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PART OF A HIGHLIGHT ON ORCHID BIOLOGY

Relationship between soil nutrients and mycorrhizal associations of two *Bipinnula* species (Orchidaceae) from central Chile

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• **Background and Aims** Mycorrhizal associations are influenced by abiotic and biotic factors, including climate, soil conditions and the identity of host plants. However, the effect of environmental conditions on orchid mycorrhizal associations remains poorly understood. The present study examined how differences in soil nutrient availability are related to the diversity and composition of mycorrhizal fungi associated with two terrestrial orchid species from central Chile.
• **Methods** For 12 populations of *Bipinnula fimbriata* and *B. plumosa*, OTU (operational taxonomic unit) richness, phylogenetic diversity and community composition of mycorrhizal fungi in root samples were estimated using internal transcribed spacer (ITS) sequences. Then, these mycorrhizal diversity variables were related to soil nutrients and host species using generalized linear models and non-metric multidimensional scaling.
• **Key Results** Variation in OTU composition of mycorrhizal fungi among sites was explained mainly by orchid host species. Fungi in Tulasnellaceae and Ceratobasidiaceae were isolated from both orchid species, but the former were more frequent in *B. fimbriata* and the latter in *B. plumosa*. Soil nutrients and orchid host species had significant effects on OTU richness and phylogenetic diversity. Mycorrhizal diversity decreased in habitats with higher N in both species and increased with P availability only in *B. fimbriata*.
• **Conclusions** The results suggest that soil nutrient availability modulates orchid mycorrhizal associations and provide support for the hypothesis that specialization is favoured by higher soil nutrient availability. Inter-specific differences in mycorrhizal composition can arise due to a geographical pattern of distribution of orchid mycorrhizal fungi, host preferences for fungal partners or differential performance of mycorrhizal fungi under different nutrient availabilities. Further experiments are needed to evaluate these hypotheses.

Key words: *Bipinnula*, central Chile, orchid mycorrhiza, mycorrhizal specialization, soil nutrients.

INTRODUCTION

One of the major questions in the study of mutualistic interactions is specificity, which refers to the number or phylogenetic range of taxa with which a particular species interacts (Molina *et al*., 1992; Thompson, 1994). Although specificity of interactions is often associated with many ecological disadvantages, such as increased intra-specific competition for mutualistic partners or reduced accessibility to alternative partners under unfavourable conditions (Poisot *et al*., 2011), cases of specialization are widespread in nature (Bronstein, 2009). High specificity might be favoured by certain environmental conditions, such as low fluctuations in partner abundance (Waser *et al*., 1996), high productivity (Poisot *et al*., 2011) or long-term stability (Phillips *et al*., 2011). Similarly habitat quality (i.e. the supply of resources that affect growth, survival and reproduction) could also play a role in mutualistic specialization (Thrall *et al*., 2006). Increasing host habitat quality might increase intra-host competition, favouring the evolution of symbiont specificity, or expand the availability of alternative hosts or symbiont partners, in turn favouring generalism (Thrall *et al*., 2006). In addition, the composition of symbiotic partners can be influenced by changes in habitat quality, where the association with different partners provides opportunities for tolerating new environmental conditions and changes in resource availability (Lilleskov *et al*., 2002; McCormick *et al*., 2006).

Mycorrhizas are widespread symbiotic associations between soil fungi and plants (van der Heijden *et al*., 2015) and are present in 92% of all land plant families (Wang and Qiu, 2006). Most mycorrhizal symbioses are generalist in nature, with plants interacting with a high number taxa or a broad phylogenetic range of fungal partners (Molina *et al*., 1992; Smith and Read, 2008), but many plant species show specialized mycorrhizal associations. Mycorrhizal associations are greatly influenced by abiotic and biotic factors, including climate (Tedersoo *et al*., 2012), soil conditions (Treseder, 2004; Lilleskov *et al*., 2002; Bunch *et al*., 2013; Huggins *et al*., 2014) and host plants (Roy *et al*., 2013). Soil nutrients are key factors regulating specificity and community composition (Parrent *et al*., 2006; Schechter and Bruns, 2008; Peay *et al*., 2009;

Polme *et al*., 2013; Roy *et al*., 2013), and soil fertility can also negatively affect mycorrhizal colonization, which is expected to be positively related to mycorrhizal diversity (Blechem and Alexander, 2012; Balzergue *et al*., 2013), and the intensity of interaction (Smith and Read, 2008).

Orchid species form mycorrhizal associations with the fungal group called *Rhizoctonia*, which includes the saprotrophic basidiomycetes in the families Tulasnellaceae, Ceratobasidiaceae and Sebacinales (Dearnaley *et al*., 2012). Although orchids interact with a relatively narrow group of mycorrhizal fungi in comparison with other vascular plant families, the composition and richness of fungal taxa can vary greatly among orchid species (Shefferson *et al*., 2007; Swarts *et al*., 2010; Phillips *et al*., 2011) and also within the distributional range of a single species (Jacquemyn *et al*., 2015), making Orchidaceae an appropriate model for studying the factors that influence mycorrhizal specificity. Little is known about how abiotic factors influence mycorrhizal association in orchids (Phillips *et al*., 2011), but limited evidence suggests that habitat (Illyés *et al*., 2009, Oja *et al*., 2015) and soil conditions (McCormick *et al*., 2012; Bunch *et al*., 2013) affect fungal community composition. Bunch *et al*. (2013), for example, showed that the composition of mycorrhizal fungi varies among populations of the orchid *Cypripedium acaule* in association with soil pH and percentage organic matter, C and N, whereas McCormick *et al*. (2006) demonstrated that drought can trigger switching of fungal partners in *Goodyera pubescens*. Even less is known about how abiotic factors influence orchid mycorrhizal specialization (Phillips *et al*., 2011); however, it has been suggested that under nutrient-poor or stressful conditions orchid species should present generalist associations in order to maximize their nutrient uptake (Jacquemyn *et al*., 2012, 2015), but this hypothesis remains untested.

