characteristics is difficult, if not impossible. With the advent of culture-independent molecular tools, however, detection and identification of fungi have become possible, providing new ways to explore orchid mycorrhizal associations. Previous studies have shown that orchids vary in mycorrhizal preferences (Otero et al. 2002, 2004). Mycorrhizal specificity may vary considerably between species, ranging from very narrow specificity in non-photosynthetic and some photosynthetic orchids (Taylor et al. 2003; Barrett et al. 2010) to broad interactions in other photosynthetic orchids (Shefferson et al. 2010; Jacquemyn et al. 2010). It was recently shown that the mycorrhizal network of the genus *Orchis* was highly nested and constrained by the plant phylogeny, but not by the phylogeny of their fungal partners (Jacquemyn et al. 2011). For epiphytic orchids, plant mycorrhiza networks showed weak, but significant phylogenetic effects, both for the plants and the fungi (Martos et al. 2012). Knowledge on how orchid fungi diversity varies between and within habitats is a key aspect for orchid recovery actions,

because symbiotic fungal isolates could be used for symbiotic orchid propagation (Brundrett 2007).

Dendrobium is a large genus of tropical epiphytic orchids. All species in this genus are photosynthetic. Some members of this genus, such as *Dendrobium officinale*, *Dendrobium fimbriatum*, *Dendrobium nobile*, and *Dendrobium chrysanthum* are used as traditional Chinese herb (The State Pharmacopoeia Commission of PR China 2010). Many *Dendrobium* species are in danger of extinction across China due to human-induced habitat loss and the lack of effective protection in the face of increasing commercial demand (Fu 1992). To conserve surviving wild populations and reintroduce plants into declining populations requires a full understanding on the fungal partners of each *Dendrobium* species. Some research associated with the endophytic fungi and mycorrhizal fungi of *Dendrobium* plants has been carried out using culture-dependent methods (Boddington and Dearnaley 2008; Hou and Guo 2009; Chen et al. 2012; Zhang et al. 2012). But no work has been done using more comprehensive, molecular approaches.

For better understanding on the natural mycorrhizal fungal population composition of *Dendrobium* plants, we have collected two *Dendrobium* species (*D. officinale* and *D. fimbriatum*) from different regions. Using culture-independent identification of mycorrhizal fungi, we asked the following questions: (a) Do the two species of *Dendrobium* have identical fungal symbionts? (b) Do the *Dendrobium* species exhibit habitat-dependent preference for mycorrhizas?

Materials and methods

Study species and sampling

Root samples were collected from two different counties of Guangxi Province, Leye (106°34′ E, 24°47′ N) and Xilin (105° 05′ E, 24°31′ N) in September–October of 2011. Both counties have subtropical climate and have high abundance of orchid species. For *D. officinale*, we sampled 12 (Leye) and nine (Xilin) roots from individual plants. For *D. fimbriatum*, we sampled five (Leye) and ten (Xilin) roots from individual plants. For each plant, we collected five root fragments (2 cm) whenever possible without dislodging the plant. Sampled roots were surface-sterilized with ethanol (70 %) for 30 s and rinsed three times in sterile water to avoid unnecessary contaminates from the velamen of the roots and surface of root epidermis. Then the root fragments were checked for the presence of orchid mycorrhizae, that is, intracellular hyphal pelotons (Rasmussen 1995). A 5-mm-long root section harboring pelotons was sampled for each root fragment, that is, five root sections per plant, and stored in −20 °C for DNA extraction.

DNA extraction, PCR, clone library, sequencing

The root samples were first surface-sterilized by dipping in 75 % ethanol for 1 min, followed by a solution of Chlorox (6 %) for 2 min, and finally 75 % ethanol for 30 s. Sterilized root fragments were washed with sterile distilled water three times and blotted with bibulous paper. The root samples were ground with a mortar and pestle in liquid nitrogen. Genomic DNA was extracted using the E.Z.N.A. ^{TM}Fungal DNA kit (Omega) following the manufacturer's instructions.

