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Mycorrhizal specificity, preference, and plasticity of six slipper orchids from South Western China

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Abstract Mycorrhizal fungi of six endangered species, *Paphiopedilum micranthum*, *Paphiopedilum armeniacum*, *Paphiopedilum dianthum*, *Cypripedium flavum*, *Cypripedium guttatum*, and *Cypripedium tibeticum*, from two closely related genera in the Orchidaceae from Southwestern China, were characterized using the nuclear internal transcribed spacer (ITS) and part of the large subunit gene of mitochondrial rDNA (mtLSU) sequences. The most frequently detected fungi belonged to the Tulasnellaceae. These fungi were represented by 25 ITS sequence types and clustered into seven major clades in the phylogenetic analysis of 5.8S sequences. Species of *Paphiopedilum* and *Cypripedium* shared no fungal ITS sequence types in common, but their fungal taxa sometimes occurred in the same major clade of the 5.8S phylogenetic tree. Although it had several associated fungal ITS sequence types in a studied plot, each orchid species had in general only a single dominant type. The fungal sequence type spectra of different species of *Paphiopedilum* from similar habitats sometimes overlapped; however, the dominant sequence types differed among the species and so did the sequence-type spectra within *Cypripedium*. Orchids of *P. micranthum* and *P. armeniacum* transplanted from the field and grown in two greenhouses had a greater number of mycorrhizal associations than those sampled directly from the field. Root specimens from *P. micranthum* taken from the greenhouses were preferably associated with mycobionts of the *Tulasnella calospora* complex, while those from the field had mycorrhizal associations of other tulasnelloid taxa. Such plasticity in mycorrhizal associations makes ex situ conservation or even propagation by means of mycorrhization of axenically grown seedlings possible.

Keywords *Cypripedium* · Molecular identification · Sequence type · *Paphiopedilum* · Tulasnellaceae · *Tulasnella calospora*

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Introduction

Mycorrhizae play a vital role in the life cycle and evolutionary history of orchids (Rasmussen 2002; Rasmussen and Rasmussen 2009). Many plants are mycorrhizal generalists with phylogenetically broad associations (Hoeksema 1999; Massicotte et al. 1999). However, studies indicate that fungal specificity is a common phenomenon in many orchids regardless of nutritional mode (Taylor and Bruns 1997; McCormick et al. 2004; Shefferson et al. 2005, 2007; Suárez et al. 2006). The specificity of orchid mycorrhizal interactions is of crucial importance to orchid ecology and conservation (Otero et al. 2007).

Closely related orchid species appear to have varying levels of mycorrhizal specificity; identity and phylogenetic breadth of mycorrhizal fungi have been shown to be evolutionary traits of some orchids (Otero et al. 2004; Shefferson et al. 2007). Studies showed that some epiphytic or terrestrial orchids are highly specific for a narrow group of mycorrhizal fungi (Otero et al. 2002; Ma et al. 2003; McCormick et al. 2004; Shefferson et al. 2005; Suárez et al. 2006). Similarly, evergreen orchids do not necessarily have greater mycorrhizal diversity than seasonally green orchids (McCormick et al. 2004). Since fungi differ in their ability to grow under different ecological conditions, plants may

switch to different mycobionts to accommodate environmental changes (McCormick et al. 2006). If orchids can switch their mycorrhizal fungi symbionts when in new environments, this would have implications for the ex situ conservation for the endangered orchids.

The genera *Paphiopedilum* and *Cypripedium*, which both belong to the subfamily Cypripedioideae of the Orchidaceae (Cox et al. 1997; Cameron et al. 1999), are very well known as slipper orchids in horticultural science. Although they are closely related phylogenetically, their biological features and geographical distributions differ significantly from each other. Species of *Paphiopedilum* are epiphytic, terrestrial or lithophytic, usually evergreen, and distributed across the tropics or subtropics, while those of *Cypripedium* are terrestrial, seasonally green with an "adult dormancy" period, and distributed widely in the north temperate regions (Cox et al. 1997; Cribb 1997, 1998; Chen and Tsi 1998; Cameron et al. 1999). All species in the two genera are listed in the Appendix of the Convention on International Trade in Endangered Species of Wild Fauna and Flora; additionally, some species are even at risk of extinction due to habitat destruction and over-harvesting for the ornamental market (Cribb 1997, 1998). Because of the importance of mycorrhiza in orchid life, it is important to understand the diversity of the fungal symbionts in order to make successful conservation strategies for the orchid. Studies on species of *Cypripedium* sampled from the North America, Europe, and East Asia (Japan and China's Taiwan) showed that they have a high specificity of association with fungi of family Tulasnellaceae (Shefferson et al. 2005, 2007; Shimura et al. 2009). Due to the limited distribution and availability of the *Paphiopedilum* species, mycorrhizal communities of *Paphiopedilum* have only sporadically been studied (Athipunyakom et al. 2004). Considering their close phylogenetic relationships, but different distributions and features in biology, it would be beneficial to detect and reveal the difference or similarity of mycorrhizal fungi between the two genera of orchids occurring in the same region in South Western China.