To evaluate the relationship between soil nutrient availability and orchid mycorrhizal specialization, we investigate the mycorrhizal associations of *Bipinnula fimbriata* and *Bipinnula plumosa*, two photosynthetic terrestrial orchid species distributed across a latitudinal range from 30 to 35°S in Mediterranean Chile, encompassing a broad gradient of soil nutrient availabilities. We sampled 12 populations of these two species, covering their entire distribution, and measured the level of mycorrhizal colonization. By sequencing the internal transcribed spacer (ITS) of nuclear ribosomal DNA, we estimated the number of operational taxonomic units (OTUs) and the phylogenetic diversity of mycorrhizal fungi. Then we related the diversity of mycorrhizal fungi to soil nutrients and orchid host species using generalized linear models and non-metric multidimensional scaling. In particular, we asked the following questions. (1) Which mycorrhizal fungi are associated with *B. fimbriata* and *B. plumosa*? (2) Do the composition, diversity and degree of colonization of mycorrhizal fungi vary among populations and between the two *Bipinnula* species? (3) To what extent is variation in fungal mycorrhizal communities related to variation in soil nutrients (particularly N and P) among sites? We tested the hypothesis that specialization in mycorrhizal associations is favoured by higher soil nutrient availability. We expected that in sites with greater soil nutrient availability, orchid species should exhibit specialized mycorrhizal associations, whereas in nutrient-poor soils orchid species should exhibit generalist associations.

MATERIALS AND METHODS

Orchid species studied

Bipinnula (sub-tribe Chloraeinae, tribe Diuridae) is a genus of terrestrial, photosynthetic orchids endemic to southern South America. The genus is distributed across Brazil, Uruguay and Argentina, with a separate group of five species endemic to Chile in south-western South America (Novoa *et al*., 2006; Cisternas *et al*., 2012). *Bipinnula fimbriata* and *B. plumosa* are both endemic to central Chile (Fig. 1A). *Bipinnula fimbriata* is relatively more frequent, and is distributed in lowland (<500 m) coastal areas from 29 to 35°S (Novoa *et al*., 2006; Fig. 1B), preferably on sandy stabilized soils, in open sites exposed to sunlight and marine breeze (Elórtegui and Novoa, 2009). It forms large, dense populations with other perennial herbs under the canopy of sclerophyllous shrubs (Steinfort *et al*., 2010). *Bipinnula plumosa* generally occurs in montane sites >1000 m, from 31 to 34°S (Fig. 1B), on south- or south-west-facing slopes of the Andean Cordillera and on some coastal hilltops. Populations of *B. plumosa* are generally more sparse or patchy, with small groups of 10–30 individuals.

Sampling

Sampling was conducted during two consecutive flowering seasons (August to December) in 2012 and 2013. We sampled seven populations of *B. fimbriata* (FJ, LV, ZP, CC, SAN, TO and CON) and five of *B. plumosa* (LA, LD, APO, EM and RC), including 115 individuals overall, encompassing almost the entire range of each species (Supplementary Data Table S1). We collected four roots per plant from ten orchid plants from each population, except for two populations of *B. plumosa* where only eight (LA) and seven (RC) individuals were sampled. Considering differences in sampling effort among populations, we performed saturation curves and we observed that they were close to saturation (see rarefaction curves in Supplementary Data Fig. S1). Collected roots were individually labelled and kept cold during transport to the laboratory, where they were processed for further analysis. In all sites where orchid populations occurred, we also collected mixed soil samples, which combined ten sub-samples of soil near each plant from each site. Mixed soil samples were dried and analysed for nitrate content ($N\text{-}NO_3$ mg kg^{-1}) and Olsen P (mg kg^{-1}). Soil analyses were performed in the Soils Laboratory of Universidad de Concepción, Chillán, Chile.

Fungal isolation

Roots were cut into 3–5 cm pieces, washed under tap water to remove soil and dirt, and sterilized as follows: samples were placed for 1 min in 1 % sodium hypochlorite and then three times consecutively for 3 min in sterile distilled water. Orchid mycorrhizal fungi form pelotons in root cortex cells. In *Bipinnula*, as in many other orchid species, groups of pelotons can be noted on the washed root surfaces as spots ranging in colour from light yellow to dark brown (Supplementary Data Fig. S2). For each root piece, the level of colonization by mycorrhizal fungi was quantified as the fraction of the root surface covered by spots (produced by the presence of pelotons).