Clone libraries were constructed following PCR amplification with the broad-spectrum basidiomycete primers internal transcribed spacer (ITS)1-OF and ITS4-OF (Taylor and McCormick 2008). This primer pair has the advantage that it does not exclude *Tulasnella* species and thus should give an accurate view of orchid associations within the Basidiomycota, representing the vast majority of the mycorrhizae found on orchid species (Taylor and McCormick 2008). In the preliminary phase of this study, the effectiveness of several primer pairs, including ITS1/ITS4-OF, ITS1-OF/ITS, and ITS1-OF/ITS4-OF, was evaluated to characterize the mycorrhizal community on the two *Dendrobium* species. ITS1-OF and ITS4-OF were most efficient and gave the most consistent amplification. PCR amplification for ITS region began with denaturation at 94 °C for 3 min, followed by 32 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, and an elongation at 72 °C for 55 s. The final cycle was followed by a 7-min extension at 72 °C. ITS regions of all samples were successfully amplified using the two primer sets. The ITS-based clone libraries were constructed for each root samples by the following proceedings: the PCR products were purified using the QiAquick PCR purification kit (Qiagen) and cloned using the pGEM-T vector (TaKaRa, Japan) and *Escherichia coli* DH5α. Ninety-six clones were randomly picked from each library and sequenced using the M13 forward primer. Sequencing reactions were performed on an ABI-310 capillary sequencer using an ABI dye-terminator kit (ABI/Perkin-Elmer) following the protocol supplied by the manufacturer. DNA sequences from the complete data set were aligned using the MEGA5 software (Tamura et al. 2011) followed by manual editing. A threshold of 97 % similarity between ITS sequences was applied to circumscribe operational taxonomic units (OTUs) among the mycorrhizal taxa, which is the usual proxy for species delimitation among basidiomycetes (Jacquemyn et al. 2011; Martos et al. 2012). To identify the different OTUs, representative sequences for each OTU were queried against GenBank by using BLAST. In the clone library analyses, 96 clones were randomly selected and sequenced, which may lead to underestimation of fungal diversity as rare fungi may be overlooked. To assess the magnitude of this effect, total species diversity as well as completeness of the sampling was assessed using rarefaction analyses (Hurlbert 1971).

Data analysis

To establish the genetic relationships between these OTUs that associated with members of the Tulasnellaceae family, a phylogenetic analysis was performed using two randomly selected sequences from each of the OTUs together with their closest related sequence found in GenBank plus additional reference sequences from known *Tulasnella* species. Sequences were aligned using Clustal X version 2.0 (Larkin et al. 2007) with minor manual adjustments. A neighbor-joining tree was constructed using MEGA version 5 (Tamura et al. 2011). The tree was rooted with sequences of the known *Thelephora* (Thelephorales, Agaricomycetes) species. For each orchid species, both the total number of OTUs across all sampled populations and the minimum, maximum, and average number of fungal OTUs detected within a single population were calculated.

Results

In all plants investigated, characteristics of orchid mycorrhizal colonization were observed. In total, over 900 high-quality ITS sequences with sequence lengths that varied between 698 and 753 bp were obtained. Most of the obtained sequences corresponded to basidiomycete sequences. Based on a 97 % cutoff value of similarity between sequences, ten different basidiomycete OTUs were distinguished (Table 1). Rarefaction analysis showed that the curve quickly reached an asymptote for the analyzed sequences (Fig. 1). Based on BLAST analysis, eight OTUs (OTU 1–8) were associated with members of the Tulasnellaceae family. Additionally, two OTUs, OTU 9 and 10, were related to the family Pluteaceae and Ceratobasidiaceae, respectively. For four OTUs (OTU 1, 5, 6, and 10), 99 % DNA sequence identity was found with a GenBank sequence (Table 1).

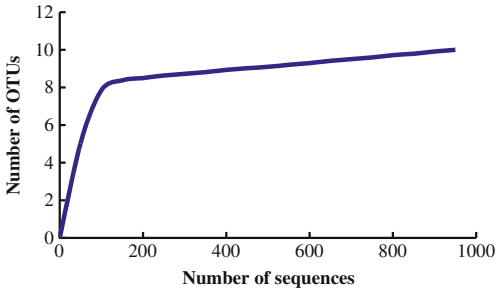

Fig. 1 Rarefaction analysis performed on the internal transcribed spacer sequence data obtained from the clone libraries for two *Dendrobium* species (962 sequences), using a 97 % sequence similarity threshold value

The phylogenetic tree is shown in Fig. 2, displaying the eight OTUs as well-supported clades (bootstrap values between 53 and 100 %). The tree constructed from ITS sequences of mycorrhizal fungi of *D. officinale* and *D. fimbriatum* clustered into four clades (I–IV). Clade I is the major clade which include four OTUs (OTU 1–4). Clade II includes two OTUs (OTU 5–6). While clades III and IV had only one OTU each, OTU 8 (clade III) and OTU 7 (clade IV). Mycorrhizal fungi of *D. officinale* were found to be associated with all clades (clade I–IV), and *D. fimbriatum* were clustered into II clades (clades I and II). The representative sequences for each OTU were deposited in GenBank (accession numbers JX545212-JX545228).

