Biological Conservation 212 (2017) 29–38

Contents lists available at ScienceDirect

Biological Conservation

journal homepage: www.elsevier.com/locate/biocon

Contrasting changes in biotic interactions of orchid populations subject to conservation introduction vs. conventional translocation in tropical China

Jason L. Downing[a,b], Hong Liu[c,d,b,*], Shicheng Shao[e], Xilong Wang[e], Melissa McCormick[f], Rongyan Deng[c], Jiangyun Gao[g,**]

[a] Florida International University, Department of Biological Sciences, International Center for Tropical Botany, 11200 SW 8th Street, Miami, FL 33199, USA
[b] Fairchild Tropical Botanic Garden, 10901 Old Cutler Road, Coral Gables, FL 33156, USA
[c] Guangxi University, College of Forestry, 100 Daxue Road, Nanning, Guangxi, China
[d] Florida International University, Department of Earth and Environment, International Center for Tropical Botany, 11200 SW 8th Street, Miami, FL 33199, USA
[e] Center for Integrative Conservation, Xishuangbanna Tropical Botanical Garden, The Chinese Academy of Sciences, Mengla 666303, Yunnan, China
[f] Smithsonian Institution, Smithsonian Environmental Research Center, Edgewater, MD, USA
[g] Lab of Ecology and Evolutionary Biology, State Key Laboratory for Conservation and Utilization of Bio-resources in Yunnan, Yunnan University, Kunming, Yunnan 650091, China

ARTICLE INFO

Keywords:
Assisted colonization
Assisted migration
Climate-change mitigation
Managed relocation
Mycorrhizal fungi
Biodiversity conservation

ABSTRACT

Conservation introduction (CI), i.e. moving a species out of its current natural range for conservation purposes, is a highly controversial and rarely attempted conservation measure. One particular concern with these conservation actions is whether species with obligate symbiotic relationships will be able to form these associations in the recipient locations. This study takes advantage of a massive conservation translocation of orchids at the Yachang National Nature Reserve in southwestern China. We compared the mycorrhizal associations of four of these targeted species. Two of the species (*Cymbidium bicolor* Lindl. and *Geodorum eulophioides* Schltr.) had narrow geographic ranges and were subjected to CI because the movement had placed them out of their natural ranges. While the other two species (*Paphiopedilum dianthum*, Tang and Wang, *and Paphiopedilum hirsutissimum*, (Lindl. ex Hook. f.) Pfitzer) had wide geographic ranges and were not moved outside of them. This movement can be considered a conventional translocation. Using DNA techniques, we identified orchid mycorrhizal fungi (OMF) associated with translocated and natural populations of these species. Our results indicated that translocated populations of both kinds (CI and conventional translocation) were able to establish new relationships with root fungi, including known OMF groups. Wide-ranging species associated with a greater number (24 OTUs) and more diverse groups of fungi (Shannon diversity (H') = 0.93) than narrow range species (15 OTUs, H' = 0.62). However, translocated populations of wide-ranging species shared fewer fungi with their natural populations than those of narrow-ranging species. This research provides the first evaluation of how critical biotic interactions may change following conservation introduction compared to that of conventional translocation, and shed light upon the feasibility of conservation introduction.

1. Introduction

Conservation introduction (sensu IUCN (2013), hereafter referred to as CI), also known as assisted colonization or managed relocation, is the movement of a species by humans to higher latitudes or elevations beyond their current native range to habitats predicted to be suitable under future climatic conditions (McLachlan et al., 2007; Hunter, 2007). CI as a viable strategy to mitigate biodiversity losses as a result of climate change remains a highly debated and rarely attempted conservation action (Hoegh-Guldberg et al., 2008; Minteer and Collins, 2010; Hewitt et al., 2011). Because of the scarcity of such controversial initiatives, only a few known examples of CI currently exist (Liu et al., 2015), and an even smaller portion of these CIs have been evaluated (Willis et al., 2009; Liu et al., 2012). Central arguments about CI include whether endangered species have the flexibility in their physiological and ecological requirements to survive in the recipient community or whether moving species out of their natural range under any circumstances is a good idea (Reichard et al., 2012), as some species introductions have led to disastrous consequences, a prime example being invasive species (Ricciardi and Simberloff, 2009).

---

* Correspondence to: H. Liu, Fairchild Tropical Botanic Garden, 10901 Old Cutler Road, Coral Gables, FL 33156, USA.
** Corresponding author.
E-mail addresses: hliu@fiu.edu (H. Liu), jiangyun.gao@ynu.edu.cn (J. Gao).

http://dx.doi.org/10.1016/j.biocon.2017.05.021
Received 9 November 2016; Received in revised form 7 May 2017; Accepted 21 May 2017

While the debate continues, it is important to take advantage of special circumstances in which actions such as relocating species to out-of-range sites have occurred. Studies of these cases can provide empirical data on the distributional requirements, both abiotic and biotic of the selected species and help to build a decision-making framework that can identify which species may be fit for CI (Liu et al., 2010; Lunt et al., 2013). One major factor to consider for CI assessments is the possible changes in the mutualistic interactions of the translocated populations. The ability to form beneficial biotic interactions in a new range is essential to the establishment of many species in a new environment (Richardson et al., 2000; Mitchell et al., 2006; Pemberton and Liu, 2008). This suggests that the degree of flexibility in biotic interactions (generalization) of a species can determine whether it will survive or not following a CI. Yet, highly specialized species may still be targeted for assisted colonization because of their intrinsic value, the unique ecosystem services they provide, or because they exhibit low ecological redundancy (Lunt et al., 2013).

Orchids have previously been identified as candidates for testing the CI strategy (see examples from Keel et al. (2011), Liu et al. (2010), Lunt et al. (2013)). Orchids can serve as model organisms to test the feasibility of moving species with a spectrum of biotic specificity because of their strong dependency on biotic interactions, including their associations with mycorrhizal fungi that are necessary for seed germination and also in later stages in life (Rasmussen, 1995; Taylor, 2004; McCormick et al., 2006). In 2006, thousands of wild orchids belonging to 29 species, and 16 genera, were moved to higher elevation sites in anticipation of the completion of the Longtan Reservoir near the Yachang National Orchid Reserve (hereafter Yachang Reserve) in Guangxi Province, southwestern China (Liu et al., 2012). With an estimated 44 genera, and 140 species in the Yachang Reserve along, this region is considered a hotspot of orchid diversity (Cribb et al., 2003; Liu et al., 2010). The impacted area consists of a 20 km stretch along the Hongshui River, where all of the low-lying areas below 400 m were to be completely inundated. The translocation recipient site in the Yachang Reserve was located < 30 km from the source sites but was 600 m higher in elevation and was 3.6 °C cooler in mean annual temperature (Liu et al., 2012).

Here we examined the mycorrhizal associations of four of these translocated species; *Cymbidium bicolor* Lindl., *Geodorum eulophioides* Schltr., *Paphiopedilum dianthum* Tang and Wang, *and Paphiopedilum hirsutissimum* (Lindl. ex Hook. f.) Pfitzer. The first two species have naturally narrow elevational ranges and are found only at lower elevations. The subsequent upward translocation of the narrow range species *C. bicolor* and *G. eulophioides*, to elevations > 1000 m above sea level (a.s.l.), exposed these species to conditions well outside their natural range (conservation introduction). In contrast, both *Paphiopedilum* species have wide elevational ranges and can be found naturally at both low and high elevations. The translocations of these two species in the Yachang Reserve were within their natural range and therefore the realm of conventional translocation.

