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The impact of life form on the architecture of orchid mycorrhizal networks in tropical forest — Source link

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The impact of life form on the architecture of orchid mycorrhizal networks in tropical forest

Abstract

Understanding the processes that determine the architecture of interaction networks represents a major challenge in ecology and evolutionary biology. One of the most important interactions involving plants is the interaction between plants and mycorrhizal fungi. While there is a mounting body of research that has studied the architecture of plant-fungus interaction networks, less is still known about the potential factors that drive network architecture. In this study, we investigated the architecture of the network of interactions between mycorrhizal fungi and 44 orchid species that represented different life forms and co-occurred in tropical forest and assessed the relative importance of ecological, evolutionary and co-evolutionary mechanisms determining network architecture. We found 87 different fungal operational taxonomic units (OTUs), most of which were members of the Tulasnellaceae. Most orchid species associated with multiple fungi simultaneously, indicating that extreme host selectivity was rare. However, an increasing specificity towards Tulasnellaceae fungal associates from terrestrial to epiphytic and lithophytic orchids was observed. The network of interactions showed an association pattern that was significantly modular (M = 0.7389, M_{random} = 0.6998) and nested (NODF = 5.53, $P < 0.05$). Terrestrial orchids had almost no links to modules containing epiphytic or lithophytic orchids, while modules containing epiphytic orchids also contained lithophytic orchids. Within each life form several modules were observed, suggesting that the processes that organize orchid-fungus interactions are independent of life form. The overall phylogenetic signal for both partners in the

interaction network was very weak. Overall, these results indicate that tropical orchids associate with a wide number of mycorrhizal fungi and that ecological rather than phylogenetic constraints determine network architecture.

Keywords

orchid life form, interaction network, modularity, nestedness, orchid mycorrhiza

Introduction

Understanding the ecological, evolutionary and co-evolutionary processes that shape the architecture of interaction networks represents one of the main challenges in ecology and evolutionary biology (Bascompte 2010). In general, two types of interaction networks have been described (summarized in Bascompte and Jordano 2014). On the one hand, interaction networks may consist of several subnetworks or modules of species that interact more with each other than with other species in the network (e.g. Olesen et al. 2007, Rezende et al. 2009, Fortuna et al. 2010, Donatti et al. 2011, Jacquemyn et al. 2015). This type of network architecture is typically encountered in species displaying antagonistic interactions, but can also be found in mutualistic systems, particularly if they contain more than 150 links (Olesen et al. 2007). Most mutualistic networks, on the other hand, lack modularity and tend to be organized in a nested pattern (Olesen et al. 2007, Thébault and Fontaine 2010).

One of the most ubiquitous interactions involving plants is the association between plants and mycorrhizal fungi. In this interaction, fungi facilitate plants with the acquisition of essential nutrients from the soil, and in return, plants generally transfer photosynthetically fixed carbon to their fungal partners (Bonfante & Genre, 2010; van der Heijden et al., 2015). Based on a mounting body of research describing the architecture of plant-fungus interaction networks (e.g. Chagnon et al. 2012, Montesinos-Navarro et al. 2012, Bahram et al. 2014, Toju et al. 2014, 2016), it has recently been suggested that the degree of nestedness and modularity are organized along a continuous gradient that is mainly driven by nutrient properties and the level of mutualism (van der Heijden et al. 2015). In general, arbuscular mycorrhizal interaction networks tend to be nested (e.g. Chagnon et al. 2012, Montesinos-Navarro et al. 2012), whereas orchid mycorrhizal networks

50 are often modular (e.g. Martos et al. 2012, Jacquemyn et al. 2015). Ectomycorrhizal networks are
51 predicted to be somewhere in between (van der Heijden et al. 2015). This simple prediction has
52 recently been challenged by Põlme et al. (2018), who summarized information on network
53 structure from a large number of studies investigating arbuscular mycorrhizal, ectomycorrhizal,
54 ericoid mycorrhizal and orchid mycorrhizal interactions. Their meta-analysis showed that the
55 degree of nestedness was not significantly affected by fungal guild, but that modularity was higher
56 in ericoid and orchid mycorrhizal fungi than in the other fungal guilds. However, the precise
57 mechanisms responsible for generating such patterns in plant-fungus interaction networks remain
58 remain poorly understood (Chagnon 2016; Põlme et al. 2018).
59 With an estimated number of >27.000 species, the orchid family encompasses a considerable
60 diversity in life forms, with approximately 30% of species being terrestrial and the remaining
61 70% being known for their potential to explore highly stressful epiphytic and lithophytic habitats
62 (Gravendeel et al. 2004, Dearnaley et al. 2012). Regardless of their life form, orchids invariably
63 rely on mycorrhizal fungi for seed germination and subsequent establishment of seedlings, and
64 most orchids retain mycorrhizal associations at adulthood as well (Rasmussen and Rasmussen
65 2009). When multiple orchids co-occur, they often tend to associate with different sets of
66 mycorrhizal fungi, leading to mycorrhizal networks that are significantly modular (Martos et al.
67 2012, Jacquemyn et al. 2015). However, it remains unclear what factors exactly drive modularity
68 in orchid mycorrhizal networks.
69 Since most orchid mycorrhizal fungi are free-living saprophytes that exhibit broad dispersal
70 patterns, their distribution is assumed to be independent of their partner plants (Smith and Read
71 2008, McCormick et al. 2012, McCormick and Jacquemyn 2014, Jacquemyn et al. 2017). Because

72 the availability of above-ground water and nutrient supplies decreases from terrestrial to
73 lithophytic habitats, it can be predicted that more stressful environments may limit the occurrence
74 and abundance of orchid mycorrhizal fungi or select for a limited set of strains that are capable of
75 surviving in these environments. Extreme host selectivity and specialization (Taylor and Bruns
76 1997, Shefferson et al. 2005) may therefore to some extent explain the significant modularity
77 typically found in orchid-fungus networks. However, many orchid species have been shown to
78 associate with multiple fungi at the same time (Waterman et al. 2011, Jacquemyn et al. 2014,
79 2015), so that extreme host selectivity cannot be the sole factor explaining modularity in orchid
80 mycorrhizal networks. The alternative hypothesis would be that modularity is driven by
81 ecological constraints (Martos et al. 2012). In this case, variation in local growth conditions and
82 the patchy distribution of compatible fungi may explain modularity (Jacquemyn et al. 2012,
83 2014). In case host selectivity and specialization are also phylogenetically conserved (Shefferson
84 et al. 2005, Xing et al. 2017), the network of interactions and modularity should also show a
85 significant phylogenetic signal (Jacquemyn et al. 2011).