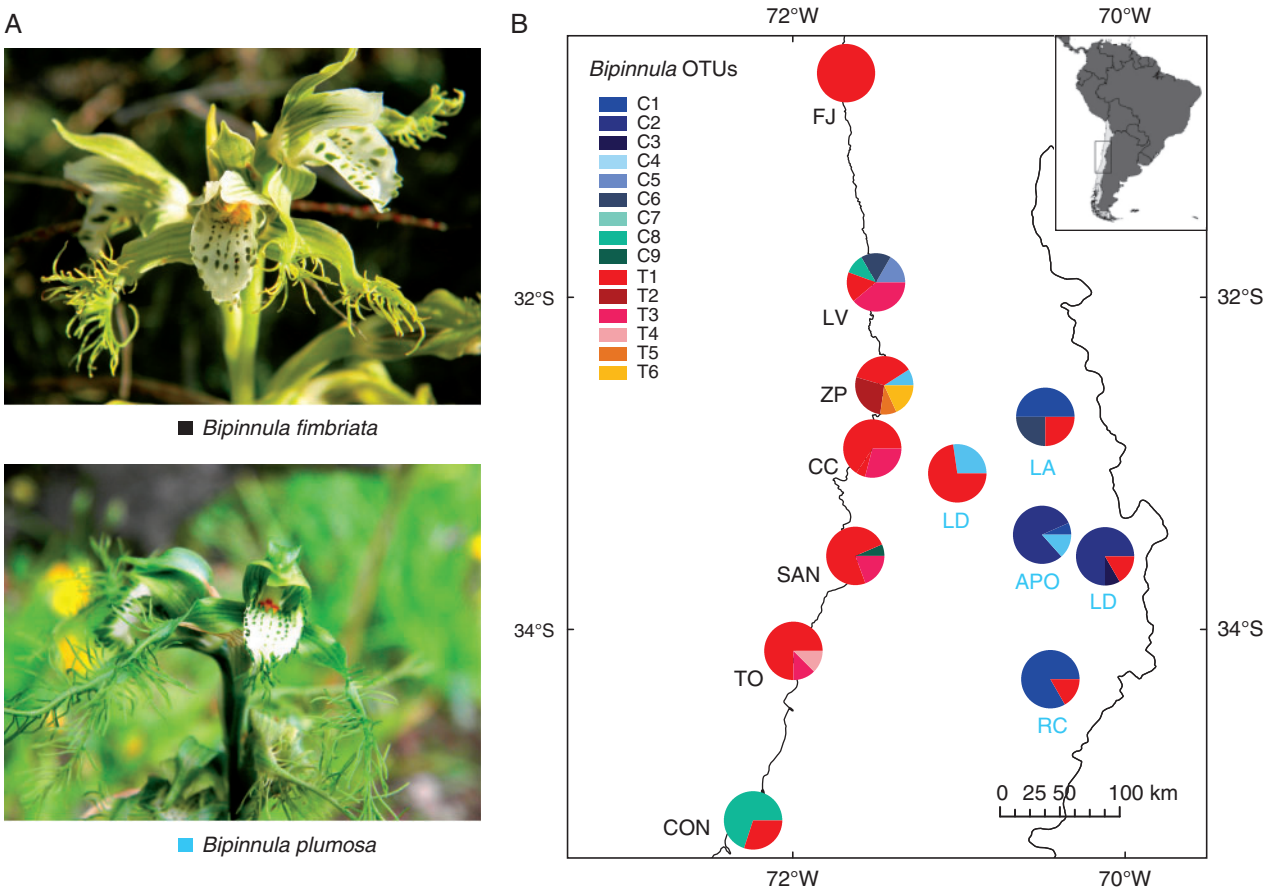

FIG. 1. Geographical distribution of mycorrhizal fungal operational taxonomic units (OTUs) associated with the orchids *Bipinnula fimbriata* (BF) and *B. plumosa* (BP) in central Chile. (A) Pictures of BF and BP. (B) Map of the sampling locations, with pie charts displaying the frequency of occurrence of each fungal OTU in each orchid population. Above each pie chart is the population name; names in black are BF populations and names in blue are BP populations. Yellow to red colours represent Tulasnellaceae OTUs, blue to green colours represent Ceratobasidiaceae OTUs.

Roots with verified presence of pelotons were cut into 3 mm sections and placed on Petri dishes with potato dextrose agar (PDA) containing 0·16 mg L^{-1} streptomycin and 0·16 mg L^{-1} penicillin, which were then stored in a dark room at 18 °C. When fungal colonies developed, fungal tips from each isolate were sub-cultured until we obtained pure fungal isolates. Adjacent root pieces with pelotons were individually placed in sterile 2 mL tubes and stored at − 20 °C until DNA was extracted (one sample per root; four roots per individual) for assessing the presence of fungal species that could not be cultured *in vitro*.

DNA extraction, amplification and sequencing

DNA was extracted from pure fungal cultures and stored root sections using a cetyltrimethylammonium bromide (CTAB) method modified from Doyle and Doyle (1990). Oligonucleotide primers ITS1 and ITS4 (White *et al.*, 1990) were used for amplification of DNA extracted from fungal isolates. To guarantee the amplification of fungal DNA rather than plant DNA from root sections, we used the specific primers ITS1F/ITS4, ITS1F/ITS4B for basidiomycetes (Gardes and Bruns, 1993), ITS1/ITS4-Tul for Tulasnellaceae (Taylor and McCormick, 2008) and CeTh1/CeTh4 for Ceratobasidiaceae (Porras-Alfaro and Bayman, 2007). For all primers, the PCR was carried out in a final volume of 100 µL, containing 10 µL of 10× buffer, 6 µL of 50 mM Mg^{2+}, 2 µL of bovine serum albumin (BSA), 2 µL of dNTP, 2 µL of each primer, 0·5 U of *Taq* polymerase and 4 µL of extracted DNA. PCR analysis was performed using the following temperature profile: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 1 min at 95 °C, 1 min at 54 °C, and 1 min at 72 °C. The cycles were terminated with a final extension at 72 °C for 10 min. The PCR products were verified on 1 % agarose gels and sent to Macrogen (Seoul, South Korea) for purification and sequencing.

Sequence editing and alignment

To determine the identity of sequences, we conducted a Blast search (www.ncbi.nlm.nih.gov/ BLAST, Altschul *et al.*, 1990) in GenBank. Only sequences corresponding to known orchid-associating mycorrhizal families (Tulasnellaceae, Ceratobasidiaceae and Sebacinaceae, according to Dearnaley *et al.*, 2012) were retained for further analysis. The sequences were aligned in BioEdit (Hall, 1999) using ClustalW

(Thompson *et al*., 1994). A separate alignment was performed for each fungal family, and phylogenetic relationships among haplotypes were inferred separately for each family.