We aimed to evaluate how mycorrhizal communities vary between natural and translocated populations and between populations subjected to CI vs. conventional translocation, both of which are critical to evaluate the feasibility of CI for orchid species and other plants that depend on similar biotic interactions. We used DNA sequencing techniques and fungal specific PCR primers to identify potential mycorrhizal root fungi. Phylogenetic analysis was used to compare the mycorrhizal communities of conspecific populations that remained in their natural range to the individuals that were translocated. We also estimated the overall richness and diversity of mycorrhizal fungi among the different orchid populations. With the notion that wide-ranging orchid species may be associated with a wide array of fungal partners, we formulated our working hypotheses as follows: 1) elevational wide-ranging species will associate with more diverse mycorrhizal fungi than narrow-ranging species; 2a) translocated populations of elevational wide-ranging species will associate with different groups of fungi than those within their natural sites; and 2b) translocated populations of elevational narrow-ranging species (the CI populations) will associate with the same groups of fungi as those in natural sites, due to the expected high level of specificity. We acknowledge that association with relatively few but wide-ranging species of mycorrhizal fungi could also enable orchids to be wide spread but for simplicity we will put this notion aside for now and take it into account in the discussion section. These hypotheses will address three major questions regarding the movement of orchid species as a conservation action; 1) can orchids form mycorrhizal associations beyond their natural elevation range? If so, 2) will translocated populations associate with similar groups of fungi in their natural and recipient sites and 3), are changes in fungal associations seen in CI populations (of elevational narrow-ranging species) similar to those conventionally translocated populations (of elevational wide-ranging species)?

2. Methods

2.1. Study species

Narrow-ranging, low elevation species (growing at a range of 350–700 m a.s.l.): *C. bicolor* and *G. eulophioides*. *Cymbidium bicolor* can be seen naturally growing on trees and limestone cliffs in southern China, Vietnam, Peninsular Malaysia, Borneo, Sulawesi, Java, Sumatra and the Philippines (Liu et al., 2012). *Geodorum eulophioides* is an extremely rare terrestrial orchid with only two disjoint occurrences globally, one near Yachang Reserve (Liu, 2010), and the other in central Myanmar (Tanaka et al., 2011). At Yachang, only two small populations consisting of a few dozen adult individuals remain and each is confined to a single hillside.

Wide-ranging elevation species (growing at a range of 350–1100 m a.s.l. in southwestern China): *P. dianthum* and *P. hirsutissimum*. *Paphiopedilum dianthum* are predominantly lithophytic, but in some instances, they can also be found growing epiphytically. This species is considered endangered by the IUCN Redlist and has a very restricted distribution in China, Laos and Vietnam (Liu et al., 2010). *Paphiopedilum hirsutissimum* is a relatively wide spread species and occurs on shaded cliffs, or in limestone forests. It can be found in Guangxi, Guizhou, and Yunnan of China, and in India, Laos, Thailand, and Vietnam.

2.2. Study sites

The Yachang Reserve, located in northwest Guangxi Province, China (Fig. 1) consists of numerous hills of limestone and non-limestone substrates and steep limestone outcrops, the highest of which are ca. 800 m a.s.l. in elevation. It has a variety of vegetation types, largely depending upon elevation. It is known for its great diversity of terrestrial and lithophytic orchids, with > 140 species in 44 genera (Shi et al., 2007; Liu et al., 2010). Here orchids occur in both monotypic and mixed-species mosaics that can dominate the understory community. The translocation site at the Yachang Reserve, the "orchid garden," was located at ~1000 m in elevation in a semi-deciduous limestone subtropical forest. Other sampled natural populations were all located within Guangxi Province (Appendix A.1) and included Dingshu Village (hereafter Dingshu), Mulun Nature Reserve (hereafter Mulun), Jingxi County rural areas (hereafter Jingxi), and Bangliang Natural Reserve (hereafter Bangliang).

2.3. Field sampling

All of the field sampling occurred in June 2014. At the Yachang Reserve, fieldwork was conducted on 6/19–6/21. There were two different sampling sites within Yachang: the "orchid garden," which is in the interior reserve and naturally rich in orchids, including natural populations of *P. hirsutissimum* and *P. dianthum*, and "Laya," a cliff-side

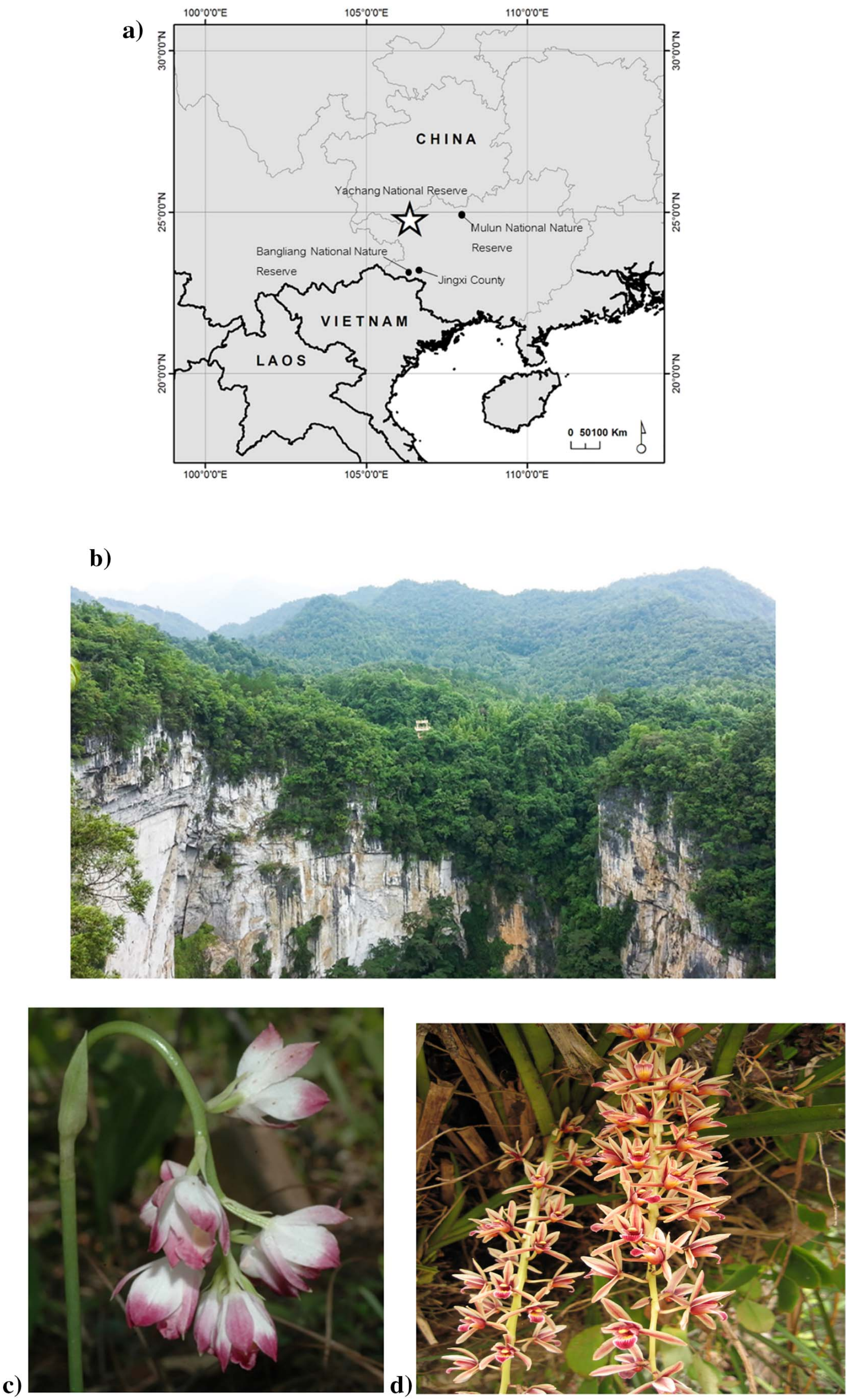


Fig. 1. (a) Map showing studies site in Guangxi Province, China. Star show the Yachang National Reserve which includes the natural sites at orchid garden and Laya cliffs, and the translocated site. Black dots show three low elevation natural sites sampled in the region; (b) (color photo) Photo showing the limestone cliff habitat at the Yachang National Nature Reserve; (c) (color photo) *Geodorum eulophiodes*, (d) *Cymbidium bicolor*, (e) *Paphiopedilum dianthum*, (f) *Paphiopedilum hirsutissimum*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 1. (*continued*)