86 To test these hypotheses, we assessed the relative importance of ecological and evolutionary
87 constraints on the structure of the network of interactions between orchids displaying various life
88 forms and their mycorrhizal fungi. More specifically, we set out to investigate the following
89 questions:

90 1. Does mycorrhizal fungal community composition significantly differ between orchids
91 with different life forms?
92 2. Is the interaction network between orchids and mycorrhizal fungi characterized by
93 significant nestedness and/or modularity?

3. Can the structure of observed orchid mycorrhizal network be explained by extreme host selectivity, phylogenetic or ecological constraints?

To answer these questions, we investigated mycorrhizal associations in 44 different orchid species that occurred in moist tropical forest of Xishuangbanna in the Yunnan province, China, and that displayed different life forms.

Materials and methods

Study sites and sampling

This study was conducted in the Xishuangbanna region (21°8´-22°36´ N, 99°56´- 101°51´ E) in the southern part of the Yunnan Province, China (Fig. S1). This region is biogeographically situated in a transitional zone from tropical South-east Asia to temperate East-Asia. Xishuangbanna has the largest area of tropical forest remaining in the country and contains approximately 5000 species of higher plants (Zhang and Cao 1995). This region is characterized by a semi-humid, tropical monsoon climate with annual temperatures varying between 15.1°C and 21.7°C and the annual rainfall between 1196 and 2492 mm. A national nature reserve (100° 16′-101° 50′ E, 21° 10′ -22° 24′ N) was established in Xishuangbanna in 1958, which consists of five subreserves: Mengyang, Menglun, Mengla, Shangyong and Manggao. The area is renowned for its high orchid diversity: Liu et al. (2015) identified 426 orchid species from 115 genera in this area.

All samples were collected in August 2016 in the Menglun subreserve (101° 25′ E, 21° 41′ N) (Fig. S1), which contains around 60 orchid species (Liu et al. unpublished data). Because about one third of these species are extremely rare (less than two individuals) (Liu et al. 2015),

mycorrhizal associations were investigated in 44 species belonging to 25 genera for which more than four individuals could be found in the study area. These orchid species include 13 terrestrial, 17 epiphytic, 6 lithophytic, and 8 species displaying both an epiphytic and lithophytic life form (Table S1). For each orchid species, four to five individual plants were selected. Individuals of a single species were selected in such a way that they were at least 15m apart. Orchid species that were both epiphytic and lithophytic were collected separately. In total, 245 plant individuals were sampled (62 terrestrial, 118 epiphytes and 65 lithophytes). For each individual plant, we collected more than 4 independent root fragments (about 2 cm long) whenever possible without dislodging the plant. Root samples were refrigerated until processing (within 3 days of sampling). Sampled roots were surface-sterilized with ethanol (70 %) for 30 s and rinsed three times in sterile water to avoid unnecessary contaminations 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.

Assessment of mycorrhizal communities

Genomic DNA was extracted from two root sections per orchid individual using the DNeasy PlantMini Kit (Qiagen) following the manufacturer's instructions. To describe the basidiomycetous mycorrhizal community, the effectiveness of several broad-spectrum basidiomycete primer pairs, including ITS1-OF/ITS4-OF (Taylor and McCormick 2008), ITS1-OF (White et al. 1990) / ITS4-Tul (Taylor and McCormick 2008) and ITS1-OF/ITS4 (White et

al. 1990) were tested. ITS1-OF and ITS4-OF gave the most consistent amplification with high yields. Clone libraries were constructed following PCR amplification with the primers ITS1-OF and ITS4-OF. PCR conditions were as follows: 94 °C for 3 min, followed by 32 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 55 s. The final cycle was followed by an extension of 7-min at 72 °C. Clone libraries were constructed for each sample using the following procedure: PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and cloned using the pGEM-T Easy Vector (TaKaRa, Japan) and competent high DH5α. Ninety-six clones were randomly selected from each library and sequenced using the M13 forward primer. Our previous studies have shown that this was a large enough clonal pool for assessing total species diversity and sequencing completeness (Xing et al. 2015, 2017). All clones were sequenced by Genewiz Inc. (Beijing, China). MEGA6 software (Tamura et al. 2013) was used to align DNA sequences from all the samples. UPARSE (Edgar 2013) was used to group the sequences into operational taxonomic units (OTUs), in which sequences exceeding 97% homology were clustered into the same OTU. This threshold is the usual proxy for species delimitation among basidiomycetes (Martos et al. 2012, Jacquemyn et al. 2015, 2017). Rarefaction analyses were used to assess completeness of the sequencing. Rarefaction analyses were conducted using EstimateS version 9.0 (Colwell 2013). The different OTUs were identified using the BLAST algorithm and deposited in GenBank (MH005840-MH005926).