Phylogenetic inference

To assess the phylogenetic relationships among mycorrhizal fungi associated with *Bipinnula*, we selected one sequence representing each haplotype. Phylogenetic relationships were inferred using maximum parsimony (MP) and maximum likelihood (ML) approaches implemented in PAUP* version 4.0b10 (Swofford, 2002). For MP, a heuristic search was undertaken using TBR branch swapping. Bootstrap support of nodes for MP and ML was computed for 10 000 repetitions. Phylogenetic trees were also constructed using the Bayesian Markov chain Monte Carlo (MCMC) inference (BI) method implemented in MrBayes v 3.1.2. The general time-reversible model of DNA substitution and shape parameter of the gamma distribution (GTR + G) was selected using JMODELTEST 0.1.1 (Posada, 2008). Four simultaneous, independent runs were performed for >10 000 000 generations, starting from random trees. Trees were sampled every 1000 generations, resulting in a total of 10 000 trees from which the first 2500 (25 %) were discarded as the burn-in phase. A 50 % majority rule consensus tree was calculated based on the remaining sampled trees enabling the use of Bayesian posterior probabilities (BPPs) for node support.

Mycorrhizal diversity

Sequences were grouped into molecular OTUs at 97 % sequence similarity. Mycorrhizal diversity was assessed by counting the number of fungal OTUs detected in each orchid population. Phylogenetic diversity was estimated using nucleotide diversity (p) and the average number of pairwise nucleotide differences per site (π; Nei and Li, 1979) in DnaSP 5.1 (Librado and Rozas 2009).

Statistical analyses

We tested the effects of orchid host species and soil nutrients on OTU composition, phylogenetic diversity, OTU richness and the level of colonization by mycorrhizal fungi associated with *B. fimbriata* and *B. plumosa*. To assess the effects on OTU composition, we calculated dissimilarity between sites using Bray–Curtis distances (Bray and Curtis, 1957) and then performed a variance analysis of these distances, using a multivariate permutation test (Adonis test) implemented in the Vegan package of R (Oksanen *et al*., 2013). We included orchid host species identity (S), soil nitrate concentrations (N), soil phosphorus concentrations (P) and the interactions between S and soil nutrients. To correct for non-normality, we used $\log_{10}$ values. Given that we detected a significant effect of the interaction between P and S, we ran an Adonis test for each orchid species separately. The effect of orchid host species on site ordination was illustrated by a non-metric multidimensional scaling (NMDS) on Bray–Curtis distances. To assess the effect of orchid host species and soil nutrients on phylogenetic diversity, OTU richness and colonization of mycorrhizal fungi, we used generalized linear models (GLMs). Each response variable was modelled separately, testing the following components: S, N, P and the interactions S and soil nutrients. In this way, we tested whether the effect of soil nutrients on mycorrhizal fungi differed between orchid species. Models were built using a bi-directional stepwise selection procedure, starting with a full model and alternately omitting and re-introducing one model component at each step (Pearce and Ferrier, 2000). Models were selected according to the lowest values of the Akaike and Bayesian information criteria (AIC and BIC, respectively). The GLM deviance was estimated as goodness of fit. Significant relationships among variables were depicted using partial residual plots of the most likely model as judged by the BIC. Given that we detect a significant effect of the interaction between P and S, we ran a GLM for each orchid species separately.

RESULTS

We isolated 149 fungi of which 88 corresponded to rhizoctonias (according to Dearnaley *et al*., 2012) and obtained 78 DNA sequences from root sections. In total, including cultivated fungi, we obtained 166 orchid mycorrhizal fungi sequences, from which 46·6 % of OTUs were obtained both by culture and as DNA extracted directly from roots sections, 33·3 % were obtained only by direct extraction and 20 % of OTUs were obtained only by culture. We identified six OTUs of Tulasnellaceae and nine OTUs of Ceratobasidiaceae. In Tulasnellaceae, we identified OTUs closely related to the genus *Tulasnella*, including *T. calospora*, *T. danica* and *T. asymmetrica* (Fig. 2A). In Ceratobasidiaceae, we obtained OTUs of the genera *Ceratobasidium* (*Ceratobasidium* sp. and *C. albasitensis*) and *Rhizoctonia* (*Rhizoctonia* sp. and *R. butinii*) (Fig. 2B). We also found in orchid root samples non-*Rhizoctonia* (*sensu lato*) fungi, mainly Ascomycetes of the genera *Peziza* (Pezizales), *Phomopsis* (Diaporthales), *Hypocrea* (Hypocreales) and *Fusarium* (Hypocreales). In addition, we detected the presence of *Neonectria* (Hypocreales), *Leptodontidium* (Helotiales), *Piromyces* (Neocallimastigales), *Cylindrocarpon* (Hypocreales), *Acremoniula* (Hypocreales) and *Pythium* (Pythiales).

The composition of orchid mycorrhizal fungi varied among sites and species (Fig. 1B). In general, Tulasnellaceae were more common in *B. fimbriata* than in *B. plumosa* (84·7 and 25 %, respectively, $\chi^2 = 55{\cdot}46$, $P < 0{\cdot}01$); the latter species associated mostly with Ceratobasidiaceae. Accordingly, NMDS showed a clear differentiation between orchid species in fungal community composition (Fig. 3), which was statistically supported by ADONIS analysis (S effect: $F_{1,11} = 2{\cdot}96$, $P = 0{\cdot}004$; Table 1). This analysis also revealed a significant effect of the interaction orchid species-by-soil P ($F_{1,11} = 1{\cdot}99$, $P = 0{\cdot}04$; Table 1). When we analysed the effect of soil nutrients on the composition of fungal communities associated with each orchid species separately, we detected a significant effect of soil P for *B. fimbriata* ($F = 2{\cdot}44$, $P = 0{\cdot}04$), but not for *B. plumosa*. In general, at high soil P availability, the relative frequency of Ceratobasidiaceae increased in *B. fimbriata*.