site with a natural population of *P. hirsutissimum*. The “orchid garden” is however, also the recipient site of translocated orchids. The translocated plants were > 50 m away from these key natural populations of orchids. At Yachang we sampled both translocated and natural individuals of *P. dianthum* and *P. hirsutissimum*. For *P. hirsutissimum*. We sampled six plants naturally occurring in the ‘orchid garden’ and an additional five plants at the Laya site. For *P. dianthum*, we sampled five naturally occurring and five translocated individuals at the “orchid garden.” We also collected samples from one of the two translocated *G. eulophioides* plants and five translocated plants of *C. bicolor* in the ‘orchid garden’.

The Dingshu site was located at low elevation (> 450 m a.s.l) near the Yachang Reserve. Here we collected samples from six plants of *G. eulophioides*. The Mulun Nature Reserve is a low elevation site where plants were located on steep limestone outcrops and occurred in lower densities, as compared to the Yachang populations. At Mulun, we sampled five naturally occurring individuals each for *P. hirsutissimum* and *C. bicolor*. One individual of *C. bicolor* was growing epiphytically. The Jingxi County study site was another low elevation area made up of two sampling sites within the county lands. Here *C. bicolor* plants sporadically occurred along the roadside in trees and limestone outcrops. We collected samples from three individuals growing epiphytically on trees of an unidentified *Ficus* species and two individuals growing as lithophytes. Bangliang is a low elevation site characterized by pristine subtropical limestone forest, located near the border of Vietnam. At this site we sampled six *P. hirsutissimum* plants, as well as one *C. bicolor* individual. All of these plants were found growing lithophytically in rocky soil. Two roots were collected from individuals of each of the four target species at each study site/population; overall, 60 samples were collected. We only selected roots that were in direct contact with the substrate. Roots were removed using a clean razor blade, and immediately rinsed with sterile water to remove any excess organic debris. Samples were placed on moist paper towels and in sterile plastic bags for transport to field stations within 24 h. Due to the remoteness of the study sites, root sample were stored at 4 °C for up to three weeks, until processing at Xishuangbanna Tropical Botanic Garden (XTBG), in Yunnan Province, China.

2.4. Fungus identification

DNA identification of fungal endophytes and the isolation of fungi from root samples were conducted at XTBG. Using a laminar flow hood and sterile microbiological techniques, we examined root samples for the presence of fungal pelotons. If present, individual pelotons were washed in a series of sterile, distilled water baths and grown in pure cultures using *E*-medium agar and liquid broth (Caldwell et al., 1991). These cultures are currently being safeguarded at 4 °C at XTBG with regular sub-culturing until cryopreservation becomes available. Subcultures of all isolates will be used for germination trials when seeds become available. To determine whether additional uncultured fungi were present in roots, DNA was extracted from the remaining root fragments using the Plant DNeasy kit (Qiagen) per manufacturer's instructions. Nuclear DNA from the ITS region was amplified using the fungal primer pairs; ITS 1F/ITS 4, ITS 1OF/ITS4OF, and ITS 5/ITS 4-Tul because they are expected to amplify the DNA of orchid fungi from culture (Gardes and Bruns, 1993; Taylor and McCormick, 2008). The PCR reactions were performed using Redmix HI fidelity TAQ (Applied Biosystems), with the following thermocycler program: 96 °C for 1 min; 35 cycles of 94 °C for 30 s, 54 °C (ITS 1F/4 and 5/4Tul) or 60 °C (ITS 1F/4) for 30 s, 72 °C for 30 s; and 72 °C for 10 min. Each PCR product was cleaned using ExoSAP-IT PCR Cleanup (Affymetrix). Sequencing reactions were completed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). All sequences were manually corrected and aligned using the MAFFT alignment plugin implemented in Geneious version 8.1.5 (Kearse et al., 2012; Biomatters). All new ITS sequences from this study are deposited in GenBank (Accession # KX387573-KX387619, KX397334-KX397339).

Recovered DNA sequences were submitted to a nucleotide BLAST search in GenBank to determine identity. Estimations of phylogenies were made using consensus trees that were produced using neighbor-joining and maximum likelihood (ML) algorithms with 500 random addition replicates. Trees were also generated in the Geneious program (Biomatters Ltd.). Sequences were grouped into operational taxonomic units (OTUs), using the ML pairwise sequence distances from the phylogenetic trees obtained from the Geneious program. To avoid alignment difficulties, we designated all OTUs as belonging to either ascomycetes or basidiomycetes and constructed separate consensus trees for the two taxonomic groups. We considered the sequences with > 97% similarity to constitute a single OTU for ascomycetes and non-Tulasnellaceae basidiomycetes. For the Tulasnellaceae, we used a > 95% similarity cutoff for these taxa because *Tulasnella* spp. are highly variable in ribosomal regions (Taylor and McCormick, 2008; Linde et al., 2014). Some studies support more conservative cut-off values (97–99%) for taxa delineation (Roche et al., 2010). However, based on the relatedness of the fungi in this study, increasing stringency for OTU delineation would not qualitatively change results. For each orchid species, the total number of sequences obtained from root samples and the total number of OTUs were calculated for each species and for each population type (natural or translocated). To compare the diversity of each fungal group for each orchid species, the Shannon-Weiner Index (*H′*) (Faith, 1992) was calculated using counts of distinct OTU sequences detected at each site type. To determine if our richness estimates were reliable, we also generated rarefied accumulation curves by plotting the number of OMF OTUs as a function of the number of samples (no. of individual sequences) over different localities. Index values were obtained using R project (version 3.0.1; R Development Core Team, 2011) and "vegan" package (Oksanen et al., 2009).

3. Results

We obtained a total of 53 ITS fungal sequences: 16 ascomycetes (12 OTUs) and 37 basidiomycetes (22 OTUs) from a total of 60 plants across all natural and translocated sites (Fig. 2). All of the fungal sequences were derived from root DNA. Some of the individuals associated with more than one fungal taxa at time and three basidiomycetes OTUs were shared between natural and translocated populations; see Appendix A.3. The majority of sequences (ca. 70%) were identified as belonging to basidiomycetes in the Tremellaceae, Ceratobasidiaceae, Thelephoraceae and Tulasnellaceae families. Ascomycete fungi represented ca. 30% of the total sequences obtained and included both putative pathogens, represented by the order Pleosporales and at least one potential OMF from the order Hypocreales.

Consensus tree topology for ascomycete sequences supported two major fungal orders (70% bootstrap value), the orders Pleosporales, and Hypocreales (Appendix A.2). The Pleosporales group was detected in translocated *G. eulophioides*, *C. bicolor* and *P. dianthum*. This type of fungus was also found in a natural individual of *P. hirsutissimum* at the Bangliang site. The Hypocreales group was strongly associated with the natural population of *G. eulophioides* (Dingshur site), where five of the six individuals sampled associated with a single *Fusarium* taxon (100% bootstrap support; Appendix A.2). This order was also well represented at the translocated site, associating with all of the species except *G. eulophioides*. The same OTU in the genus *Dactylonectria* (Hypocreales) was detected in natural and translocated individuals of *C. bicolor*; if this is a functional mycorrhiza (beneficial), it suggests an established evolutionary relationship with this fungal genus.

