# DIVERSIDAD DE HONGOS MICORRÍCICOS EN EL GÉNERO *BIPINNULA* (ORCHIDACEAE) EN CHILE

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Por

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*Me subí a la ácida montaña,*

*busqué las flores donde albean,*

*entre las rocas existiendo*

*medio dormidas y despiertas.*

*Yo la encontré por mi destino,*

*de pie a mitad de la pradera,*

*gobernadora del que pase,*

*del que le hable y que la vea.*

(La flor del aire, Gabriela Mistral)

Mi nombre es María Isabel Mujica, nací en Santiago el 12 de diciembre de 1988. Mis primeros años los pasé en Curi-Ruca, cerca de Malalhue, XIV Región. Creo que en ese tiempo nació mi amor por la naturaleza. Fui al colegio Monte Tabor y Nazaret, en Santiago, y después entré a estudiar Biología a la Pontificia Universidad Católica, donde me titulé el año 2011. El 2012 comencé el Magíster en Ciencias de la Universidad de Chile, para estudiar las micorrizas de las orquídeas chilenas. Pretendo continuar con esta línea de investigación en el doctorado, estudiando el papel del mutualismo en la ecología y evolución de las especies, usando a las orquídeas como modelo. A continuación entrego esta tesis para cumplir los requisitos conducentes al grado de Magíster en Ciencias, mención Ecología y Biología Evolutiva.

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### Capítulo II

**Fig. 1** Bayesian majority consensus tree based on transcribed spacer (ITS) sequences of Ceratobasidiaceae and Tulasnellaceae fungi. Trees were constructed with operational taxonomic unit (OTU) sequences from (a) Ceratobasidiaceae (b) Tulasnellaceae obtained from *Bipinnula fimbriata* and *Bipinnula plumosa* roots collected from different populations in central Chile. The Ceratobasidiaceae tree (b) was rooted with *Sebacina vermifera* (EU625999.1 genbank). Values on each branch represent Parsimony bootstrap values/ Maximum likelihood bootstrap values /Bayesian posterior probabilities (BPP).

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## RESUMEN

Las interacciones biológicas tienen un rol clave en la distribución y diversificación de las especies; siendo las micorrizas -asociación simbiótica entre las raíces de una planta y un hongo del suelo- un ejemplo de ello. Un tipo particular de micorrizas son las micorrizas de orquídeas, las semillas de las orquídeas no poseen reservas energéticas, por lo que deben asociarse con un hongo micorrícico que les proveerá los nutrientes necesarios para germinar. El requerimiento de formar una asociación simbiótica con los hongos puede limitar la distribución y abundancia de las orquídeas. En este sentido, se ha estudiado ampliamente la relación entre la especialización micorrícica y la distribución de las orquídeas. Por otro lado, los cambios en el nivel de especialización son comunes en la evolución de las orquídeas y debido a las consecuencias de la especialización en la ecología de estas especies, es importante estudiar los factores ambientales que podrían promover la especialización. En esta tesis se abordaron las siguientes preguntas ¿Está la distribución de las orquídeas limitada por la asociación simbiótica? ¿Qué factores promueven la especialización micorrícica? Para responderlas, se estudiaron cuatro especies del género *Bipinnula* que poseen distribuciones geográficas contrastantes. Estudiamos la diversidad de hongos micorrícicos asociada a estas cuatro especies aislando y cultivando los hongos desde las raíces y extrayendo el ADN de los hongos directamente de la raíces de las plantas. Como resultado, se obtuvo información básica acerca de las micorrizas de orquídeas en el sur de Sudamérica, tanto acerca de la identidad y distribución de los hongos micorrícicos, como del nivel de especialización en esta asociación. Además, se observó una relación entre la especialización y el rango de distribución en *Bipinnula*, y una interacción extremadamente especialista en las especies raras, sugiriendo que las micorrizas podrían limitar la distribución de estas

especies. Por otro lado, se observó un efecto de la disponibilidad de nitrógeno y fósforo sobre la especialización micorrícica, indicando que los nutrientes del suelo juegan un rol importante modulando esta interacción. Estos resultados sugieren la incorporación de variables ambientales en el estudio de micorrizas de orquídeas para tener un entendimiento más completo de esta asociación. En conclusión, esta tesis contribuye al conocimiento de la ecología de las micorrizas de orquídeas, y por lo tanto provee pistas para la conservación de estas especies.