All of the 115 plants sampled from 12 orchid populations showed signs of colonization by mycorrhizal fungi, but the intensity differed between populations. The best model for

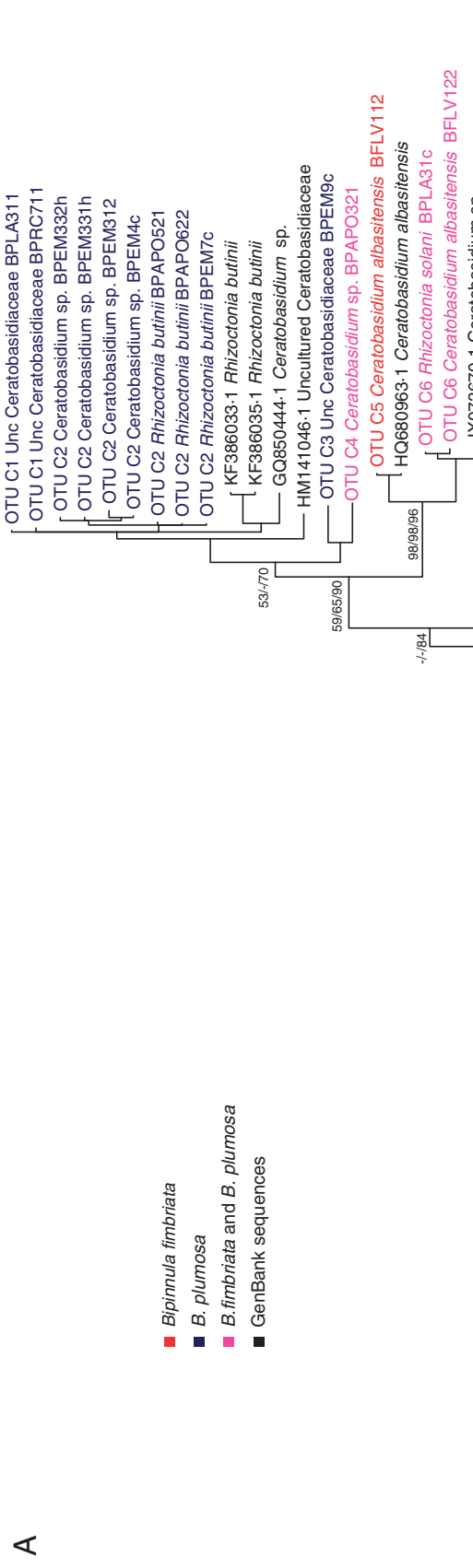

FIG. 2. Bayesian majority consensus trees based on internal transcribed spacer (ITS) sequences of Ceratobasidiaceae (A) and Tulasnellaceae (B) fungi. Trees were constructed with fungal operational taxonomic unit (OTU) sequences obtained from *Bipinnula fimbriata* and *B. plumosa* roots. The Ceratobasidiaceae tree was rooted with *Sebacina vermifera* (GenBank accession no. EU625999.1) and the Tulasnellaceae tree was rooted with *Tulasnella albida* (GenBank accession no. AY373294.1) and *T. violea* (GenBank accession no. DQ457643.1). Values on each branch represent: parsimony bootstrap values/maximum likelihood bootstrap values/Bayesian posterior probabilities.