The most dominant group of basidiomycete fungi detected belonged to the core Tulasnellaceae (14 OTUs), and were found to associate with all of the study species. We only failed to detect any basidiomycete associates in the single translocated individual of *G. eulophioides*. Within the Tulasnellaceae, we detected three distinct fungal OTUs (A, B and C) that were shared among different individuals and/or across site types, with OTUC (Accession no. JQ713581.1) being detected in all of the study species and in both natural and translocated sites (Appendix A.3). These *Tulasnella* fungi were closely-related to known OMF that were previously identified from southwestern China and Yachang Reserve (> 97% sequence similarity; Accession nos. JX545218.1 and JQ713581.1). We also detected three closely-related OTUs belonging to the Tremellaceae group. These three OTUs were detected in both natural and translocated individuals at Yachang. Of the two translocated individuals that associated with a fungus in Tremellaceae, one species had a narrow range, *C. bicolor*, and one had a wide range, *P. dianthum*. A naturally occurring individual of *P. hirsutissimum* in Yachang Reserve also associated with this fungal group. Ceratobasidiaceae fungi were detected in two individuals of *P. hirsutissimum*, one from a natural individual at Bangliang, and the other from a translocated individual at Yachang; these sites represent two spatially separated populations. The OTUs were found to be closely related to an orchid-associated *Ceratobasidium* sp. (see Accession no. GQ850444.1; Appendix A.3). The final Basidiomycete family identified was the Thelephoraceae. Similar to the results for the Tremellaceae, the fungal sequences belonging to the Thelephoraceae were exclusive to individuals at Yachang Reserve and included both translocated and naturally occurring individuals.

Together the elevational narrow-ranging species associated with fewer and less diverse fungal taxa in both the natural and translocated sites and shared more fungal OTUs between the two site types (Figs. 2 and 3). Similar to the individual species breakdown for the elevational narrow-ranging species (not shown here), the majority of fungal sequences from the natural sites belonged to the Tulasnellaceae and

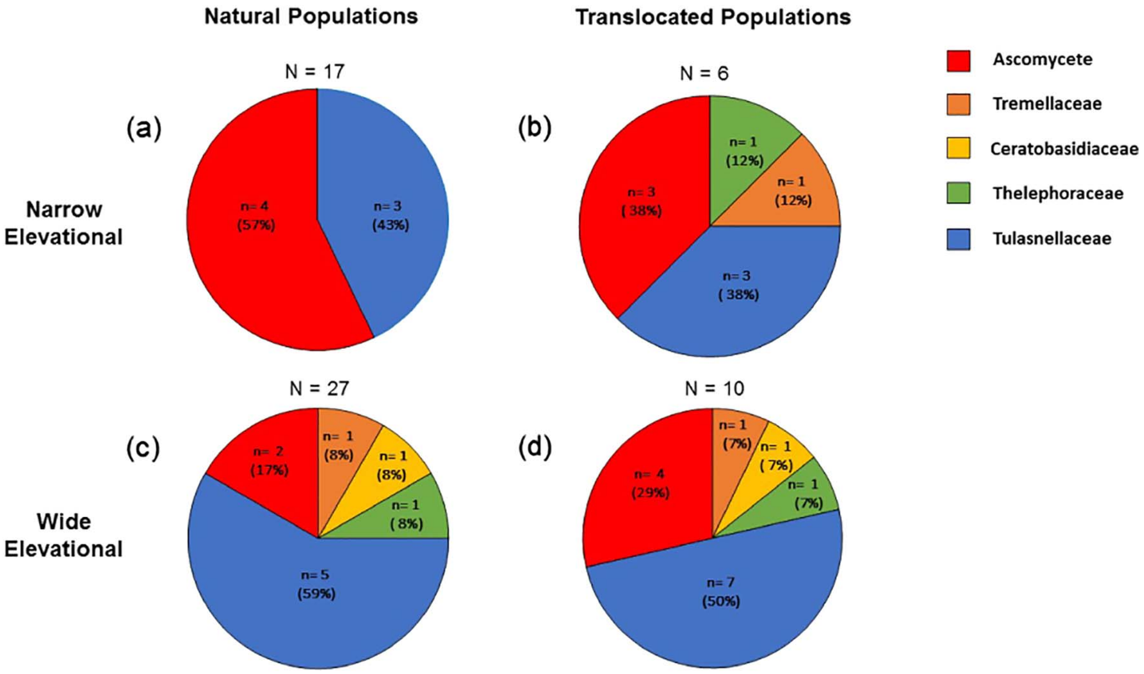


Fig. 2. Pie charts showing the combined differences in fungal communities for the natural and translocated populations; narrow elevational species (a and b) and wide elevational species (c and d) populations. N = number of plants samples, and n = total number of distinct fungal taxa (OTUs).

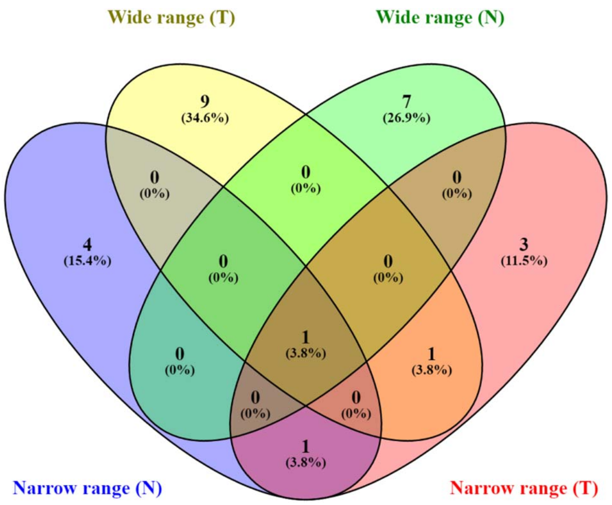


Fig. 3. Four-way Venn diagram showing the total number of OMF OTUs detected for wide elevational range species and narrow elevational range species. (N) represents all natural sites combined and (T) represents all translocated sites combined. Note that for narrow ranging species two taxa were shared between natural and translocated populations (2/10, 20%) and wide ranging species shared only one taxa (1/18, 5%).

Table 1
Number of OMF sequences identified in from narrow and wide range elevational orchid species at six sites. H' = Shannon-Weiner diversity index and J' = the evenness index. Numbers in bold represent mean (H') and (J') values.

Site	No. of plants	No. of OMF sequences	OTU richness	H'	J'
Narrow species					
Orchid garden	6	5	5	1.79	1.11
Dingshu	6	3	2	0.63	0.91
Mulun	5	2	2	0.69	0.96
Jingxi	5	0	0	0	0
Bangliang	1	0	0	0	0
	23	**10**	**9**	**0.62**	**0.59**
Wide species					
Orchid garden	21	20	15	2.60	0.96
Laya	5	5	1	0	0
Mulun	5	0	0	0	0
Bangliang	6	3	3	1.098	1.0
	37	**28**	**21**	**0.93**	**0.49**

Table 2
Number of OMF sequences identified from four orchid species at six natural and one translocated sites. H' = Shannon-Weiner diversity index and J' = the evenness index. Numbers in bold represent mean (H') and (J') values.