The genus *Paphiopedilum* is divided into three subgenera; *Paphiopedilum micranthum* and *Paphiopedilum armeniacum* are closely related and treated in the same subgenus but allopatric in distribution, while *P. micranthum* and *Paphiopedilum dianthum* are sympatric in distribution yet belong to different subgenera (Cribb 1998). It would be interesting to elucidate the phylogenetic breadth of symbionts of closely related species within the same subgenus of *Paphiopedilum* but from different localities and comparatively remotely related species in different subgenera yet from similar habitats. Orchids of *Paphiopedilum* can grow relatively well and flower normally when transplanted from the wild to a greenhouse, while plants of *Cypripedium* were either flowerless or even died in the same greenhouse. It is interesting to detect and reveal the differences of mycobionts or the plasticity in mycorrhizal association of the same species from the field and also in a greenhouse environment.

In the present paper, we asked the following questions: (1) Do species of *Paphiopedilum* and *Cypripedium* have similar specificity in mycorrhizal association and identical fungal symbionts? (2) Do the orchid species have preference in their mycorrhizal association in a habitat? (3) Do particular orchids possess plasticity to enable a switch of mycorrhizal fungi under different growing habitats? In order to answer those questions, we sampled three species of *Paphiopedilum* and three species of *Cypripedium* from South Western China and used phylogenetic analysis of nuclear internal transcribed spacer (ITS) sequences and part of the large subunit gene of mitochondrial rDNA (mtLSU) sequences to identify and characterize their mycorrhizal fungi.

Materials and methods

Root sampling

We used roots as sampling units, and two roots were sampled per plant. Roots of *P. micranthum* and *P. dianthum* were sampled from Wenshan, South Eastern Yunnan. *P. micranthum* was also collected from two additional localities: one greenhouse in Kunming Institute of Botany in Central Yunnan and the other greenhouse in Xingyi, South Western part of Guizhou Province. Roots of *P. armeniacum* were collected from the field in Baoshan, Western Yunnan and the greenhouse in Kunming Institute of Botany. Roots of *Cypripedium flavum*, *Cypripedium guttatum*, and *Cypripedium tibeticum* were collected from the same location in Shangri-la, north western part of Yunnan Province. All of the orchids from the greenhouses were transplanted from the field for at least 3 years prior to root-tip sampling. In total, 148 root samples were collected and analyzed (Table 1). Sampled mycorrhizal roots were surface sterilized with ethanol (70%) to avoid unnecessary contaminates from the velamen of the roots and surface of root epidermis. Mycorrhizal zones of root samples of 5–8 mm length from different plants were kept in 1.5-ml Eppendorf tubes separately stored in −20°C for DNA extraction.

DNA extraction, PCR, cloning, sequencing

DNA was extracted from roots using a CTAB method modified from Doyle and Doyle (1987). Mycorrhizal fungal ITS was amplified by using the primer sets ITS1F–ITS4F (White et al. 1990; Gardes and Bruns 1993) and ITS1OF–ITS4OF (Taylor and McCormick 2008). Fungal mtLSU was amplified with primers ML5 and ML6 (Bruns et al.

Table 1 Number of Plant individuals, root samples, fungal ITS sequences, ITS sequences types directed PCR amplified from the orchid roots, and the dominant fungal sequences types well as their percents

Orchid (site)	Plant individuals	Root samples	ITS sequences	ITS sequence types	*H′*	*E′*	Dominant type (%)
PA (Kunming)	11	14	12	5	1.358	0.844	Type 7 (50.000)
PA (Baoshan)	12	16	16	3	0.838	0.756	Type 7 (62.500)
PM (Kunming)	13	14	14	4	1.233	0.889	Type 12 (50.000)
PM (Xingyi)	11	18	18	8	1.767	0.850	Type 15 (38.889)
PM (Wenshan)	18	25	25	4	0.711	0.513	Type 25 (80.000)
PD (Wenshan)	10	19	18	6	1.455	0.812	Type 16 (50.000)
CF(Shangri-la)	16	16	17	7	1.681	0.864	Type 24 (35.294)
CT(Shangri-la)	7	9	9	3	0.937	0.853	Type 3 (55.556)
CG(Shangri-la)	15	17	17	3	0.804	0.732	Type 4 (70.588)