In total, five (OTU 2, 3, 5, 7, and 9) and four (OTU 4, 5, 8, and 10) fungal OTUs were found in the *D. officinale* collected from Leye and Xilin, respectively (Fig. 3). The number of OTUs observed within a single individual varied between three and five for Leye and between three and four for Xilin (Table 2). Among these OTUs, only OTU 5 was found in *D. officinale* collected from both sites. For *D. fimbriatum*, however, only one OTU was found in both populations (Fig. 3). The myocrrhizal fungi exhibit high host specificity toward *D. fimbriatum*. The dominant fungal species were quite different

Table 1 Phylogenetic affiliation of operational taxonomic units (OTUs) in the phylum of Basidiomycota

		Phylogenetic relationship		
OTU	Sequence length (bp)	Family	Closest match in GenBank (accession no.)	Sequence identity (%)
OTU 1	706	Tulasnellaceae	Uncultured Tulasnellaceae PD563 (GQ241838)	99
OTU 2	715	Tulasnellaceae	Uncultured Tulasnellaceae isolate P213 (DQ925494)	83
OTU 3	710	Tulasnellaceae	Uncultured Tulasnellaceae PD559 (GQ241836)	88
OTU 4	704	Tulasnellaceae	Uncultured Tulasnellaceae PA274 (FJ786674)	86
OTU 5	707	Tulasnellaceae	*Epulorhiza* sp. Nq (AJ313446)	99
OTU 6	708	Tulasnellaceae	*Tulasnella calospora* isolate Da-KP-0-1 (GU166410)	99
OTU 7	698	Tulasnellaceae	Tulasnellaceae sp. Pv-QS-0-1 (GU166411)	85
OTU 8	753	Tulasnellaceae	*Epulorhiza* sp. MO 043 (AB369928)	86
OTU 9	765	Pluteaceae	*Pluteus seticeps* vorcher Shaffer 798 (HM562199)	90
OTU 10	725	Ceratobasidiaceae	*Ceratobasidium* sp. AG-G isolate Str14 (DQ102402)	99