Site	No. of plants	No. of OMF sequences	OTU richness	H'	J'
Natural					
Orchid garden	11	9	6	1.67	0.93
Laya	5	5	2	0	0
Dingshu	6	3	2	0.636	0
Mulun	10	2	2	0.693	1.0
Jingxi	5	0	0	0.00	0
Bangliang	7	3	3	1.099	1.0
	44	**22**	**15**	**0.683**	**0.49**
Translocated					
Orchid garden	**16**	**16**	**13**	**2.630**	**0.948**

Ascomycota, with lesser relationships gained at the CI site with the Tremellaceae and Thelephoraceae fungi. There were a total of nine OTUs that were identified as known OMF for the elevational narrow-ranging species (Fig. 2a and b), three of which were unique to the natural sites, four unique to the translocated site, and two shared between site types (data include one Hypocreales OTU for *C. bicolor* that was conserved between sites). In contrast to the elevational narrow-ranging species, combined breakdowns of the fungal communities for the wide-ranging species showed little difference in the types of associations between the natural and translocated populations (Fig. 2c and d). We also saw a dramatic increase in the total number of OMF OTUs detected (24 total OTUs; data include one Hypocreales OTU for *P. hirsitissimum*) as compared to the elevational narrow-ranging species, and a large portion of these OTUs were unique to each site, with only one shared taxon (Fig. 3).

The diversity of the OMF associations for the elevational narrow-ranging species was significantly lower than for the wide-ranging species, as well as for natural versus translocated populations (Tables 1 and 2). Only ten OMF sequences (nine OTUs) were identified overall across the elevational narrow-ranging species sites, and the Shannon-diversity index (H') and evenness (J') were also low; $H' = 0.62$ and $J' = 0.59$, respectively (Table 1). The OMF richness doubled for the wide-ranging species, which together associated with 19 distinct taxa over four study sites; the diversity index was also much higher ($H' = 0.93$) and the evenness estimate was slightly lower ($J' = 0.49$; Table 2). The greater OTU richness and higher diversity estimates for the elevational wide-ranging species are partially attributable to the large number of distinct OMF sequences detected at the 'orchid garden' translocation site. Shannon diversity values were nearly four times as high at the single translocated site as the six natural sites (Table 2). Our rarefication analyses generated curves for translocated individuals at Yachang site, and for the wide ranging species sites that were non-linear and did not flatten at the tail end, suggesting that we likely underestimated our total species richness for these species (Appendix A.4).

4. Discussion

Our results support the concept that wide-ranging orchid species associate with a greater number and more diverse groups of fungi (generalists). Here translocated orchids were not hampered by the ability to establish mycorrhizal relationships with new fungi in the recipient ranges; this was true for both elevational narrow-ranging species (subjected to CI) and wide-ranging species (subjected to conventional translocation). This empirical evidence supports the feasibility of managed relocation of orchids. Since the translocation in 2006, orchid survivorship remains at 0–70% across all species, with extreme weather events being the major reason for mortality (Liu et al., 2015). It appeared from our study that specificity in mycorrhizal interactions might have been less of a limiting factor in the successful establishment of translocated orchids than one would expect.

Mycorrhizal association is a strong factor that determines where and in what abundance orchids can be found (Zettler et al., 2003; Swarts et al., 2010; McCormick et al., 2009, 2012; Jacquemyn et al., 2012) and a lack of appropriate beneficial fungal partners in the recipient sites could prevent the establishment of translocated individuals. In the absence of accurate assessments of the abundance and distribution of OMF across the different environments, it is logical to expect that orchids with a higher degree of mycorrhizal specificity and/or narrow range limits may have more difficulty acquiring appropriate fungal partners than those with less specificity. We have documented orchid species that are rare in their native ranges, successfully establishing in introduced ranges, such as *Cyrtopodium polyphyllum* (Liu and Pemberton, 2010). However, it is not clear whether seeds of the studied species can germinate at the translocation site. Keel et al. (2011), demonstrated that seeds of *Habenaria repens*, a very wide-ranging species from a southern ecotype at sea-level were capable of germinating at higher latitudes, using the mycorrhizal fungi already present in the recipient habitat. This showed evidence of recruitment potential for certain translocated orchids, a fundamental condition in creating a self-sustaining orchid population.

We did find that the extremely rare endemic *G. eulophioides*, associated with the fewest and least diverse groups of mycorrhizal fungi even when compared to the other narrow-ranging species *C. bicolor*. However, the single translocated *G. eulophioides* individual was transplanted one year prior to sampling and might not have been able to complete the process of acquiring its symbiotic partners, whereas the other species that had been translocated several years earlier might have had sufficient time to do so. Although we detected fewer overall fungal taxa for *C. bicolor*, the community assemblage was more similar to the fungal communities of the wider ranging species which utilized diverse groups of basidiomycetes in the translocated populations. Interestingly, only one of the wide-ranging species' fungal associates was present in both the natural and translocated ranges. Despite the narrow-ranging species association with a lower diversity of OMF overall, two of their fungal taxa were shared between the native and translocated ranges. This suggests that the elevational narrow-ranging species, particularly *C. bicolor*, may be using a narrower group of fungi that are available over different habitats while the wide-ranging species can associate with a broader group of mycorrhizal fungi and multiple taxa within Tulasnellaceae that may be more site or habitat specific. We cannot rule out the possibility of root fungi being transported from the natural population sites. However, since we detected so few fungi that were shared across the different location types, the results still support the conclusion that these species are able acquire new fungi from the environment. High rates of adaptive radiation for fungi in this warm and humid region are not implausible, particularly in core *Tulasnella* because of the high ITS sequence variation in these fungi even within the same taxa (Binder et al., 2005; Moncalvo et al., 2006; Linde et al., 2014). Yet, all of the orchid species had a strong relationship with Tulasnellaceae and in particular with the *Tulasnella* taxa OTU C (Accession no. JQ713581.1), which was widely detected. Due to the broad compatibility of this distinct fungal taxon, further research to identify its full life history and environmental requirements are highly recommended. Efforts to safeguard living cultures for this taxa have been undertaken at Xishuangbanna Tropical Botanic Garden. Furthermore, preservation of those habitats that support the OTU C fungus is of conservation importance to Yachang Reserve. This strategy should also be applied to other fungal taxa that are shared between natural and translocated sites.

In addition to the associations formed with likely beneficial fungal groups such as Tulasnellaceae, interactions with fungal groups that are pathogenic or detrimental to plant survivorship must also be considered when translocating species. However, very little research has been done regarding the identification of non-beneficial root fungi in orchids, particularly in ascomycete fungi and the effects of these associations on overall plant health and survivorship. Ascomycete fungi that associate with orchids are generally considered to be either endophytic (root-colonizing symbionts) or pathogens, with the latter being the more common relationship (Queloz et al., 2011). These fungi are rarely considered mycorrhizal, and are commonly detected as intercellular hyphae within the root (Těšitelová et al., 2012). One rare confirmed mutualist in the ascomycetes is the order Helotiales, which was detected in our study. It has also been detected in other terrestrial orchids (Těšitelová et al., 2012), and is known to enhance plant performance (Tedersoo et al., 2009). Ultimately we can only surmise the ability of any of the fungi to serve as functional mycorrhizae and/or pathogens if we can establish them with orchids under controlled conditions. Here we have chosen a conservative approach and considered only the basidiomycete fungi, and the Hypocreales and Ascomycete fungi that were detected in more than one site to be potential OMF.