Plant ITS amplification and sequencing

From each orchid species, one healthy leaf was selected for genomic DNA extraction. Plant DNA was extracted using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's

158 instructions. The rDNA's ITS region was amplified with the primers ny43 and ny47 (Cameron
159 2005). The PCR conditions were as follows: 94°C for 3 min, followed by 94°C for 30 s, 55°C for
160 30 s and 72°C for 55 s, 32 cycles, and extension at 72°C for 7 min. Amplification products were
161 checked by electrophoresing on a 1.0% agarose gel to ensure that a single DNA band of the
162 expected size was produced. For sequencing, a QIAquick PCR purification kit (Qiagen, Germany)
163 was used to purify PCR products from unincorporated nucleotides, excess primer and salts, as
164 well as primer dimers. Purified PCR products were sequenced by GENEWIZ Inc. (Beijing,
165 China).

166 **Data analysis**

167 *Fungal diversity*

168 To compare the phylogenetic diversity of fungal associations between orchid species, we first
169 constructed a ML tree for all the fungal OTUs identified in this research. The 87 fungal OTU
170 sequences were aligned using Clustal X version 2.0 (Larkin et al. 2007). The T92+G model of
171 evolution was identified as the best-fit model for the fungal OTU dataset using the Akaike
172 Information Criterion implemented in jModelTest 2 (Darriba et al. 2012). The ML phylogeny was
173 constructed with RAxML 7.2.8 (Stamatakis et al. 2008). Clade support was estimated with
174 RAxML through a nonparametric bootstrap analysis of 1,000 pseudo-replicate datasets.

175 The phylogenetic distances between the OTUs from this tree were used to calculate the
176 phylogenetic diversity (PD; Faith 1992) and mean pairwise distance (MPD; Webb et al. 2002) of
177 the OTUs associated with each orchid species. All calculations were done using the software
178 package 'picante' (Kembel et al. 2010) in R (R Development Team 2016). Univariate analysis of

179 variance (ANOVA) was used to test the hypothesis that the number of OTUs, phylogenetic
180 diversity (PD) and the mean pairwise distance (MPD) differed significantly between terrestrial,
181 epiphytic and lithophytic orchids.

182 *Network architecture*

183 To describe the properties of the interaction network, we first assembled all interactions between
184 orchid species and fungal OTUs based on individual occurrences of fungal OTUs on orchid roots
185 and applied all subsequent network analysis to the species-level matrix. Two frequently used
186 network measures were used to describe the architecture of the network: nestedness and
187 modularity. We used a nestedness metric that is based on overlap and decreasing fill (NODF) to
188 calculate the degree of nestedness. This measure is less dependent on the size of the shape of the
189 interaction matrix than other measures of nestedness and therefore provides an unbiased measure
190 to estimate the degree of nestedness (Almeida-Neto et al. 2008). To assess the significance of
191 nestedness, two different null models were used (Guimarães and Guimarães 2006). In the first
192 null model, each cell in the interaction matrix has the same probability of being occupied. This
193 null model is very general and does not take into account the fact that the number of connections
194 per species may vary substantially. A more conservative null model would therefore be a model
195 in which the probability of drawing an interaction is proportional to the degree of specialization
196 (Bascompte et al. 2003). In this null model, the probability of each cell being occupied is the
197 average of the probabilities of occupancy of its row and column (Almeida-Neto et al. 2008). All
198 nestedness analyses were performed using the software package ANINHADO 3.0 (Guimarães
199 and Guimarães 2006).

To estimate the degree of modularity and the number of modules, we used the simulated annealing algorithm developed by Guimerà and Amaral (2005), which identifies modules whose nodes have the majority of their links inside their own module. This algorithm provides an index of modularity *M*:

$$M = \sum_{s=1}^{N_M} \left[\frac{l_s}{L} - \left(\frac{d_s}{2L} \right)^2 \right]$$

where N_M is the number of modules, L represents the number of links in the network, l_s is the number of links between nodes in module s, and d_s is the sum of the number of links of the nodes in module s. This measure of modularity has been used before to describe the properties of bipartite networks (e.g. Olesen et al. 2007, Fortuna et al. 2010, Thébault and Fontaine 2010). To determine the significance of the observed modularity index, 999 random networks with the same species degree distribution as the original network were constructed and the observed modularity index was compared with indices from random networks (Guimerà et al. 2004).

Phylogenetic constraint analysis

Finally, we used a phylogenetic signal strength to test whether the phylogenetic relatedness of orchid species correlated with a similar set of mycorrhizal fungi, that is whether the observed interaction network structure was significantly affected by the phylogeny of the plants or the fungi. The fungi that were involved in the studied orchids mainly belonged to three fungal clades (Tulasnellaceae, Ceratobasidiaceae and Sebacinales), and have been called *rhizoctonia* for convenience (Dearnaley et al. 2012). For better understanding the interaction network of orchid and mycorrhizal fungi, we only used the *rhizoctonia* dataset (53 OTUs) for further analysis. Because phylogenetic signal measurements are based directly on evolutionary rates (branch

lengths) estimated by phylogenetic inferences, we constructed a ML tree for the orchid species and the rhizoctonia fungi, respectively. Branch lengths were estimated without a molecular clock assumption in the ML trees. The ITS sequences of 40 orchid species and 39 Tulasnellaceae OTUs were aligned using Clustal X version 2.0 (Larkin et al. 2007). The K2+G+I and K2+G evolution models were identified as the best-fit models for the orchids and Tulasnellaceae datasets, respectively, using the Akaike Information Criterion implemented in jModelTest 2 (Darriba et al. 2012). For both data sets, an ML phylogeny was constructed with RAxML 7.2.8 (Stamatakis et al. 2008). Clade support was estimated with RAxML through a nonparametric bootstrap analysis of 1,000 pseudo-replicate data sets. We then evaluated the strength of the phylogenetic signals of the two phylogenies on the orchids-Tulasnellaceae fungi interaction network using a linear model approach that fits the phylogenetic variance-covariance matrix to the plant-fungi interaction matrix (Ives and Godfray 2006). We applied the phylogenetic bipartite linear model of Ives and Godfray (2006). We calculated the independent phylogenetic signals of the orchids (d_O) and Tulasnellaceae (d_T) phylogenies on the interaction matrix and the strength of the signal of both phylogenies combined (MSE_d). The significance of the phylogenetic structure was determined by comparing the mean square error (MSE) of this model of evolution (MSE_d) with the MSE derived under the assumption of no phylogenetic signals (i.e., a star phylogeny) and with the MSE derived under the assumption of a maximum phylogenetic signal (i.e., Brownian motion evolution, MSE_b). The model minimizing the MSE was considered the best fit. Bipartite linear models were performed using the *pblm* function in the picante R package (Kembel et al. 2010).