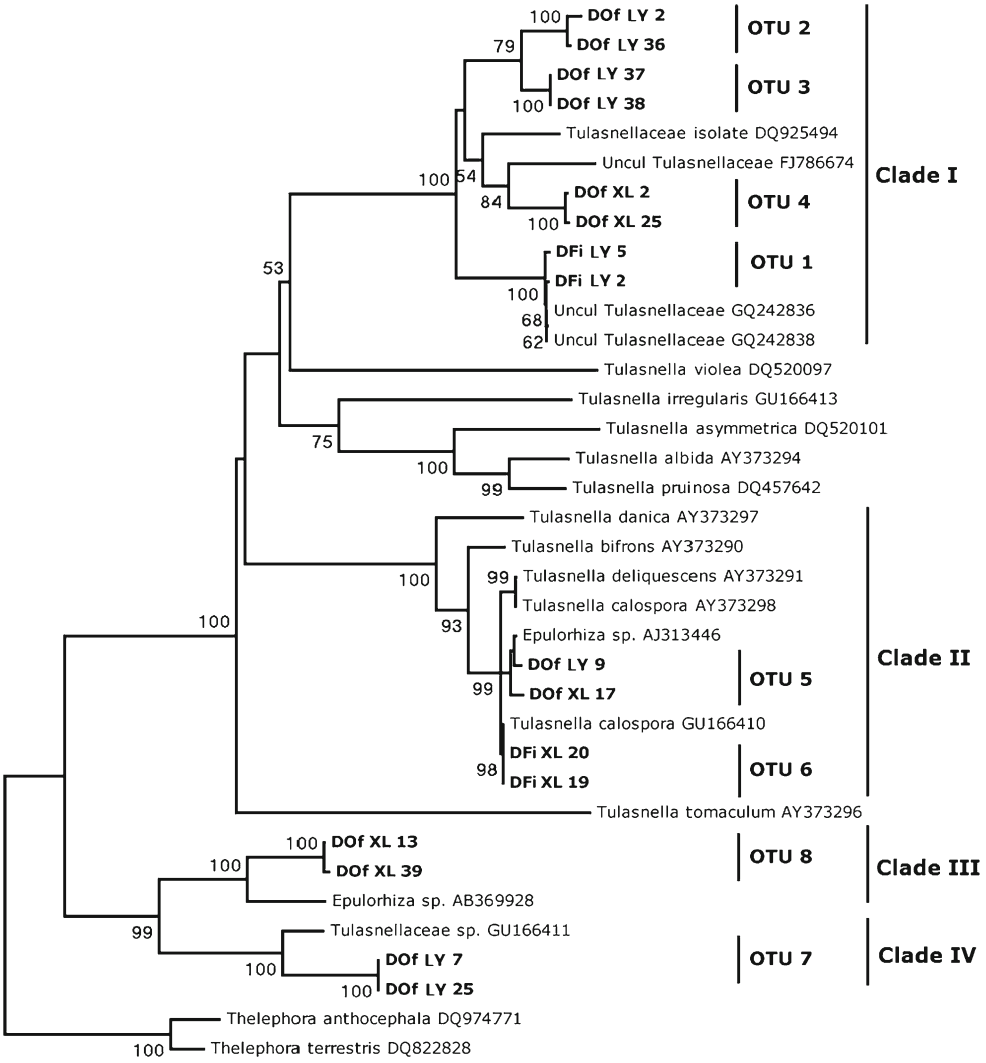

Fig. 2 Internal transcribed spacer (ITS) phylogeny of the *D. officinale* and *D. fimbriatum* mycobionts associated with the Tulasnellaceae family (OTU 1–8). For each OTU, two representative sequences were, together with their closest GenBank match and other reference sequences from know *Tulasnella* species found in GenBank, used to construct the phylogenetic tree. The tree was rooted with sequences of two *Thelephora* (Thelephorales, Agaricomycetes) species. Sequences are annotated by the sample name (DOf=*D. officinale*, DFi=*D. fimbriatum*), the collecting site (LY=Leye, XL=Xilin), and the number of the clone. Sequences downloaded from GenBank are shown with accession numbers. Bootstrap ≥50 % are shown

in the two *Dendrobium* species. For *D. officinale* samples from Leye and Xilin, the dominant mycorrhizal fungi were OTU 2 (50 %) and OTU5 (36.3 %). For *D. fimbriatum*, the dominant mycorrhizal fungi were OTU 1 (100 %) and OTU 6 (100 %). The differences in dominant fungi occurred despite the fact that the orchids co-occurred in the same habitat.

Discussion

Mycorrhizal fungi associate with *Dendrobium* plants

Researches on the mycorrhizal fungi associated with some species of *Dendrobium* have been carried out (reviewed by Rasmussen 2002; Dearnaley 2007; Liu et al. 2010), such as *D. nobile* (*Rhizoctonia* sp., *Mycena orchidicola*), *Dendrobium sinense* (*Mycena orchidicola*), *D. officinale* (*Mycena dendrobii*, *Mycena* sp., *Epulorhiza* sp.), *Dendrobium hancockii* (*Epulorhiza anaticula*), *Dendrobium crumenatum* (Tulasnellaceae sp.), *Dendrobium speciosum*, *Dendrobium dicuphum* (*Tulasnella irregularis*), and *Dendrobium* sp. (*Tulasnella pinocola*, *T. deliquescens*). Based on the literature, most of the identified mycorrhizal fungi associated with *D. officinale* belong to one genus *Mycena* (Guo et al. 1999; Zhang et al. 2012). To date, the mycobionts of *D. fimbriatum* have never been reported. Here, we investigated the orchid mycorrhizal associations in adult *Dendrobium* plants, *D. officinale* and *D. fimbriatum* (36 individual plants) sampled at two different habitats in Guangxi Province, China; they were used for DNA-based analysis of the root-inhabiting mycorrhizal community. Contrary to the literature, our results indicated that *D. officinale* associated with a wide range of basidiomycetous mycorrhizal partners, with a total of eight different OTUs identified in total and only one OTU (OTU 5) was shared by *D. officinale* at both collecting sites.

Most fungal OTUs found in our study were affiliated with the family Tulasnellaceae, species of which have been described as mycorrhizal symbionts in many other orchids (Kristiansen et al. 2004; McCormick et al. 2004; Shefferson et al. 2008; Yuan et al. 2010; Jacquemyn et al. 2010, 2012b).