For ITS sequence types, see Fig. 1

H′ Shannon–Weiner diversity index; *E′* evenness index; *PA P. armeniacum*; *PM P. micranthum*; *PD P. dianthum*; *CF C. flavum*; *CT C. tibeticum*; *CG C. guttatum*

1998). 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 7-min extension at 72°C. The PCR procedure for mtLSU region was identical to the PCR for ITS except the annealing temperature was 56°C. PCR products were purified with Bioteke multifunction DNA clean Kit (Bioteke, China) then cloned using PMD-18T vector (Takara, China) following manufactures' instructions. Discrete colonies were directly amplified and sequenced with an ABI 3730 automated sequencer using the PCR primers. New sequences are deposited at GenBank under accession numbers and are listed in Figs. 1 and 2.

Phylogenetic analysis

All sequences were submitted to a BLAST search in GenBank on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/; Altschul et al. 1997). Closely related published fungal sequences were downloaded for phylogenetic analysis. Sequences were aligned using ClustalX with minor manual adjustments using BioEdit. For broad phylogenetic analyses of the DNA sequences, only 5.8S and mtLSU sequences were used because of the high divergence of the ITS1 and ITS 2 regions (Suárez et al. 2006; Porras-Alfaro and Bayman 2007; Shefferson et al. 2007, 2008). Based on the analyses of the 5.8S sequences, several major clades (clades I–VII in Fig. 1a) were revealed. Within four of the clades (clades I, II, IV, and V), the relationships of the mycobionts could be resolved. However, the relationships of the mycobionts with the remaining three clades (III, VI, and VII) were poorly resolved; thus, ITS1 and/or ITS2 plus 5.8S sequences were further analyzed. Phylogenetic analyses were carried out with the Maximum Likelihood (ML) method as implemented in the online version of Bootstrap RAxML (RAxML-HPC on Abe 7.0.4) (Stamatakis et al. 2008) available on the CIPRES portal (http://www.phylo.org/sub_sections/portal/) under the GTRMIX model of DNA substitution with 1,000 rapid bootstrap replicates. In addition, to validate the ML results, phylogenetic relationships were also estimated using the maximum parsimony (MP) method of the computer package PAUP* version 4.0 (Swofford 2002) for each data alignment. MP analysis in PAUP* used a heuristic search strategy with the following settings: gaps as missing data; multistate taxa interpreted as uncertainty; starting tree(s) obtained via stepwise addition; addition sequences with random option of 1,000 replicates; held one tree at each step during stepwise addition; tree-bisection-reconnection branch-swapping; steepest descent and "MulTrees" options not in effect. Five hundred MP bootstrap replicates were completed using heuristic search with the same search parameters as above.

Fungal sequence types were determined by ITS sequences with at least 97% similarity (Mühlmann and Peintner 2008), and the results were incorporated in the phylogenetic trees. To compare the specificity and distribution of tulasnelloid ITS sequence types of the six slipper orchids among species and collected sites, the Shannon–Weiner diversity index (*H′*) and evenness index (*E′*) were calculated using the number of fungal sequences distributed in each ITS sequence type.

Results

PCR results and sequences BLAST

One hundred fifty-four ITS and 61 mtLSU sequences from mycorrhizal fungi were amplified from orchid root tip