5. Conclusions

This research provides an important assessment of the changes in the biotic associations of taxa subjected to a rarely attempted conservation introduction project. Our results support the notion that conservation translocations, including CI can be a viable tool to mitigate extinction risk, including species with high biotic dependency. Based upon the high diversity of fungi detected in the roots, our findings also suggest that the Yachang Reserve site is exceptionally rich in OMF diversity when compared to the surrounding natural sites we sampled. However, it is important to acknowledge that we did not estimate the abundance and distribution of the OMF communities within the environment independent of the orchids. Regardless, the high diversity of OMF detected in the roots is likely one of the main drivers of the extreme and highly localized diversity of orchid species observed there, as well as the success observed in these orchid translocations thus far.

Future studies should focus on the seed germination ability of translocated taxa at the recipient site and to determine whether the fungi we identified as OMF are indeed functional mycorrhiza and capable of facilitating seed germination and seedling development. Studies have shown that in some orchids the fungi associated with adult plants are more diverse that the fungi necessary for seed germination (McCormick et al., 2006, 2012). When seeds become available, it will be critical to conduct germination trials on the recovered fungal isolates as well as sowing seeds in situ to determine whether translocated populations will be viable in the future. This study provides additional support for the strong relationship between fungal specificity and plant diversity and distribution. Yet in the face of anthropogenic-driven change, this heavy dependence upon other organisms may put specialist plants like orchids at more risk than other generalist plant species. The study may also shed light to determine how mycorrhizae may vary between orchid species, between and within habitats, both of which are critical to understanding orchid ecology and to develop a more comprehensive decision making framework for future conservation introductions.

Acknowledgments

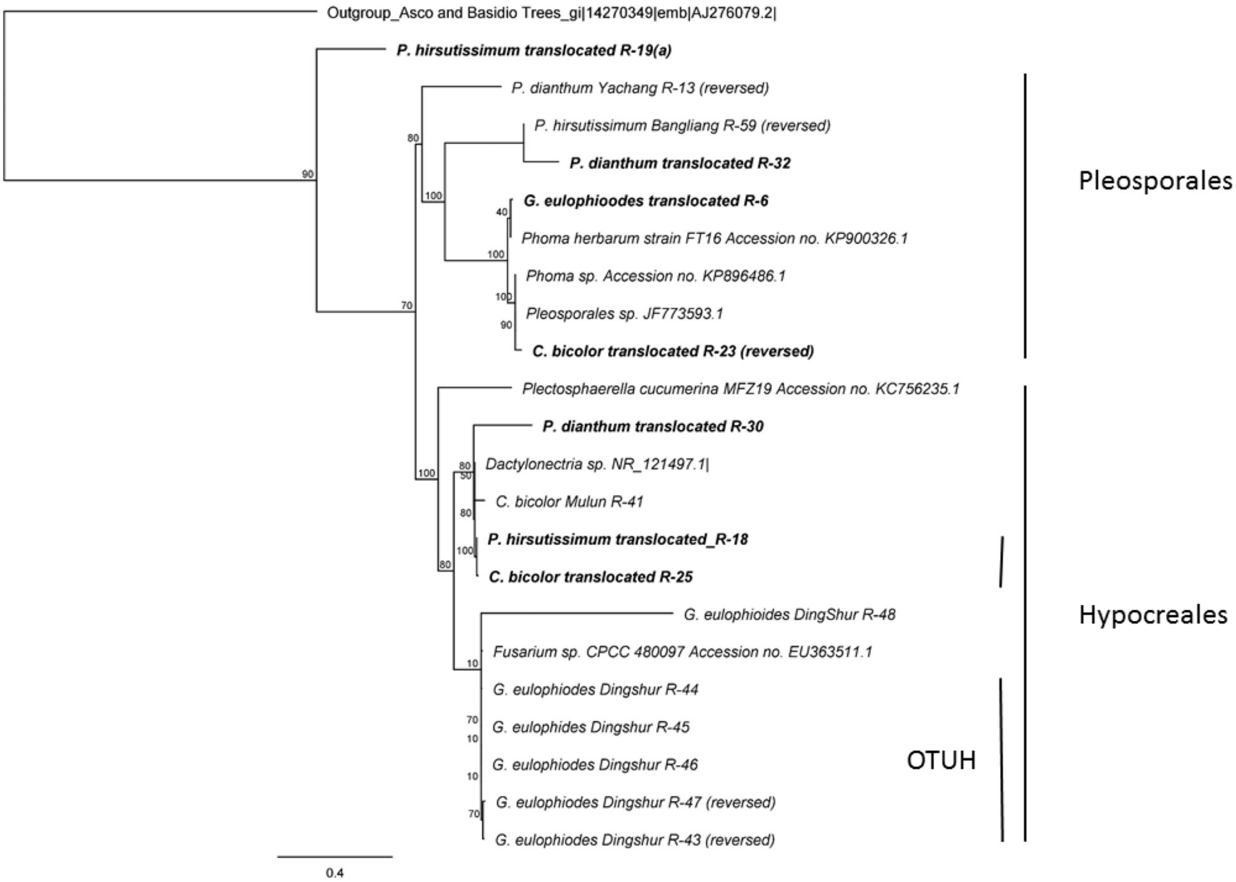
Thanks to Xishuangbanna Tropical Botanical Garden and Chinese Academy of Sciences who kindly hosted our research. Funding was provided by the National Science Foundation of China (grant no. 31360146 to H. Liu, and no. 31470450 to J.Y. Gao) and from the Guangxi Science and Technology Bureau (grant no.12217-04) to H. Liu, and the National Science Foundation's East Asian Pacific Islands Summer Institute Grant Award #1414633 to JL Downing. The research was also supported through resources and funds provided by Fairchild Graduate Fellowship, Kelly Foundation Tropical Botany Fellowship and the McKnight Dissertation Fellowship. Thanks to the rangers at Yachang and Mulun Nature Reserves. Special gratitude goes out to Jessie Han from the Chinese Academy of Sciences; we thank you for your support throughout the fieldwork period in Guangxi. We also acknowledge and thank Dennis Giardina for his contributions toward the development of the manuscript. Special thanks to Yuying Zhang for help with figures.