Results

Fungal diversity

In all orchid species investigated, typical characteristics of orchid mycorrhiza were observed in the roots and for each species one or more ITS sequences were obtained, resulting in a total of 1343 diverging sequences. Almost all of the obtained sequences corresponded to basidiomycete fungi (1324 sequences), except for a few sequences that belonged to ascomycete fungi (19 sequences). The 1324 basidiomycete sequences yielded a total of 87 OTUs at a sequence similarity threshold of 97% (Table S2). Rarefaction analysis showed that the curve quickly reached an asymptote for the analyzed sequences (Fig. S2). Among them, 53 OTUs (1157 sequences) were assigned to *rhizoctonia* fungi according to Dearnaley *et al*. (2012). Thirty-nine OTUs (1065 sequences), 11 OTUs (64 sequences) and 3 OTUs (28 sequences) were assigned to members of Tulasnellaceae, Ceratobasidiaceae and Sebacinales, respectively. Besides, other fungal taxa known to associate with orchids were retrieved, including members of the Thelephoraceae (2 OTUs, 5 sequences), Cortinariaceae (2 OTUs, 24 sequences), Marasmiaceae (1 OTU, 10 sequences), Russulaceae (1 OTU, 3 sequences), unknown Cantharellales (4 OTUs, 16 sequences), and Atractiellales (3 OTUs, 21 sequences). Additionally, a number of possibly endophytic fungi belonging to Tricholomataceae and Septobasidiaceae were only sporadically detected.

Epiphytic orchids associated with 50 different OTUs, whereas terrestrial and lithophytic orchids associated with 25 and 24 OTUs, respectively (Table S1; Fig. S3). When comparing fungal communities between terrestrial, epiphytic and lithophytic orchids, it is apparent that

members of the Tulasnellaceae were the dominant species in all life forms. However, the relative frequency of Tulasnellaceae increased from terrestrial (47.33%), over epiphytic (83.87%) to lithophytic orchids (96.71%) (Fig. 1). Moreover, distinct guilds of fungal OTUs associated with the different life forms of orchids. Out of 87 OTUs, no OTU was shared between the three life forms, indicating that they associate with distinct mycorrhizal fungi. Epiphytic and lithophytic orchids shared one OTU with terrestrial orchids, whereas epiphytic and lithophytic orchids shared 10 OTUs (Fig. S3). Orchid species that occurred both in epiphytic and lithophytic habitats associated with 29 different OTUs in total, of which eight OTUs were shared between the two life forms. On average, 42.1% of all OTUs found in an orchid species displaying both life forms were shared between the epiphytic and the lithophytic life form.

The average number of fungal OTUs retrieved per orchid species did not differ significantly ($P > 0.05$) between life forms (Fig. 2a). Epiphytic orchids interacted on average with 3.36 ± 0.50 OTUs, whereas terrestrial and lithophytic orchids associated with 2.69 ± 0.36 and 2.85 ± 0.40 OTUs. Average phylogenetic diversity, on the other hand, was highest in terrestrial orchids (PD = 0.6252 ± 0.0545) and was significantly ($P < 0.05$) higher than that of lithophytes (0.4921 ± 0.0413), but not of that of epiphytes (0.6018 ± 0.0592) (Fig. 2b). Finally, the highest MPD was detected in the epiphytic orchids (0.3491 ± 0.0636), but it was not significantly higher than that observed in terrestrial (0.3212 ± 0.0823) or lithophytic orchids (0.2154 ± 0.0548) (Fig. 2c).

Nestedness and modularity

The overall network comprised 52 orchid species (13 terrestrial, 17 epiphytic, 6 lithophytic, and 8 species displaying both an epiphytic and lithophytic life form) and 87 fungal OTUs, and showed

159 established links (connectance C: 0.035). The overall network appeared to be significantly (P < 0.01) nested (NODF = 5.53, E_r = 3.98, C_e = 4.82). The modularity analysis indicated that the network was significantly modular (M = 0.7389, M_{random} = 0.6998) and that 15 distinct modules were identified (Fig. 3). These modules had, on average, 9 links within modules and 1.6 links to other modules. The largest module consisted of 10 orchid species and contained eight epiphytic and two lithophytic orchids. The second largest module contained eight orchid species, five of which were lithophytic orchids and three epiphytic species. Terrestrial orchids formed a set of five distinct modules, which had almost no links to modules containing epiphytic or lithophytic orchids (Fig. 3).

When only *rhizoctonia* fungi were considered, very similar results were obtained, indicating that results are not biased due to sporadic occurrence of non-*rhizoctonia* fungi. In this case, the interaction network consisted of 52 orchid species and 53 OTUs and contained 117 binary links (C = 0.043) (Fig. 4). The network was again significantly (P < 0.05) nested (NODF = 7.78, E_r = 4.79, C_e = 6.10). The modularity index was high (M = 0.7128), and significantly larger than that of random matrices (M_{random} = 0.685 ± 0.013). There were 15 modules that varied in size between 1 and 9 orchids (average number of orchid species within a module: 3.5) (Figure S4). Modules containing terrestrial orchids were almost completely isolated from modules containing epiphytic or lithophytic orchids and vice versa. Modules containing epiphytic orchids contained lithophytic orchids, confirming our previous analyses that epiphytic and lithophytic orchids share some of their fungal partners and that they are grouped in several modules (Fig. S4).