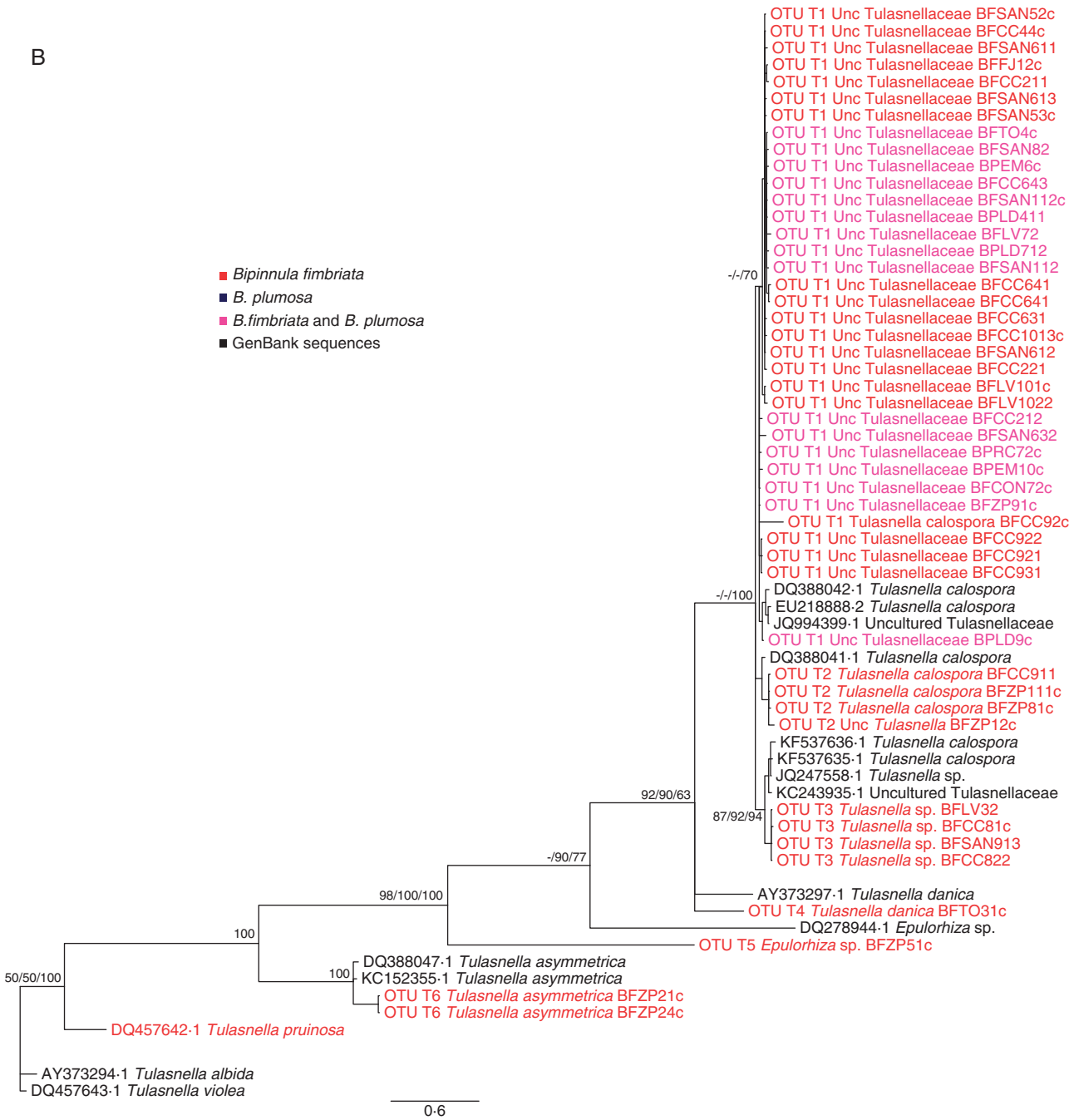

FIG. 2. Continued

explaining the variation in mycorrhizal colonization, as judged by AICs, included orchid species and soil P (null model, AIC = 80·61; best model, AIC = 70·17, R^2 = 0·69). Colonization intensity was higher in *B. frimbiata* than in *B. plumosa* (19·74 and 13·32 %, respectively; P = 0·001) and was positively affected by soil P (B = 0·36, P = 0·04) (Fig. 4B).

Mycorrhizal diversity also varied considerably among populations. The number of fungal OTUs found ranged between one in the FJ population (*B. fimbriata*) and five in the ZP and LV populations (*B. fimbriata*) (Fig. 1B). The best model for fungal OTU richness included the interaction between orchid species and soil nutrients (null model, AIC = 40·6; best model, AIC = 31·8, R^2 = 0·79) and therefore OTU richness associated with each orchid species was analysed separately. For *B. fimbriata*, we found a positive effect of soil P (B = 0·3, P = 0·01) and a negative effect of soil N (B = −2·7, P = 0·03) on fungal OTU richness, whereas for *B. plumosa* there were no significant relationships (Fig. 4A). Results for phylogenetic diversity of mycorrhizal fungi differed from those for OTU richness. Populations LA (*B. plumosa*) and LV (*B. fimbriata*) showed the

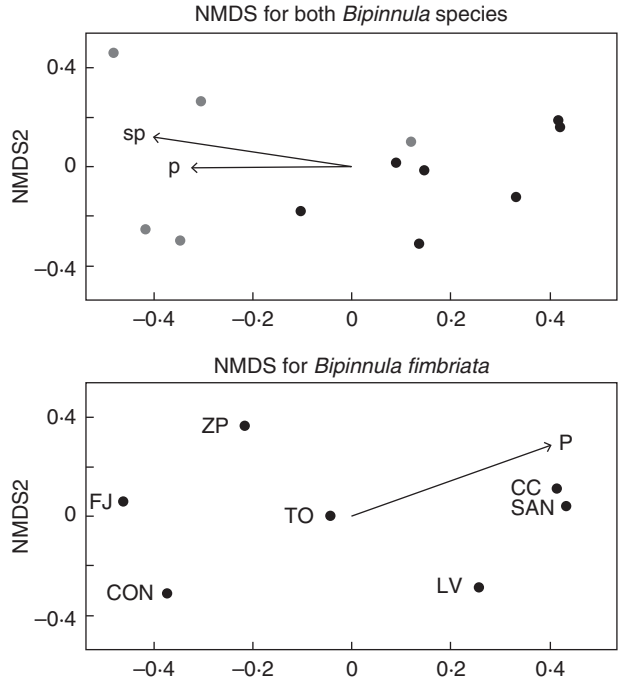

FIG. 3. Non-metric multidimensional scaling (NMDS) ordination plots based on the composition of the fungal mycorrhizal community associated with: (A) orchids *B. fimbriata* (black dots) and *B. plumosa* (grey dots); (B) only orchid *B. fimbriata*. Explanatory variables (S, orchid species; N, soil N, and P, soil P) found to be significant ($P \leq 0{\cdot}05$) in an Adonis test are represented as vectors in each diagram.

TABLE 1. *Effects of orchid host species and soil nutrients on operational taxonomic unit (OTU) composition for mycorrhizal fungi associated with 12 populations of* B. fimbriata *and* B. plumosa

	d.f.	*F*	R^2	*P*-value
Orchid species (S)	1	2·9662	0·19905	0·005
P	1	1·75405	0·11771	0·105
N	1	1·19411	0·08013	0·296
S × P	1	1·99589	0·13394	0·045
S × N	1	0·99159	0·06654	0·485

P, phosphorus; N, nitrate.

greatest phylogenetic diversity (π = 122, pi = 0·279 and π = 118, pi = 0·276, respectively), and populations FJ and CC (*B. fimbriata*) had the lowest phylogenetic diversity (π = 1·3, pi = 0·002 and π = 8·4, pi = 0·016, respectively). The best model for fungal phylogenetic diversity included only soil N (null model, AIC = −17·1; best model, AIC = −21·1, R^2 = 0·38), and showed negative effects of soil N on phylogenetic diversity ($B = -1{\cdot}6$, $P = 0{\cdot}03$) (Fig. 4D).