Appendix A

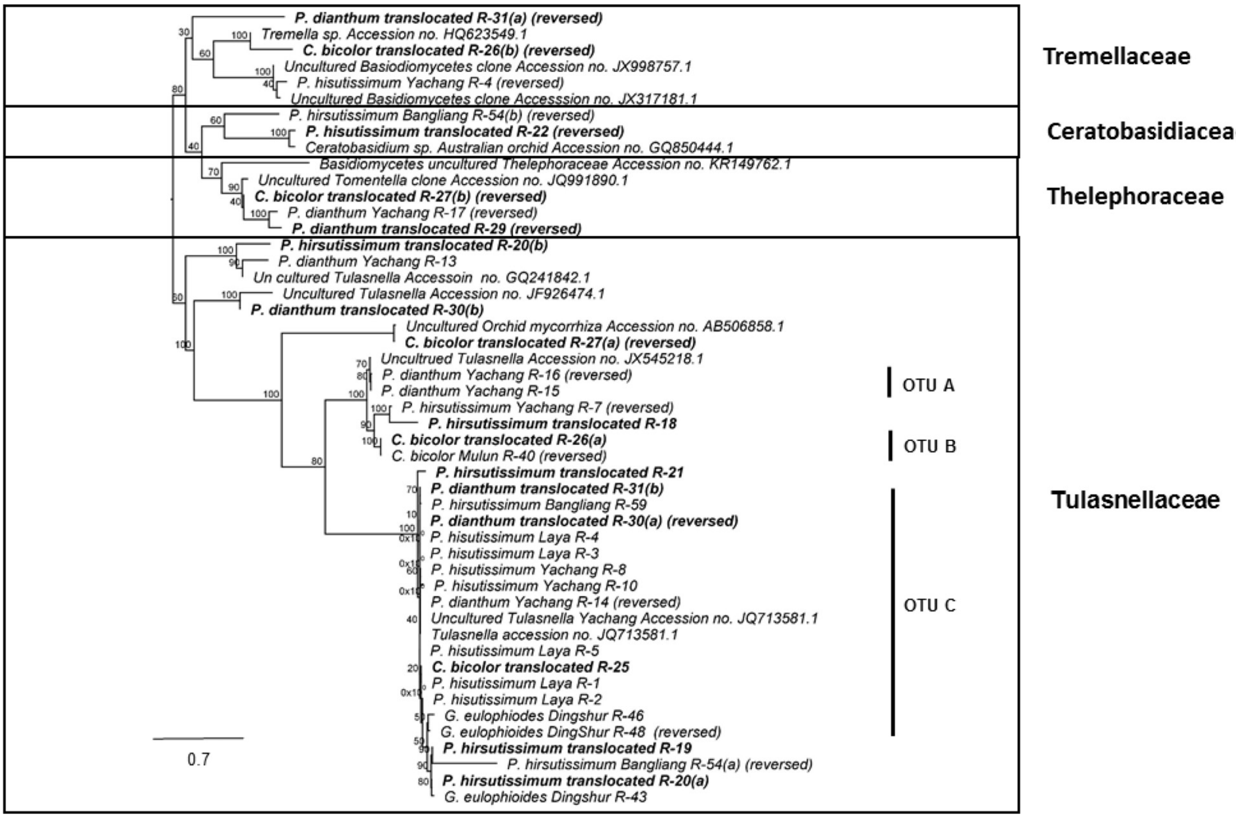
Appendix A.1

Number of plants sampled for each orchid species included in this study at each site sampled. Note that only samples of **natural populations** were included here because all translocated populations were located in the "orchid garden" site in the Yachang Orchid National Nature Reserve.

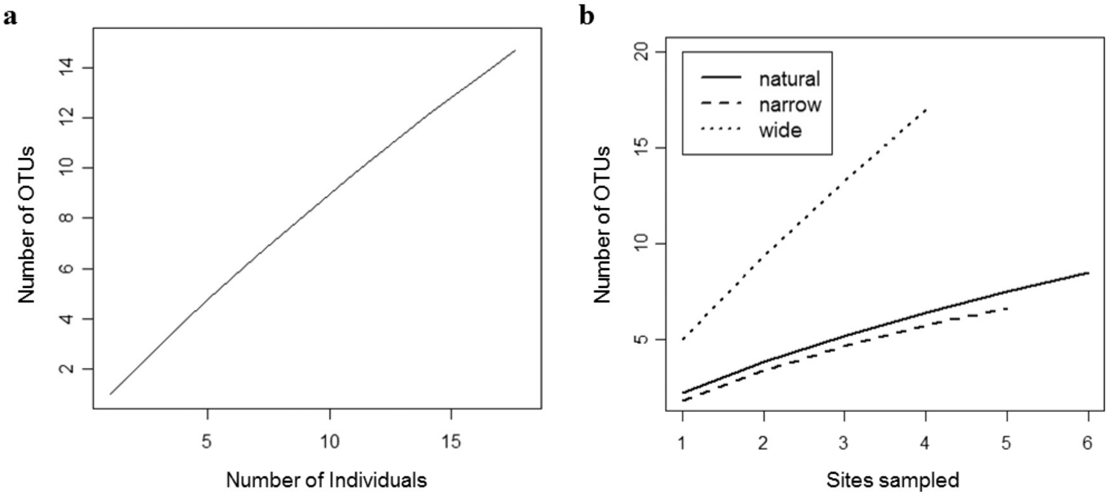
Species	Site					
	Yachang		Dingshu	Mulun	Bangliang	Jingxi
	Orchid garden	Laya				
Geodorum eulophioides	–	–	**6**	–	–	–
Cymbidium bicolor	–	–	–	**5**	**1**	**5**
Paphiopedilum dianthum	**5**	–	–	–	–	–
Paphiopedilum hirsutissimum	**6**	**5**	–	**5**	**6**	–



Appendix A.2. Maximum- likelihood trees (500 bootstraps) of ITS sequences of Ascomycete fungi isolated from four orchid species, *Geodorum eulophioides* and *Cymbidium bicolor* (narrow elevational ranges), and *Paphiopedilum dianthum* and *Paphiopedilum hirsutissimum* (wide elevational ranges) at natural and translocated sites in Guangxi Province. Sequences in bold and italics represent translocated plants. Clade named OTUH represents ≥ 97% pairwise sequence similarities.



Appendix A.3. Maximum- likelihood trees (500 replicates with bootstrapping percentages) of ITS sequences of Basidiomycete fungi isolated from four orchid species, *Geodorum eulophioides* and *Cymbidium bicolor* (narrow elevational ranges), and *Paphiopedilum dianthum* and *Paphiopedilum hirsutissimum* (wide elevational ranges) at natural and translocated sites in Guangxi Province. Sequences in bold and italics represent translocated plants. Tulasnellaceae OTU clades represent ≥ 95% pairwise sequence similarities.



Appendix A.4. Rarefaction curves showing the number of OTUs observed for (a) translocated individuals sampled at Yachang Orchid Reserve, and (b) sample-based rarefaction curves for natural, narrow range, and wide range species.

References

Binder, M., Hibbett, D.S., Larsson, K.H., Larsson, E., Langer, E., Langer, G., 2005. The phylogenetic distribution of resupinate forms across the major clades of mushroom-forming fungi (Homobasidiomycetes). Syst. Biodivers. 3 (2), 113–157.

Caldwell, B.A., Castellano, M.A., Griffiths, R.P., 1991. Fatty acid esterase production by ectomycorrhizal fungi. Mycologia 83 (2), 233–236.

Cribb, P.J., Kell, S.P., Dixon, K.W., Barrett, R.L., 2003. Orchid conservation: a global perspective. In: Dixon, K.W., Kell, S.P., Barrett, R.L. (Eds.), Orchid Conservation. Natural History Publications, Kota Kinabalu, Sabah, pp. 1–24.

Faith, D.P., 1992. Conservation evaluation and phylogenetic diversity. Biol. Conserv. 61 (1), 1–10.

Gardes, M., Bruns, T.D., 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol. Ecol. 2 (2), 113–118.

Hewitt, N., Klenk, N., Smith, A.L., Bazely, D.R., Yan, N., Wood, S., MacLellan, J.I., Lipsig-Mumme, C., Henriques, I., 2011. Taking stock of the assisted migration debate. Biol. Conserv. 144, 2560–2572. http://dx.doi.org/10.1016/j.biocon.2011.04.031.

Hoegh-Guldberg, O., Hughes, L., McIntyre, S., Lindenmayer, D.B., Parmesan, C., Possingham, H.P., Thomas, C.D., 2008. Assisted colonization and rapid climate change. Science 321 (5887), 345–346.

Hunter, M.L., 2007. Climate change and moving species: furthering the debate on assisted colonization. Conserv. Biol. 21 (5), 1356–1358.

International Union for Conservation of Nature (IUCN), 2013. Guidelines for reintroductions and other conservation translocations. In: Version 1.0. IUCN Species Survival Commission, Gland, Switzerland and Cambridge, UK.

Jacquemyn, H., Brys, R., Lievens, B., Wiegand, T., 2012. Spatial variation in below-ground seed germination and divergent mycorrhizal associations correlate with spatial segregation of three co-occurring orchid species. J. Ecol. 100 (6), 1328–1337.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Mentjies, P., Drummond, A., 2012. Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28 (12), 1647–1649.