Phylogenetic signal

304 Finally, we used a linear model approach to evaluate the phylogenetic signal of both the orchid
305 and the fungal phylogenies on the orchid-*rhizoctonia* network. For both the orchids and the fungi,
306 the phylogenetic signal was very weak (d_o < 0.001, 95% CI 0-0.023; d_f < 0.001, 95% CI 0-6.612e-
307 06). The strength of the overall phylogenetic signal (MSE_d = 0.044) was similar to that of a star
308 phylogeny (MSE_s = 0.044) and lower than that of the maximal inertia (MSE_b = 0.0471).
309 Therefore, neither phylogenetic relationships among orchids nor among fungi imposed some
310 structure on the association matrix (Fig. 4). When we analyzed the phylogenetic signal of the
311 orchid-*rhizoctonia* subnetworks in different life forms, the phylogenetic signal was small and not
312 significantly different from zero for lithophytic (d_o = 0.1411, [0.04-0.22]; d_f < 0.001, [0-0.020]),
313 epiphytic (d_o < 0.001, [0-0.002]; d_f < 0.001, [0-0.001]) and terrestrial orchids (d_o = 0.007, [0-
314 0.209]; d_f < 0.001, [0-0.031]). Similar results were obtained when only *Tulasnella* fungi were
315 taken into account (data not shown).
316
317 **Discussion**
318 Non-random associations have been commonly observed in plant-fungus interaction networks
319 (e.g. Montesinos-Navarro et al. 2012; Chagnon et al. 2012, Martos et al. 2012, Toju et al. 2014;
320 2016; Bahram et al. 2015), but the precise mechanisms leading to non-random interactions are
321 less well understood. Here, we investigated the architecture of the network of interactions between
322 a large number of orchids and mycorrhizal fungi and asked whether the observed patterns were
323 the result of ecological, evolutionary and/or co-evolutionary processes. Our analyses showed that
324 the observed interaction network was significantly modular and to a much lesser extent nested.
325 Terrestrial and epiphytic/lithophytic orchids clearly associated with distinct sets of mycorrhizal

326 fungi, but within each life form several modules were discerned as well, indicating that strong
327 partner selectivity and high turnover of mycorrhizal partners were the main factors explaining the
328 observed network architecture.

329 **Host specificity and selectivity in terrestrial, epiphytic and lithophytic orchid species**

330 Within tropical ecosystems, orchids with different life forms (in this case terrestrial, epiphytic and
331 lithophytic orchids) are capable of occupying different niches and therefore coexisting in one
332 habitat. Because the three life forms represent largely different environments, they may select for
333 different mycorrhizal fungi. Our results showed that members of the Tulasnellaceae were the most
334 dominant fungi in the three life forms, supporting previous findings that Tulasnellaceae symbionts
335 are ubiquitous in terrestrial orchids worldwide (Jacquemyn et al. 2017) as well as in some
336 epiphytic orchids (Kartzinel et al. 2013, Xing et al. 2017). Besides members of the Tulasnellaceae,
337 other *rhizoctonia* fungi of the Ceratobasidiaceae and Sebacinales (Dearnaley et al. 2012) were
338 observed, particularly in terrestrial and epiphytic orchids, but not in the lithophytic species, which
339 almost exclusively associated with fungi from the Tulasnellaceae. Terrestrial orchids further had
340 sporadic associations with members of Thelephoraceae, Cortinariaceae, Marasmiaceae, unknown
341 Cantharellales and Atractiellales.

342 The factors that drive mycorrhizal specificity are not clear, but it has been suggested that it
343 might be affected by environmental factors (Jacquemyn et al. 2010, Kartzinel et al. 2013).
344 Associating with multiple fungi could confer symbiotic assurance when mycorrhizal fungi show
345 a patchy distribution or are only stochastically available, which may be crucial in dynamic or
346 disturbed habitats such as forest canopies (Kartzinel et al. 2013). Although the number of fungal

associates did not significantly differ between life forms, terrestrial and epiphytic orchids interacted with more diversified fungi than lithophytic orchids and an increasing specificity towards Tulasnellaceae fungal associates was observed from terrestrial to epiphytic and lithophytic orchids. Epiphytic orchids showed levels of phylogenetic diversity similar to that of terrestrial orchids. Compared to terrestrial and epiphytic habitats, lithophytic habitats represent harsh environments that are characterized by lower availability of above-ground water and nutrient supplies, which may explain the increased dependency and specificity on mycorrhizal fungi. Moreover, lithophytic habitats most likely also not support fungi that are involved in associations with other organisms such as trees. The lower phylogenetic diversity of mycorrrhizal fungi observed in lithophytic orchids therefore most likely arises from ecological factors associated with lithophytic habitats.

Network architecture

Extreme host selectivity and specialization may lead to significant turn-over in orchid-fungus associations in co-occurring orchid species and therefore explain the low nestedness values that were observed in this study and the significant modularity that is typically found in orchid-fungus networks (Jacquemyn et al. 2015). Our results showed that some orchids (e.g. *Nervilia plicata*, *Oberonia variabilis* and *Epigeneium amplum*) associated with only a single fungal taxon, confirming previous research that extreme host specialization can be observed in orchids (e.g. Warcup 1985, Taylor and Bruns 1997, Shefferson et al. 2005, Swarts et al. 2010). However, the majority of the orchid species investigated here interacted with several partners at the same time,

367 indicating that extreme host specialization cannot be the sole explanation for the observed
368 network structure.