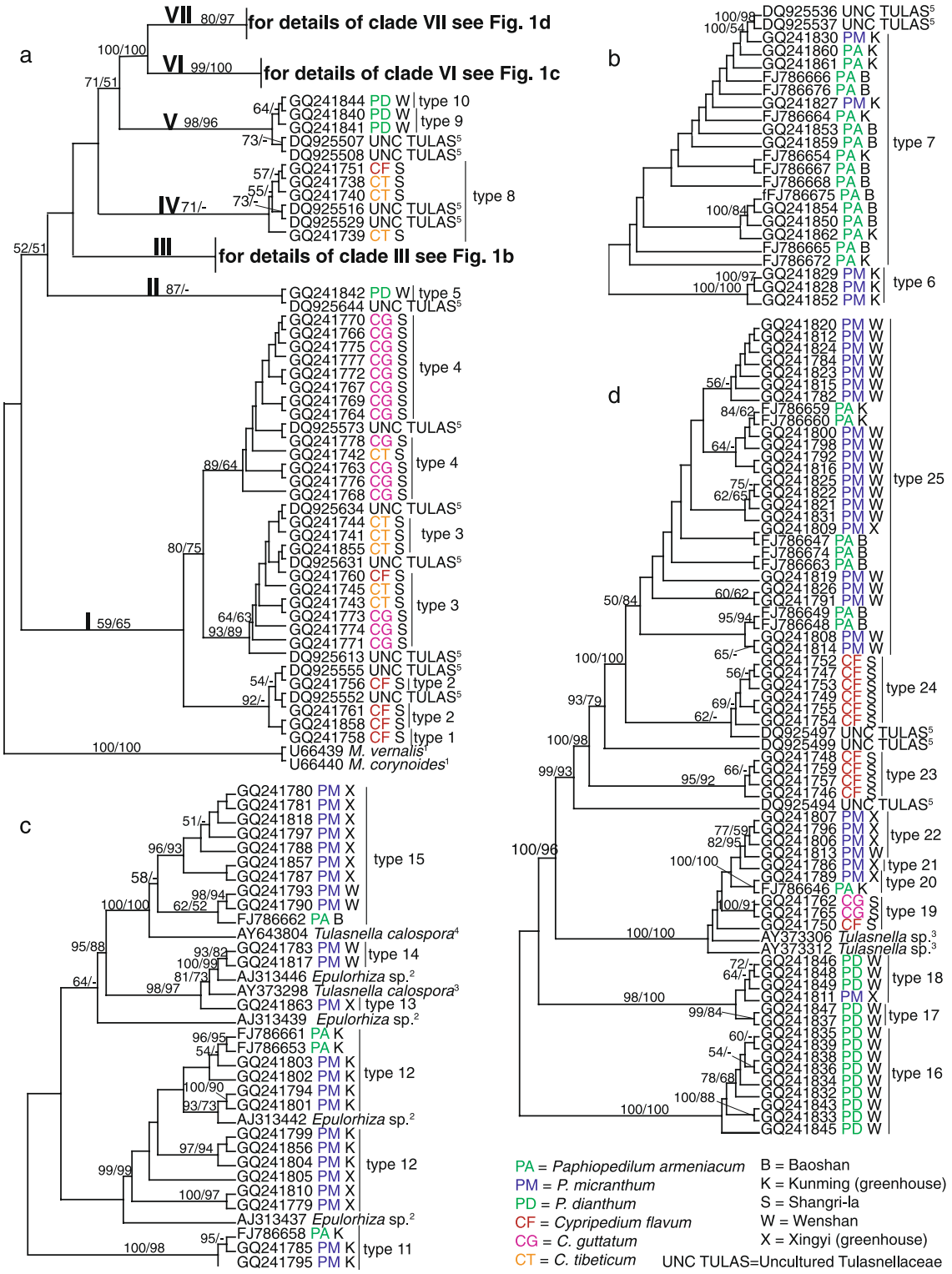

Fig. 1 ML cladograms inferred from DNA sequences of symbionts amplified from roots of orchids and sequences of related fungi from the GenBank. **a** Relationships among clades I–VII inferred from the 5.8S sequences of mycorrhizal fungi detected from roots of six species of slipper orchids and related sequences of fungal taxa from the GenBank. *Multiclavula vernalis* and *Multiclavula corynoides* were used as outgroup as suggested by Shefferson et al. (2007). **b** Relationships of the mycobionts clustered in clade III of **a** based on the analysis of the dataset of ITS1+5.8S+ITS2. **c** Relationships of the mycobionts clustered in clade VI of **a** based on ITS1+5.8S+ITS2. **d** Relationships of the mycobionts clustered in clade VII of **a** based on 5.8S+ITS2. *Numbers above branches* are ML/MP bootstrap values (values <50 % not shown). Sequences generated from fungal symbionts in this study are those accession numbers with *FJ* and *GQ* initials. Sources for reference sequences used in the figures are as follows: *1* Lutzoni (1997); *2* Ma et al. (2003); *3* McCormick et al. (2004); *4* Bougoure et al. (2005); *5* Shefferson et al. (2007)