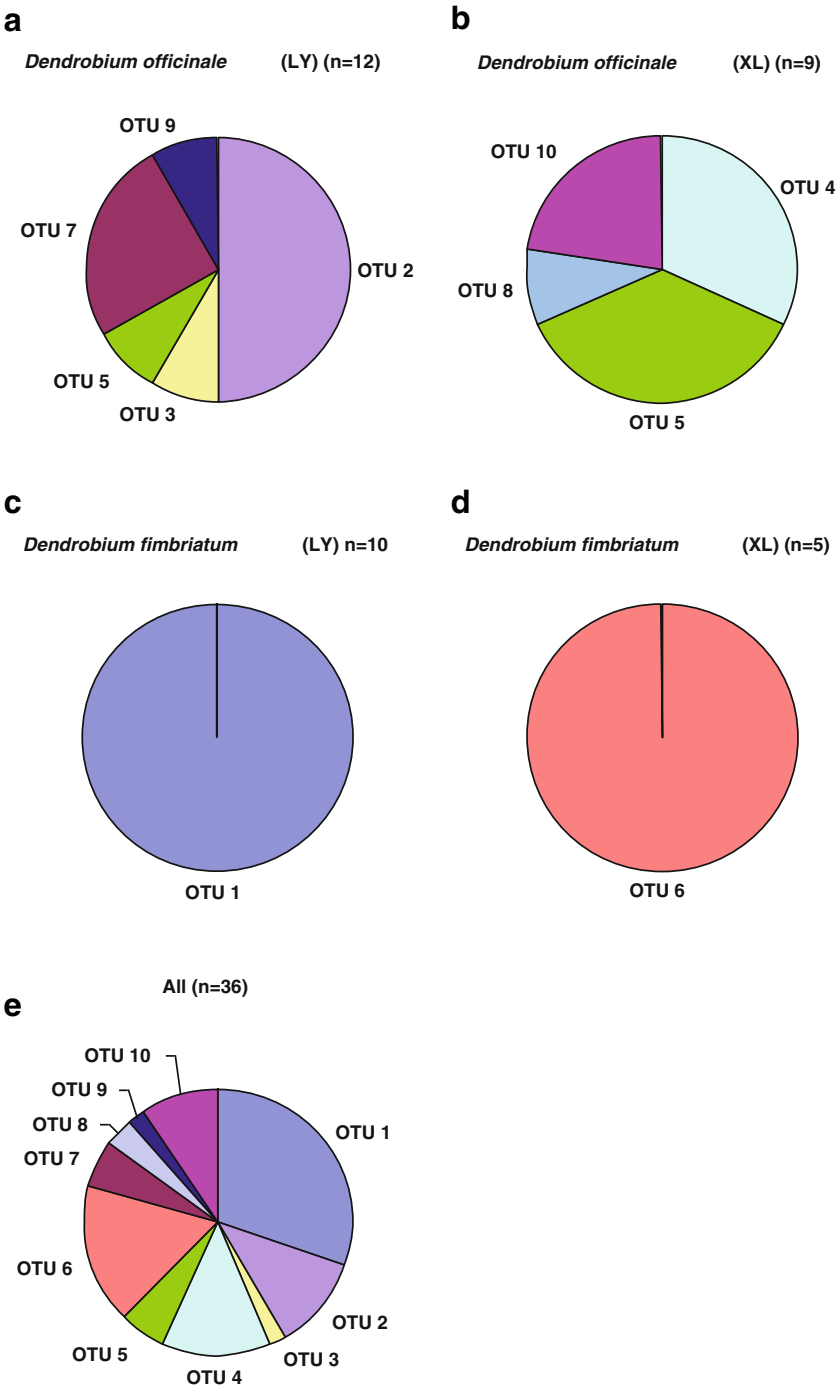

Fig. 3 Frequency distribution of the operational taxonomic units (OTUs) in *D. officinale* and *D. fimbriatum* collected from Leye and Xilin (**a**–**d**) and across the two species (**e**)

Table 2 Minimum, maximum, and average (±SD) number of operational taxonomic units (OTUs) found in individual plant of two species of the genus *Dendrobium* collected from different habitats. The total number of OTUs found across all sampled populations is also given

Species (collecting site)	Min	Max	Average	Total
Dendrobium officinale (Leye)	3	5	3.4 (±1.2)	5
Dendrobium fimbriatum (Leye)	1	1	1 (±0)	1
Dendrobium officinale (Xilin)	3	4	3.2 (±0.2)	4
Dendrobium fimbriatum (Xilin)	1	1	1 (±0)	1

In addition, OTUs related to members of Ceratobasidiaceae and Pluteaceae were observed, although only very sporadically. Nonetheless, the Ceratobasidiaceae fungi have been recognized as important associates of other orchid genera, such as *Goodyera* (Shefferson et al. 2010), *Tolumnia* (Otero et al. 2004), and *Pterostylis* (Bougoure et al. 2005; Bonnardeaux et al. 2007), but the Pluteaceae fungi have been described as non-mycorrhizal agaric fungi (Justo et al. 2011). Mycena species have been reported as mycorrhizal fungi of *D. officinale* (Guo et al. 1999). After

inoculating, the seedlings of *D. officinale* with the *Mycena* sp., a typical structure of orchid mycorrhiza has been observed (Zhang et al. 2012). But in this study, we did not find any fungal OTUs related to *Mycena*. This may be due to the fact that samples were collected from different habitats.