369 One possible other explanation for the observed variation in network structure is based on the
370 concept of forbidden links, i.e. ecological constraints that prevent the occurrence of certain
371 pairwise interactions among those possible in the entire network (Jordano et al. 2006, Olesen et
372 al. 2011). Our results indicated very little overlap in fungal associations between terrestrial
373 orchids on the one hand and epiphytic/lithophytic orchids on the other hand. The observed
374 differences in mycorrhizal partners between life forms suggest a clear ecological barrier between
375 terrestrial and epiphytic/lithophytic habitats, which is somewhat similar to temporal uncoupling
376 of flowering and pollinator activity or size mismatching between plants and animals in plant-
377 pollinator networks (Jordano et al. 2003, Olesen et al. 2011). The presence of forbidden links may
378 therefore explain the strong modular structure when all interactions are analyzed across different
379 life forms. Strong evidence of modularity was also found in a mycorrhizal network of orchids on
380 Réunion Island. Similar to the results presented here, the modularity was correlated with an
381 ecological barrier between terrestrial and epiphytic orchids (Martos et al. 2012).

382 Within each life form, several sub-modules were observed that had very few links to other
383 modules. For example, several terrestrial orchid species interacted with fungi that were not shared
384 by other terrestrial species. Similarly, several epiphytic orchid species associated with fungi that
385 were not encountered in other epiphytic species. These results suggest that the processes that
386 organize orchid-fungus interactions do not depend on orchid life form and that within each life
387 form link specificity (Lewinsohn et al. 2006) and strong turnover in mycorrhizal partners have
388 further contributed to the observed modular structure of the entire network. Similar patterns have

been found for orchid species in species-rich Mediterranean orchid communities (Jacquemyn et al. 2015) and may reflect spatial or mutual selective limitations acting between different orchids and fungi (Jacquemyn et al. 2012, 2014). Strong turnover in mycorrhizal partners may decrease resource competition and therefore lead to niche partitioning and stable coexistence of multiple orchid species (e.g. Jacquemyn et al. 2014; 2015). Seed germination experiments have indeed shown that strong partner selectivity and non-random spatial distribution of mycorhizal fungi in the soil leads to stable co-existence of orchid species (Jacquemyn et al. 2012, 2014; Waud et al. 2016).

Phylogenetic constraints

Apart from differences in ecological conditions, the architecture of interaction networks can also be determined by phylogenetic relationships between species (Bascompte and Jordano 2007). In this case, it can be expected that phylogenetically closely related species tend to exhibit similar physiological or ecological properties and therefore may have similar network properties (Freckleton et al. 2002, Blomberg et al. 2003, Garland et al. 2005, Ives and Godfray 2006). Previous research has shown that in several orchid genera closely related species associated with more similar fungal communities (e.g. *Cypripedium* (Shefferson et al. 2007), *Goodyera* (Shefferson et al. 2010), *Orchis* (Jacquemyn et al. 2011), *Dendrobium* (Xing et al. 2017), suggesting that phylogenetic constraints may influence the mycorrhizal community an orchid associates with. However, in this research no phylogenetic signal on the overall network structure was detected. These results are in line with findings of Martos et al. (2012), who also showed a

409 weak overall phylogenetic signal in the interaction matrix of a large number of tropical orchids
410 and their associated mycorrhizal fungi.

411 Our results contrast with those from Martos et al. (2012), in that no strong phylogenetic
412 signal of both partners was found in the subnetworks of any of the life forms studied. The stronger
413 phylogenetic signal in the epiphytic sub network of Martos et al. (2012) might be explained by
414 the phylogenetic depth of epiphytic orchid taxa on Réunion Island. Most orchids belonged to the
415 sub tribe Angraecinae, which diversified in Madagascar and the Indian Ocean islands, whereas at
416 our study site, the sampled orchids were a more phylogenetically diverse assemblage of epiphytic
417 orchids on the one hand, and terrestrial orchids on the other hand.

418 **Conclusions**

419 To conclude, our results showed that orchids displaying different life forms associated with
420 different fungal symbionts, which resulted in a network structure that was significantly modular.
421 Within life forms, multiple modules were found, suggesting that the processes that organize
422 orchid-fungus interactions are independent of life form. Our results further showed an increasing
423 specificity towards Tulasnellaceae fungi from terrestrial over epiphytic to lithophytic orchids,
424 suggesting that more stressful environments limit the potential pool of mycorrhizal partners and
425 thus the potential for associations with diverse fungi. Significant modularity in the network may
426 point to a high interaction intimacy between orchids and fungi and a strong ecological barrier
427 between terrestrial and epiphytic/lithophytic habitats. Phylogenetic relationships, on the other
428 hand, did not affect network patterns, indicating that ecological factors were more important than
429 past evolutionary history in explaining the observed network architecture. To gain better insights

into the precise mechanisms leading to the modular structure, we eagerly anticipate future studies that describe the total pool of mycorrhizal symbionts occurring in different habitats and compare patterns of partner choice between orchids with different life forms using seed germination experiments and fungal identifications.