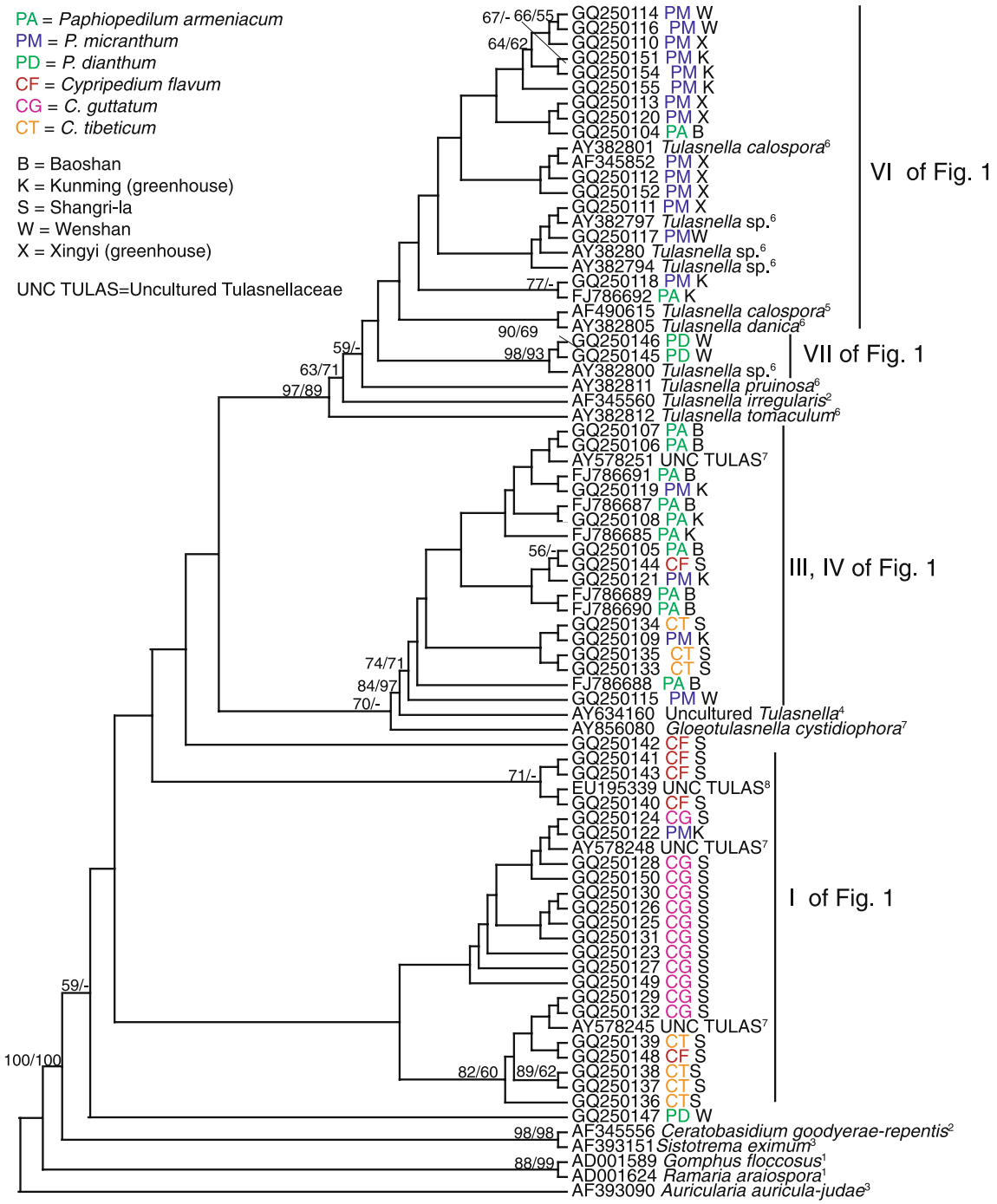

Fig. 2 ML cladogram inferred from mtLSU sequences of mycorrhizal taxa amplified from roots of six species of slipper orchids and published sequences of related fungal taxa from the GenBank. *Auricularia auricula-judae* was used as outgroup followed Shefferson et al. (2005). *Numbers on branches* designate ML/MP bootstrap values (values <50% not shown). Sequences generated from fungal symbionts in this study are those accession numbers with *FJ* and *GQ* initials. Sources for sequences obtained from the GenBank are indicated as follows: *1* Bruns et al. (1998); *2* Kristiansen et al. (2001); *3* Binder and Hibbett (2002); *4* Bidartondo et al. (2004); *5* Kristiansen et al. (2004); *6* McCormick et al. (2004); *7* Shefferson et al. (2005); *8* Shefferson et al. (2008)

DNA. BLAST analyses identified 146 ITS and 58 mtLSU sequences as fungi of Tulasnellaceae.

Apart from the Tulasnellaceae, there were few other fungal sequences (GQ280335–GQ280345) identified by BLAST searches as closest to other fungal taxa, such as *Fusarium* spp. and *Penicillium* spp. The ecological significances between these taxa and orchids are yet not clear, and some of which maybe contaminants in the studied roots. Because

of the low frequencies of these fungi detected in the orchid roots, we considered their mycorrhizal partnerships to the orchids as uncertain and excluded them from further analysis (also see Bayman et al. 1997, 2002; Suárez et al. 2006).