DISCUSSION

Fungal identity

This study showed that the southern South American orchids *B. fimbriata* and *B. plumosa* associated mainly with rhizoctonia fungi from Tulasnellaceae and Ceratobasidiaceae. As reported by Steinfort *et al.* (2010) in a previous study of *B. fimbriata*, we did not detect the presence of Sebacinales, which are often found in orchid roots (Oberwinkler *et al.*, 2014). In addition to Tulasnellaceae and Ceratobasidiaceae, we found members of Pezizaceae, that have previously been detected in orchid roots from Europe (Selosse *et al.*, 2004) and South Africa (Waterman *et al.*, 2011), the mycorrhizal status of which deserves further analysis. We also found numerous additional fungal taxa that have been identified as orchid root endophytes, including members of Hypocreales and *Leptodontidium* (Fernando and Currah, 1996), or as plant pathogens, such as *Neonectria* (Halleen *et al.*, 2006; Lovett *et al.*, 2006), *Cylindrocarpon* (Unestam *et al.*, 1989) and *Fusarium* (Benyon *et al.*, 2000). These results are consistent with recent molecular studies showing a broad range of non-rhizoctonia fungi associated with orchid roots (Dearnaley *et al.*, 2012).

Mycorrhizal composition

The composition of mycorrhizal fungi differed significantly between the two orchid host species. We observed a predominance of Tulasnellaceae in *B. fimbriata* populations and a predominance of Ceratobasidiaceae in populations of *B. plumosa*. These differences in fungal partners might reflect distinct mycorrhizal preferences for host species or, alternatively, this pattern could result from differential performance of mycorrhizal fungi in sites with different levels of soil P. In accordance with the latter hypothesis, we found that *B. plumosa*, which grows in the Andean foothills where soil P availability is higher due to volcanic activity (Instituto Geográfico Militar, 1984), was often associated with Ceratobasidiaceae. In contrast, for *B. fimbriata*, which grows in coastal range sites where soil P concentration is lower, mycorrhizal composition varied with soil P concentration, with a tendency for a higher frequency of Cerobatosidaceae in sites with greater soil P. These results are consistent with the growing evidence showing that soil conditions influence mycorrhizal composition in orchids (McCormick *et al.*, 2006; Illyés *et al.*, 2009; Bunch *et al.*, 2013). In particular, Bunch *et al.* (2013) found that soil nutrients strongly affected the fungal associations of *Cypripedium acaule* and suggested that this could result from different fungal communities available in soils or from fungal selection by orchids imposed by different soil conditions across sites. Experimental studies have demonstrated that plants can discriminate and reward the best fungal partners depending on resources supplied (Kiers *et al.*, 2011), and the best partner could change depending on soil availability.

Mycorrhizal diversity

Diversity of mycorrhizal fungi associated with *Bipinnula* species varied widely among sites and was well correlated with soil nutrients. OTU richness and phylogenetic diversity of mycorrhizal fungi decreased with increasing soil N availability (Fig. 4B, D). A similar negative relationship between soil N and mycorrhizal diversity has also been documented for ectomycorrhizal (Lilleskov *et al.*, 2002) and arbuscular mycorrhizal fungi (Liu *et al.*, 2012). Increased diversity of mycorrhizal