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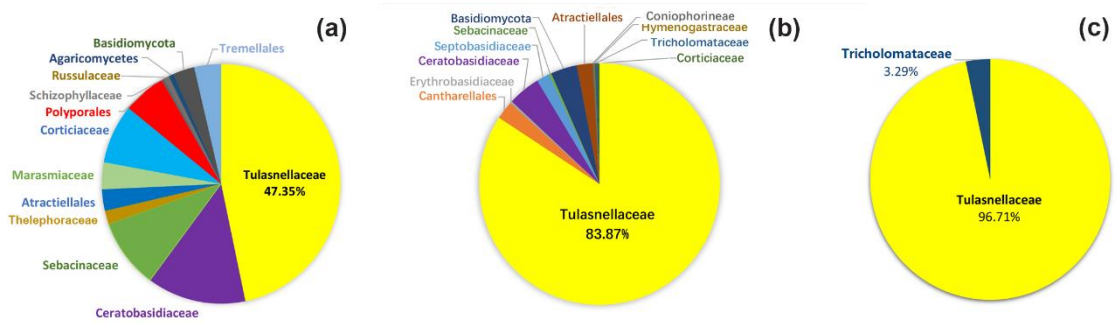
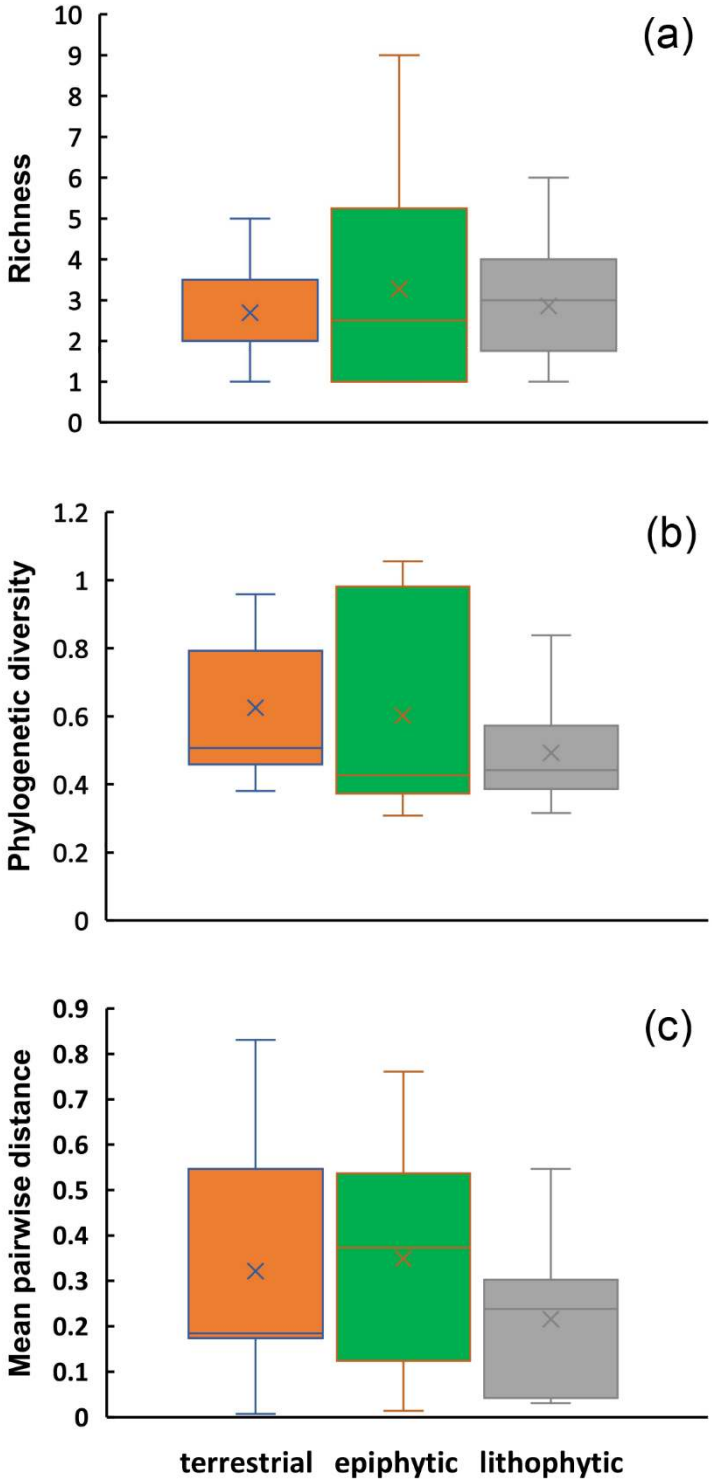


Figure 1. Frequency distribution (based on number of sequences) of fungal families detected in orchids displaying different life forms. (a) Terrestrial orchids; (b) epiphytic orchids; (c) lithophytic orchids.



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590 Figure 2. Fungal diversity in terrestrial, epiphytic and lithophytic orchids. (a) OTU richness; (b)
591 Phylogenetic diversity; (c) Mean pairwise distance.

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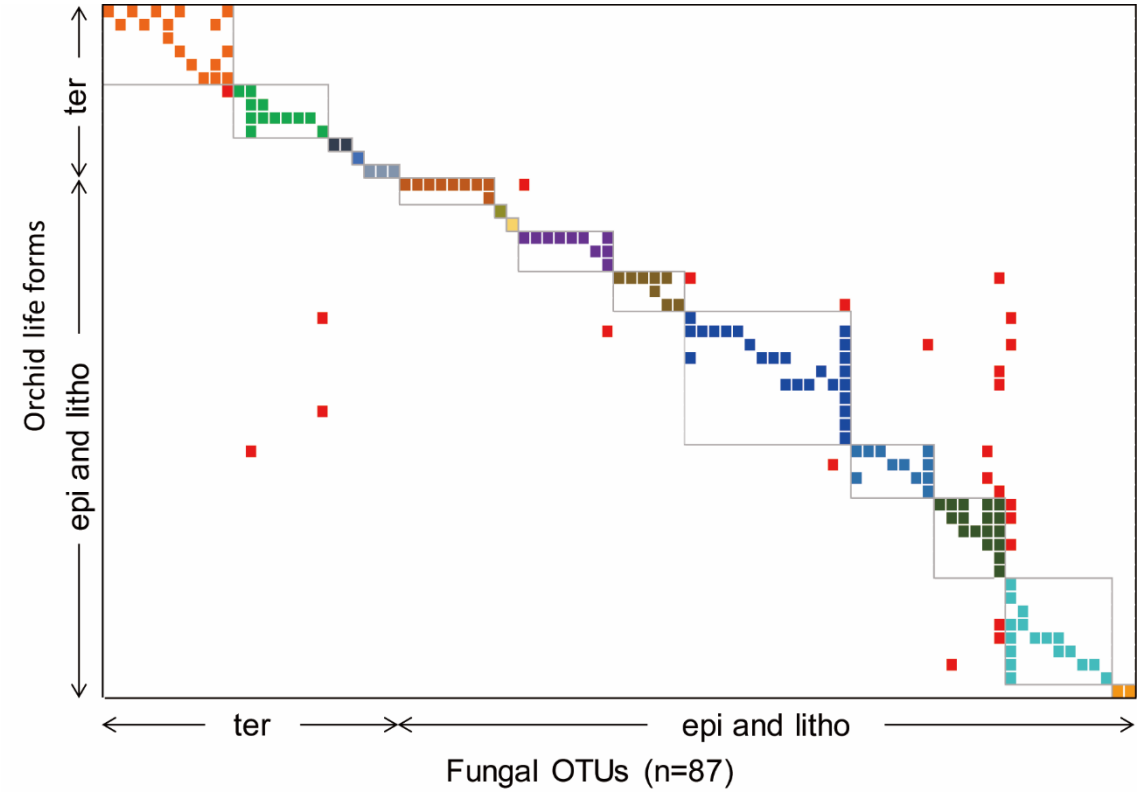