Phylogenetic analyses

One hundred seventy-four 5.8S sequences, including 146 amplified by us and a further 28 downloaded from the GenBank, were analyzed, and the alignment contained 174 nucleotide sites (70 were parsimony informative). Parsimony analysis resulted in 307 most parsimonious trees of 166 steps, with Consistency Index (CI)=0.747 and Retention Index (RI)=0.987. The final alignment of mtLSU sequence data, consisting of 58 amplified sequences and 22 downloaded from the GenBank, contained 275 nucleotides (81 sites were parsimony informative). Parsimony analysis resulted in 59 parsimonious trees of 261 steps, with CI=0.690, RI=0.915. For clades III, VI, and VII clustered in the analysis of 5.8S sequences (Fig. 1a), the sequences of 5.8S plus ITS1 and/or ITS2 were further analyzed. In the dataset for clade III, the sequences alignment of ITS1+5.8S+ITS2 contained 649 nucleotides, among them 520 were constant and 109 sites were parsimony informative. Parsimony analysis resulted in one most parsimonious tree of 140 steps, with CI=0.950 and RI=0.969. In clade VI, the dataset of ITS1+5.8S+ITS2 alignment contained 614 nucleotides; 90 sites were parsimony informative. Parsimony analysis resulted in 73 most parsimonious trees of 232 steps, with CI=0.703 and RI=0.897. In clade VII, the dataset of 5.8S+ITS2 containing 432 nucleotides were analyzed; among them, 139 sites were parsimony informative. Parsimony analysis resulted in two most parsimonious trees of 377 steps, with CI=0.647 and RI=0.940. Maximum parsimony analyses of the sequences datasets yielded topographies similar to those from the ML analysis (trees not showed). Figures 1 and 2 represent cladogram versions of the ML trees from all the sequences dada analysis.

The tree constructed from 5.8S sequences of mycorrhizal fungi of *Cypripedium* and *Paphiopedilum* clustered into seven major clades (I–VII), representing 25 ITS sequence types (types 1–25; Fig. 1a–d). The mtLSU region revealed less resolutions of the relationships among the detected fungal sequences than the ITS. The phylogenetic tree constructed from mtLSU sequences clustered into five clades in Fig. 2, which correspond to clades I, III, IV, VI, and VII in Fig. 1a. Attempts to amplify the mtLSU region for the taxa in clades II and V of Fig. 1a were unsuccessful.

Mycorrhizal association analysis

Based on phylogenetic analysis of 5.8S sequences and ITS sequence types, each species of orchid in this study had associations with at least three fungal ITS sequence types (Table 1, Fig. 1a–d). Species of *Paphiopedilum* in this study were found to be associated with 17 types (types 5–7, 9–18, 20–22, 25), which clustered into five different clades (II–III, V–VII). All types of mycobionts of *P. armeniacum* were found in the fungal sequences amplified from *P. micranthum*. Only one of the 57 sequences amplified from *P. micranthum* was identical to a type of the fungal sequences from *P. dianthum*, type 18.

Mycorrhizal fungi of *C. flavum*, *C. guttatum*, and *C. tibeticum* clustered into three clades (I, IV, and VII), consisting of eight ITS sequence types (types 1–4, 8, 19, 23–24). All three species of *Cypripedium* shared type 3, *C. flavum* and *C. tibeticum* were associated with type 8, *C. flavum* and *C. guttatum* were associated with type 19, and both *C. guttatum* and *C. tibeticum* had the mycorrhizal symbionts of type 4. Types 1, 2, 23, and 24, were exclusively detected in the roots of *C. flavum*.

Mycorrhizal fungi of *Cypripedium* and *Paphiopedilum* never shared a common ITS sequence type (Fig. 1a–d). However, clade VII consisting of fungal sequence types sampled from both genera in this study. Fungal taxa from clades II, III, and V were associated with species of *Paphiopedilum* from this study plus five *Cypripedium* sequences (DQ925507, DQ925508, DQ925536, DQ925537, and DQ925644) from the study of Shefferson et al. (2007), although no fungal sequence types of *Cypripedium* from this study were clustered in these clades. Fungal types of clade VI were only sampled from species of *Paphiopedilum*. Some of these sequences are most closely related to fungal sequences from *Spathaglotis plicata* (AJ313437, AJ313439; Ma et al. 2003) and *Microtis parviflora* (AY643804; Bougoure et al. 2005).