Keel, B.G., Zettler, L.W., Kaplin, B.A., 2011. Seed germination of *Habenaria repens* (Orchidaceae) in situ beyond its range, and its potential for assisted migration imposed by climate change. Castanea 76 (1), 43–54.

Linde, C.C., Phillips, R.D., Crisp, M.D., Peakall, R., 2014. Congruent species delineation of *Tulasnella* using multiple loci and methods. New Phytol. 201 (1), 6–12.

Liu, H., 2010. Geodorum Eulophioides – challenges for conserving one of the rarest Chinese orchids. Orchids (March issue), 161–162.

Liu, H., Pemberton, R., 2010. Pollination of an invasive orchid, *Cyrtopodium polyphyllum* (Orchidaceae), by an invasive oil-collecting bee, *Centris nitida*, in southern Florida. Botany 88 (3), 290–295.

Liu, H., Feng, C.-L., Luo, -B., Chen, B.-S., Wang, Z.-S., Gu, H.-Y., 2010. Potential challenges of climate change to orchid conservation in a wild orchid hotspot in southwestern China. Bot. Rev. 76, 174–192.

Liu, H., Feng, C.L., Chen, B., Wang, Z., Xie, X.Q., Deng, Z., Wei, X., Liu, S., Zhang, Z., Luo, Y., 2012. Overcoming extreme weather challenges: successful but variable assisted colonization of wild orchids in southwestern china. Biol. Conserv. 150 (1), 68–75.

Liu, H., Ren, H., Liu, Q., Wen, X.Y., Maunder, M., Gao, J.Y., 2015. Translocation of threatened plants as a conservation measure in China. Conserv. Biol. 29, 1537–1551.

Lunt, I.D., Byrne, M., Hellmann, J.J., Mitchell, N.J., Garnett, S.T., Hayward, M.W., Zander, K.K., 2013. Using assisted colonisation to conserve biodiversity and restore ecosystem function under climate change. Biol. Conserv. 157, 172–177.

McCormick, M.K., Whigham, D.F., Sloan, D., O'Malley, K., Hodkinson, B., 2006. Orchid-fungus fidelity: a marriage meant to last? Ecology 87 (4), 903–911.

McCormick, M.K., Whigham, D.F., O'Neill, J.P., Becker, J.A., Werner, S., Rasmussen, H.N., Bruns, T.D., Taylor, D.L., 2009. Abundance and distribution of *Corallorhiza odontorhiza* reflect variations in climate and ectomycorrhizae. Ecol. Monogr. 79 (4), 19–635.

McCormick, M.K., Taylor, D.L., Juhaszova, K., Burnett Jr., R.K., Whigham, D.F., O'Neill, J.P., 2012. Limitations on orchid recruitment: not a simple picture. Mol. Ecol. 21 (6), 1511–1523.

McLachlan, J.S., Hellmann, J.J., Schwartz, M.W., 2007. A framework for debate of assisted migration in an era of climate change. Conserv. Biol. 21 (2), 297–302.

Minteer, B.A., Collins, J.P., 2010. Move it or lose it? The ecological ethics of relocating species under climate change. Ecol. Appl. 20 (7), 1801–1804.

Mitchell, C.E., Agrawal, A.A., Bever, J.D., Gilbert, G.S., Hufbauer, R.A., 2006. Biotic interactions and plant invasions. Ecol. Lett. 9, 726–740.

Moncalvo, J.M., Nilsson, R.H., Koster, B., Dunham, S.M., Bernauer, T., Matheny, P.B., Danell, E., 2006. The cantharelloid clade: dealing with incongruent gene trees and phylogenetic reconstruction methods. Mycologia 98 (6), 937–948.

Oksanen, J., et al., 2009. Vegan: Community Ecology Package. R package Version 1.15-4. http://CRAN.R-project.org/package5veganæ .

Pemberton, R.W., Liu, H., 2008. Naturalized orchid bee pollinates resin-reward flowers in Florida: novel and known mutualisms. Biotropica 40 (6), 714–718.

Queloz, V., Sieber, T.N., Holdenrieder, O., McDonald, B.A., Grünig, C.R., 2011. No biogeographical pattern for a root-associated fungal species complex. Glob. Ecol. Biogeogr. 20, 160–169.

R Development Core Team, 2011. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available in: http://www.r-project.org.

Rasmussen, H.N., 1995. Terrestrial Orchids: From Seed to Mycotrophic Plant. Cambridge University Press.

Reichard, S., Liu, H., Husby, C., 2012. Is managed relocation another pathway of biological invasion? In: Maschinski, J., Haskins, K.E. (Eds.), Managing Eden: Plant Reintroduction's Promises, Perils, and Uses in a Changing Climate. Island Press, Washington, D.C., pp. 243–262.

Ricciardi, A., Simberloff, D., 2009. Assisted colonization is not a viable conservation strategy. Trends Ecol. Evol. 24 (5), 248–253.

Richardson, D., Allsopp, N., D'Antonio, C., Milton, S., Rejmanek, M., 2000. Plant invasions - the role of mutualisms. Biol. Rev. 75 (1), 65–93.

Roche, S.A., Carter, R.J., Peakall, R., Smith, L.M., Whitehead, M.R., Linde, C.C., 2010. A narrow group of monophyletic Tulasnella (*Tulasnellaceae*) symbiont lineages are associated with multiple species of Chiloglottis (*Orchidaceae*): implications for orchid diversity. Am. J. Bot. 97 (8), 1313–1327.

Shi, J.J., Cheng, D., Luo, S.Y., Liu, Tan H.S., Luo, Y.B., 2007. An orchid paradise: the Yachang orchid nature reserve, in Guangxi, China. Orchid Rev. 115, 188–195.

Swarts, N.D., Sinclair, E.A., Francis, A., Dixon, K.W., 2010. Ecological specialization in mycorrhizal symbiosis leads to rarity in an endangered orchid. Mol. Ecol. 19 (15), 3226–3242.

Tanaka, N., Yukawa, T., Htwe, K.M., Koyama, T., Murata, J., 2011. New or noteworthy plant collections from Myanmar (7): fourteen additional species of Orchidaceae. *Acta phytotaxonomica et geobotanica*. APG 61 (3), 161–165.

Taylor, D.L., 2004. Myco-heterotroph-fungus marriages - is fidelity over-rated? New Phytol. 163 (2), 217–221.

Taylor, D.L., McCormick, M.K., 2008. Internal transcribed spacer primers and sequences for improved characterization of Basidiomycetous orchid mycorrhizas. New Phytol. 177 (4), 1020–1033.

Tedersoo, L., Pärtel, K., Jairus, T., Gates, G., Põldmaa, K., Tamm, H., 2009. Ascomycetes associated with ectomycorrhizas: molecular diversity and ecology with particular reference to the Helotiales. Environ. Microbiol. 11 (12), 3166–3178.

Těšitelová, T., Těšitel, J., Jersáková, J., Řihová, G., Selosse, M.A., 2012. Symbiotic germination capability of four Epipactis species (*Orchidaceae*) is broader than expected from adult ecology. Am. J. Bot. 99, 1020–1032.

Willis, S.G., Hill, J.K., Thomas, C.D., Roy, D.B., Fox, R., Blakeley, D.S., Huntley, B., 2009. Assisted colonization in a changing climate: a test-study using two UK butterflies. Conserv. Lett. 2 (1), 46–52.

Zettler, L.W., Sharma, J., Rasmussen, F.N., 2003. Mycorrhizal diversity. In: Dixon, K.W., Kell, S.P., Barrett, R.L., Cribb, P.J. (Eds.), Orchid Conservation, pp. 205–226.