Figure 3. Matrix representation of the interactions between 44 orchid species (including 8 epi-/lithophytic species) (columns) and 87 orchid mycorrhizal fungal OTUs (rows). The overall network was significantly modular. The clusters displaying the largest modularity include terrestrial, epiphytic and lithophytic orchid–fungus interactions. The 15 identified modules are shown in different colors. Red cells are species links to other modules, and non-red cells are links within modules.

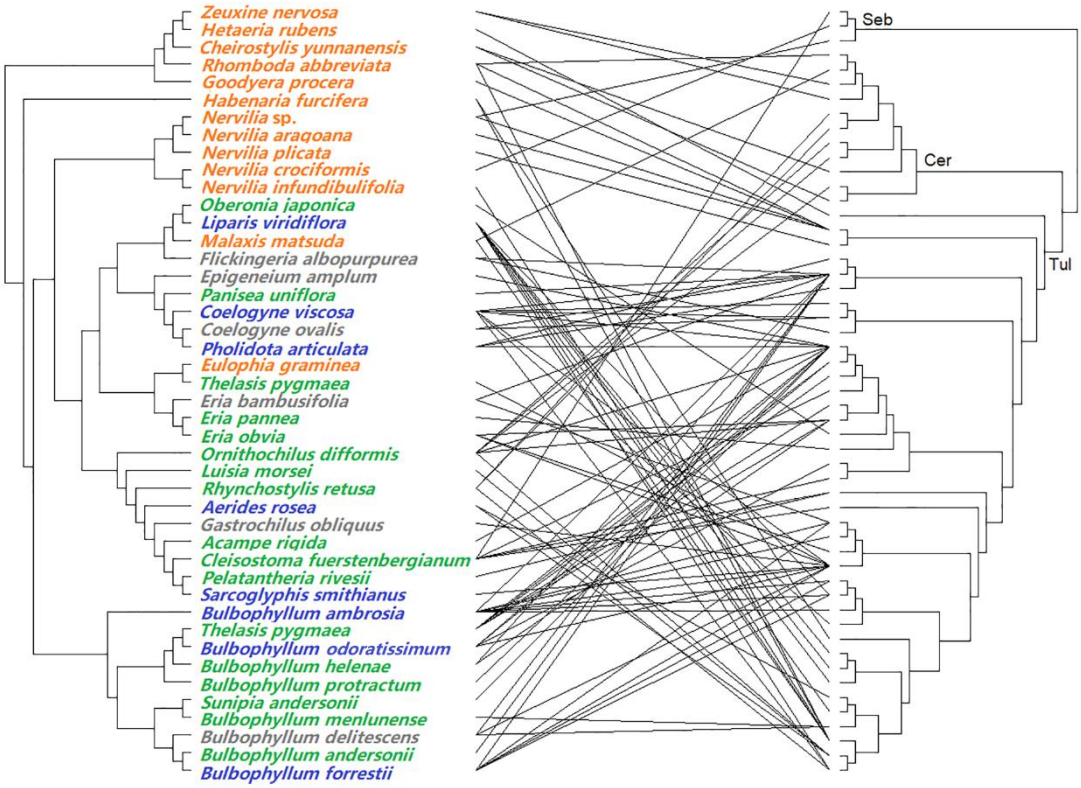

Figure 4. Interaction network between orchids and *rhizoctonia* fungi. The network shows all links between 53 *rhizoctonia* OTUs and 44 orchid species (13 terrestrial, 17 epiphytic, 6 lithophytic and 8 epiphytic/lithophytic orchids) (103 binary links in total). On the orchids phylogenetic tree, terrestrial, epiphytic, lithophytic and epiphytic/lithophytic are shown in orange, green, grey and blue, respectively. Seb, Sebacinales; Cer, Ceratobasidiaceae; Tul, Tulasnellaceae.

Supporting information

TABLE S1 Different life forms of orchid species collected from Xishuangbanna, Yunnan province, China and their fungal associates.

TABLE S2 List of fungal operational taxonomic units (OTU) identified using cloning techniques

FIGURE S1 Map of Xishuangbanna showing the National Natural Reserve and Menglun subreserve where 44 orchid species (including 8 epi-/lithophytic species) were sampled.

FIGURE S2 Rarefaction analysis performed on the internal transcribed spacer sequence data obtained from the clone libraries for all orchid species (1324 sequences), using a 97 % sequence similarity threshold value.

FIGURE S3 Sharing of orchid mycorrhizal OTUs between terrestrial, lithophytic and epiphytic orchids.

FIGURE S4 Matrix representation of the interactions between 44 orchid species (including 8 epi-/lithophytic species) (columns) and 53 *rhizoctonia* OTUs (rows). The overall network was significantly modular. The clusters displaying the largest modularity include terrestrial, epiphytic and lithophytic orchid–fungus interactions. The 15 identified modules are shown in different colors. Red cells are species links to other modules, and non-red cells are links within modules.