Greenhouse specimens of *P. armeniacum* and *P. micranthum* had a higher diversity index and evenness index for mycorrhizal symbionts than those sampled in the field (Table 1, Fig. 1b–d). In the field samples of the six species of orchids, *P. dianthum* and *C. flavum* showed higher diversity index and evenness index of symbionts taxa than the other four species. Based on the abundance of ITS sequence types (Fig. 1a–d), the fungal type observed the most was from a single orchid species and is labeled as the "dominant mycorrhizal taxon." Mycorrhizal fungal sequence type spectra of associated orchid species within *Paphiopedilum* sometimes overlapped, but dominant sequence types differed among the orchid species and so did the sequence type spectra within *Cypripedium* (Table 1).

The sequence types of dominant mycorrhizal fungi of *C. flavum*, *C. tibeticum*, and *C. guttatum* were types 24 (35.29%), 3 (55.56%), and 4 (70.59%) respectively. These dominance differences were found where the orchids occurred in the same habitats and had the same mycorrhizal taxa in common. Dominant mycorrhizal taxa were different

between the wild and transplanted plants of *Paphiopedilum* (see Fig. 1b–d and Table 1). Near 80% of the fungi sampled from the wild plants of *P. micranthum* were clustered in sequence type 25 from clade VII. However, more than 60% mycorrhizal fungi of transplanted plants of *P. micranthum* both in the greenhouses in Kunming and Xingyi clustered in clade VI. The difference between wild and greenhouse mycorrhizal communities of *P. armeniacum* were not as dramatic. Sequence type of the dominant mycorrhizal fungus of *P. armeniacum* in the wild (62.5%) and greenhouse (50%) was type 7 from clade III. Mycorrhizal fungi sequences (three of 12) of *P. armeniacum* sampled from the greenhouse clustered in clade VI were higher than that sampled from the wild habitats (one of 16).

Discussion

Specificity and preference of mycorrhizal associations in slipper orchids

Species of Tulasnellaceae have been described as mycorrhizal symbionts of *Cypripedium* (Shefferson et al. 2005, 2007), and many other orchids (e.g., Warcup and Talbot 1967, 1971; Kristiansen et al. 2001, 2004; McCormick et al. 2004; Suárez et al. 2006; Porras-Alfaro and Bayman 2007; Shefferson et al. 2008). Athipunyakom et al. (2004) isolated some fungal strains from several species of *Paphiopedilum* and identified them as *Epulorhiza repens*, *Rhizoctonia* spp., and *Ceratorhiza ramicola*, some of which are anamorphic states of species of *Tulasnella*. Our analysis of the mycorrhizal fungal sequences showed that species of *Paphiopedilum* also associate with fungi from the Tulasnellaceae (Figs. 1, 2). Despite the close phylogenetic relationship between *Cypripedium* and *Paphiopedilum* (Cox et al. 1997), these two genera almost never shared identical fungal ITS sequence types. Among the fungal ITS sequences from *Cypripedium* roots, 1,030 samples of Shefferson et al. (2007) and 42 of us, only two sequences, DQ925536 (from *C. candidum*) and DQ925537 (from *C. parviflorum*) (Shefferson et al 2007), showed 96.6–98.2% similarity to our sequences in type 7, which were amplified from *P. micranthum* and *P. armeniacum* (Fig. 1b).

Specificity in mycorrhizal associations has been defined not only by the number of fungal species that a plant can be associated with but also by the phylogenetic breadth of symbionts (McCormick et al. 2004; Shefferson et al. 2005, 2007). Species of *Cypripedium* have been shown to associate with tulasnelloid fungi as indicated by Shefferson et al. (2005, 2007) and confirmed by our data. In our study, three species of *Cypripedium* collected from the same habitat shared some common fungal associates. We also found that three species of *Paphiopedilum* associated with a limited group of tulasnelloid fungi. Mycorrhizal fungi of *P. armeniacum* and *P. micranthum*, classified in the same subgenus and occurs in similar habitats with allopatric (Cribb 1998), shared the majority of identical ITS sequence types of fungi even when the samples were collected from different locations. In contrast, *P. dianthum* and *P. micranthum*, belonging to different subgenera, rarely associate with identical fungal taxa, even if their root samples were collected from plants within a distance less than 5 m from each other in the field. This comparative study clearly demonstrates that the six species of orchids form associations with a narrow phylogenetic range of mycorrhizal taxa in the Tulasnellaceae.