Specificity and preference of mycorrhizal associations in *Dendrobium* plants

The specificity of orchid mycorrhizal associations has important implications for orchid biology, conservation, and restoration of orchid populations (Dearnaley 2007). Specificity in mycorrhizal associations has been defined by both the number of fungal species that a plant can be associated with and the phylogenetic breadth of symbionts (McCormick et al. 2004; Shefferson et al. 2007). It has been reported that fungi associated with orchids are most likely to be similar when the orchids grow in close proximity to each other (McKendrick et al. 2002; McCormick et al. 2004). Our study revealed that an individual *D. officinale* associated with three to five fungal OTUs. This result supports earlier findings that a single orchid individual can be associated with more than one fungal species at the same time (Otero et al. 2002; McCormick et al. 2004; Shefferson et al. 2008; Jacquemyn et al. 2010, 2012b). Additionally, we found that *D. fimbriatum* only hosted one fungal OTU, regardless of habitat. These data suggest that *D. fimbriatum* may have more specificity than *D. officinale* in its association with mycorrhizal fungi. However, we still know little about the specificity of the mycorrhizal plant symbiosis. Here, we described specificity in terms of fungal identity only, but specificity can also encompass functioning of mycorrhizal partners toward the plants.

Previous studies have indicated a preference among some orchids for specific fungal partners (Otero et al. 2004). Some photosynthetic orchids, even when sampled over a wide range, have a single dominant mycorrhizal fungus (Shefferson et al. 2005; McCormick et al. 2006; Irwin et al. 2007). There is also evidence that the degree of specificity can vary among species of terrestrial orchids (McCormick et al. 2004; Jacquemyn et al. 2010) and in neotropical, epiphytic orchids (Otero et al. 2002, 2004, 2007; Suárez et al. 2008). In this study, we found that the dominant mycobionts of each *Dendrobium* species differed. For *D. officinale* growing in Leye and Xilin, the dominant mycorrhizal fungus was OTU 2 and OTU 5, respectively. For *D. fimbriatum*, the dominant mycorrhizal fungus was OTU 1 and OTU 6, respectively. A single Tulasnellaceae species predominated in each habitat, suggesting that *D. fimbriatum* displays narrow fungal specificity. Recent work on the relationship between mycorrhizal network and plant phylogeny had been carried out (Jacquemyn et al. 2010, 2012b; Martos et al. 2012). Jacquemyn et al. (2012b) found that closely related orchid species tended to interact with a similar set of fungal partners. In this research, we only analyzed the mycorrhizal network of two species in the genus *Dendrobium* and found that the fungal partners were different between the two *Dendrobium* species. This result supports previous findings that co-occurring orchid species tend to use different mycorrhizal partners (Waterman et al. 2011). Jacquemyn et al. (2012a) also showed that divergent mycorrhizal associations may explain coexistence of orchid species. However, because *Dendrobium* is so rare, we were only able to use small sample size, which may have led to an underestimation of fungal diversity. It will be necessary to sample these plants in more locations to fully describe the mycorrhizal specificity in these two orchid species.

Although the molecular is powerful for understanding the mycorrhizal community associated with these *Dendrobium* species, isolation and cultivation of the mycorrhizal fungal strains are still needed for the mycorrhizal fungi be further used in the conservation procedures for these orchid species.

In summary, we have examined the mycorrhizal associations for two species of the genus *Dendrobium* at two sites in Guanxi Province, China. The result suggested that *D. officinale* associated with a wide range of basidiomycetous mycorrhizal partners, while *D. fimbriatum* displayed a more narrow fungal specificity. Future studies should focus on other *Dendrobium* species that occur in other environmental conditions to elucidate the nature and specificity of mycorrhizal associations in this genus. Furthermore, the relationship between mycorrhizal network architecture and plant phylogeny in the genus *Dendrobium* should also be analyzed.

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