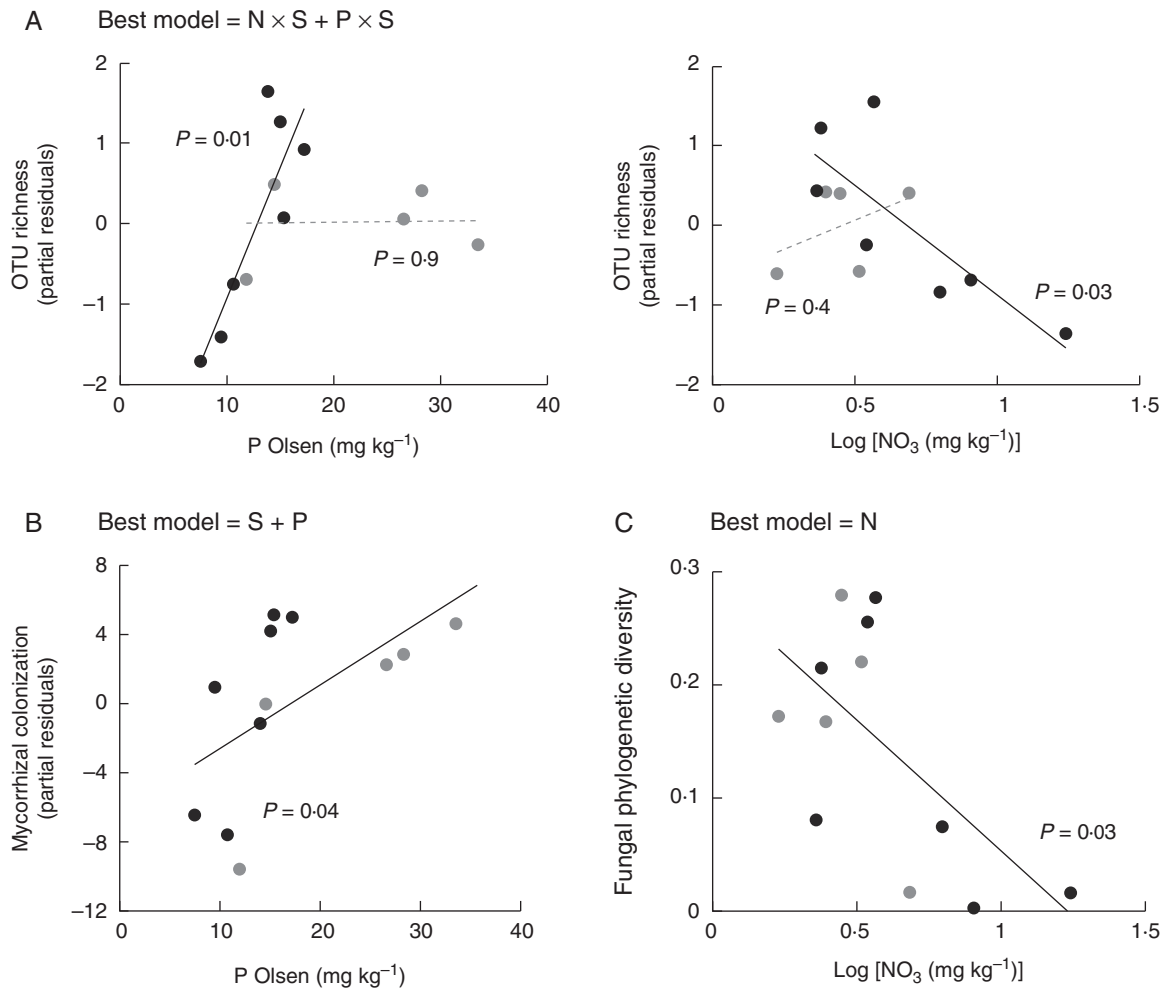

FIG. 4. Partial residual plots showing the relationships between soil nutrients and mycorrhizal fungi associated with 12 populations of *B. fimbriata* (black circles) and *B. plumosa* (grey circles): (A) fungal operational taxonomic unit (OTU) richness as a function of soil P (left) and soil N (right); (B) fungal phylogenetic diversity as a function of soil N; (C) fungal colonization intensity as a function of soil P. The best model obtained for each dependent variable is shown: S, orchid species; N, soil N; P, soil P. Partial residuals were obtained after removal of the other predictors in the best models. Differences between trends in *B. fimbriata* (solid line) and *B. plumosa* (dashed line) are indicated when the interactions S × P or S × N had a significant effect.

fungi in sites with lower soil N could reflect a tendency towards generalization under reduced soil nutrient availability. As proposed by Jacquemyn *et al.* (2012), given that different fungal lineages can exploit different nutritional resources, the ability of plants to associate with multiple partners at the same time could maximize their nutrient uptake, and, therefore, favour generalist associations under poor nutrient conditions. Likewise, under low nutrient availability, there could be a relaxation of partner choice by orchid which could result in more generalist associations (Kiers *et al.*, 2011). In turn, in fertile soils, plants could tend to associate with lower mycorrhizal diversity or to avoid the interaction. This tendency is not expected to occur in non-mycorrhizal endophytic fungi, because plants do not receive nutrients from these fungi. In agreement with this, our data show no correlation between OTU richness of non-mycorrhizal fungi and soil N ($r = 0{\cdot}03$, $P =$ n.s., data not shown), but a higher number of DNA sequences are needed to draw definitive conclusions.

In contrast to what we observed regarding soil N, we documented a positive relationship between OTU richness and P availability, but only for *B. fimbriata* (Fig. 4A). The effects of nutrients on mycorrhizal fungi can vary greatly depending on soil nutrient availability (Treseder and Allen, 2002). When the growth of plant and fungal partners is limited by soil N or soil P, fungal abundance is expected to increase with additions of N or P, but when plant growth is not nutrient limited, fungal abundance could become C limited and might decline with soil nutrient availability (Treseder and Allen, 2002). In the case of *Bipinnula* species, we found a positive relationship between mycorrhizal colonization and soil P, suggesting that fungal growth may be limited by soil P, but not by soil N. Under these conditions, an increment in soil P may enhance fungal abundance, supporting a higher OTU richness. Instead, if soil N is not limiting for mycorrhizal fungi, N availability might have a negative effect on mycorrhizal fungal diversity because of plant adaptations towards generalism in nutrient-poor soils. To explore these hypotheses, experimental studies of orchid populations to assess the effects of nutrient additions under P and N limitation are needed. In this way, we could test whether the effect of nutrients on mycorrhizal diversity and composition

depend on the initial nutrient status of populations, and also whether the effect is mediated by different fungal abundances in soils and/or by different orchid preferences.

Overall, a high diversity of mycorrhizal fungi associated with two *Bipinnula* species and a high variability in the diversity and composition of mycorrhizal partners between the two species and among conspecific populations are reported here. Differences in mycorrhizal composition between orchid species can arise due to differences in host plant preferences for fungal partners, due to contrasting distribution of mycorrhizal fungi, or because of differential performance of mycorrhizal fungi in soils with different nutrient availability. Further studies of the abundance of soil fungi abundance are needed to distinguish between the hypotheses. In our study, the differences in mycorrhizal diversity among sites were explained by the identity of orchid host species and differences in soil P and N content. These results support the hypothesis that higher soil nutrient availability promotes specialization in orchid–mycorrhizal associations, in particular for soils with high N availability. Soil P availability in turn was positively related to mycorrhizal diversity, probably because under low soil P availability, plants and fungi are both P limited. These two hypotheses must be tested by further experimental work.

Data accessibility

DNA sequences: GenBank accession numbers KP306566–KP306727

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: rarefaction curves generated for populations of the orchid species *Bipinnula fimbriata* and *B. plumosa* in central Chile. Figure S2: orchid root sections showing different levels of mycorrhizal colonization. Table S1: list of geographical locations and ecological descriptions for *Bipinnula* populations included in this study. Table S2: list of fungal taxa associated with the orchids *Bipinnula fimbriata* and *B. plumosa*; their taxonomic affiliation was inferred from the closest match in GenBank and the occurrence of each fungal taxon in each orchid population. Table S3: polymorphic sites and haplotypes of Tulasnellaceae and Ceratobasidiaceae sequences.

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