Recent studies showed that some orchids have their preferences for fungal partners (e.g., Otero et al. 2004; Suárez et al. 2006). We found that one species of orchid often has a single dominant fungal sequence type in a habitat (Table 1). It was reported that the 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 three species of *Cypripedium* sampled from the same locality shared some fungal taxa (e.g., types 3, 4, 8, and 19). However, the dominant fungal taxa in each species were different. The ITS sequence types 24, 3, and 4 were the most frequently sampled from *C. flavum*, *C. tibeticum*, and *C. guttatum*, respectively. Similarly, the most frequently sampled taxa on the roots of *P. armeniacum* and *P. micranthum* from the same greenhouse (Kunming) were types 7 and 12, respectively. If several fungal taxa can form mycorrhizae with one orchid species at the same habitat, the more taxa that are likely to be associated with the orchid might depend on its preference for fungal partners.

Plasticity of mycorrhizal association

The ability to switch between fungal symbionts is a potential adaptation to severe environments (McCormick et al. 2006). *P. armeniacum* and *P. micranthum* sampled from greenhouses showed higher diversity and evenness index of fungal symbionts than samples from the field (Table 1). In comparison to the dominant mycorrhizal taxa of the field samples distributed in clade VII, *P. micranthum* in the two greenhouses, all dominantly associated with taxa in clade VI (Fig. 1b–d), defined as the *T. calospora* complex, a group of fungi known to form mycorrhizal associations with many orchids (e.g., Hadley 1970; Masuhara and Katsuya 1994; McCormick et al. 2004; Suárez et al. 2006). This suggests that fungal-orchid associations are sensitive to environmental stimuli and can possibly adjust to favor survival of the plant partner (Dearnaley 2007). A similar strategy for survival was observed in *Erythrorchis cassythoides*, which associates

predominately with ECM fungi that interact with the living host, but its main mycobiont becomes a saprotrophic species when the tree host is dead (Dearnaley 2006). Mycorrhizal specificity of an orchid may limit the distribution of the species and also the survivability of transplanted plants. However, such plasticity in mycorrhizal association makes the ex situ conservation of them or even propagation of them by means of mycorrhization of their axenically grown seedlings possible.

Taxa of the *T. calospora* complex are probably a dominant group of mycobionts in the two greenhouses. Our analysis showed that axenically grown seedlings of *P. armeniacum* were colonized predominantly with fungal taxa of the *T. calospora* complex when transplanted to greenhouses in substrates which previously harbored *P. armeniacum* plants collected from the natural habitats (data unpublished). Surprisingly, taxa from the *T. calospora* complex were not identified in the 42 root samples of *Cypripedium* (Figs. 1, 2), nor in the 1,030 *Cypripedium* mycorrhiza DNA samples in the study of Shefferson et al. (2007). When plants of *Cypripedium* were transplanted from the wild to the Kunming greenhouse, most of them either died or did not flower. Even if flowering, their flowers were always smaller than those in the field. In comparison, over 80% of plants of *Paphiopedilum* grow well and flowered normally when transplanted from the wild to the same greenhouse. One reason for the above phenomenon may be that there are no mycobionts available in the greenhouse for the plants of *Cypripedium* to thrive. Another reason is the probability that plants of *Cypripedium* have no plasticity to switch to other fungal taxa in the greenhouse.

When studying both the number of sampled roots per plant and the number of sampled plant individuals (see Table 1), it should be noted that due to limited resources in the field and the strict laws of protection, some mycobionts in the roots of our studied species may well not be uncovered. Our data showed that orchids of *Paphiopedilum* and *Cypripedium* shared no fungi of identical sequence types. However, we could not exclude the possibility that mycorrhizal fungi species of *Paphiopedilum* are identical in a few of sequence types of the fungal symbionts of *Cypripedium* orchids when additional root samples are studied. Nevertheless, the chances of finding mycorrhizal partners of *Cypripedium* orchids identical to those fungal taxa in clade VI, with which *Paphiopedilum* species associated, seem to be low, considering the fact that many species of *Cypripedium* were studied but no identical mycobionts were found in the clade (Shefferson et al. 2005, 2007; Shimura et al. 2009). In addition, considering the fact that mycobionts of Atractiellomycetes and Sebacinales were found from other orchids (Suárez et al. 2008; Tao et al. 2008; Kottke et al. 2009), there might be mycobionts from other fungal groups in the roots of slipper orchids, for which PCR amplification using the given settings failed. Further sampling and sequencing with additional primers, data from culture studies with new isolating techniques (Zhu et al. 2008), and ultra structural analyses will give a more detailed and complete picture about the mycorrhizae of the orchids.

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