## ABSTRACT

Biological interactions have a key role on species distribution and diversification; mycorrhizas -an association between soil fungus and plants roots- are an example of this. A particular kind of mycorrhizas are orchid mycorrhizae, orchid seeds are very small and lack of energetic reserves so they depend on mycorrhizal fungi for germintation and early development. The requirement to form a symbiotic association with fungi could constrain the distribution of orchids. This way, it has been largely studied the relation between mycorrhizal specialization and rarity in orchids. On the other hand, changes on specialization are common in the Orchidaceae family, and -considering the consequences of mycorrhizal specialization- it is important to study the environmental factors that may promote specialization. In this thesis we assessed the following questions ¿Orchid distribution is limited by mycorrhizal association? ¿Which factors promote mycorrhizal specialization in orchids? To answer them, we studied four species of the genus *Bipinnula*, that present contrasting distributional ranges. We study the mycorrhizal diversity associated to these species by isolating fungi from roots and direct fungal DNA extraction from orchid roots. We obtained basic knowledge about mycorrhizal associations in South America, about fungal distribution and identity, and level of mycorrhizal specialization. Our results showed a relation between specialization and distributional range in Bipinnula, and an extremely specialist association in the rare species, these results suggest that the mycorrhizal interaction can be constraining the distribution of these species. On the other hand, we observed that soil content of P and N affect the mycorrhizal specialization in *Bipinnula*, indicating that soil nutrients play a key role on modulating orchid mycorrhizas. These results suggest that environmental factors should be included in the research on orchid mycorrhizas to improve our

understanding of this interaction. In conclusion, this thesis contributes to orchid mycorrhizal knowledge and therefore, provides clues for orchid conservation.

# INTRODUCCIÓN

La familia Orchidaceae es una de las familias más diversas. Posee alrededor de 25000 especies, lo que representa un 10% de las angiospermas (Cribb *et al.*, 2003).Ocurren en todos los continentes y se presentan en diferentes hábitats y hábitos (terrestres, epífitas y litófitas) (Chase *et al.*, 2003). Las orquídeas han sido sujeto de fascinación para los biólogos desde hace siglos (Bronstein *et al.*, 2013) e incluso ocuparon gran parte del tiempo de Charles Darwin, quien en una carta a Joseph Hooker confesaba *"I never was more interested in any subject in my life, than in this of Orchids"* (Roberts & Dixon, 2008)

Además de la sorprendente riqueza y la diversidad de formas, esta familia se caracteriza por depender fuertemente de sus interacciones mutualistas, tanto de polinizadores, como de hongos micorrícicos (Leake & Cameron, 2012; Selosse, 2014; Waterman *et al.*, 2011). Debido a esto, las orquídeas se han propuesto como un excelente modelo para el estudio de las interacciones biológicas y su rol en la ecología y evolución de las especies (Bronstein *et al.*, 2013, Selosse, 2014). Muchas de las características claves de las orquídeas están relacionadas con sus asociaciones simbióticas (Waterman & Bidartondo, 2008) y se ha sugerido que la gran diversificación de las orquídeas es un resultado de su interacción con hongos micorrícicos (Otero & Flannagan, 2006, Swarts *et al.*, 2010) y polinizadores (Cozzolino & Widmer, 2006).

Las semillas de las orquídeas son extremadamente pequeñas y son producidas en grandes cantidades (una cápsula puede contener entre 1000 hasta 4 millones de semillas), de ahí que sean llamadas "polvo de semillas" (Arditti & Ghani, 2000). No poseen reservas energéticas, por lo que dependen de hongos micorrícicos para la provisión de los nutrientes necesarios para la germinación y el crecimiento inicial de la plántula. Al encontrarse el hongo y la semilla, el hongo coloniza al embrión y la semilla germina, desarrollándose un protocormo (Janes 2009) -tejido indiferenciado no-fotosintético

compuesto por la planta y el hongo, que depende del hongo para su nutrición (McCormick *et al.*, 2006)- hasta la aparición de las primeras hojas. En esta etapa la orquídea ya es autótrofa y le transfiere compuestos carbonados al hongo, mientras que el hongo le transfiere nutrientes como nitrógeno y fósforo a la planta (Cameron *et al.*, 2006).

Además de tener que encontrar las condiciones abióticas adecuadas para establecerse, las orquídeas tienen la limitación de encontrar hongos micorrícicos compatibles (Rasmussen, 2002). De esta manera, el requerimiento de formar una asociación simbiótica con los hongos puede limitar la distribución y abundancia de las orquídeas (Phillips *et al.*, 2011, 2014). Las orquídeas se caracterizan por presentar patrones de distribución contrastantes. Así, algunas especies poseen distribuciones geográficas amplias, mientras que otras son raras con rangos de distribución muy restringidos (Batty *et al.*, 2002; Cribb *et al.*, 2003; Phillips *et al.*, 2011), lo que ha sido situado como una consecuencia de la interacción micorrícica. Las orquídeas con asociaciones más generalistas tendrán mayores probabilidades de encontrar un hongo compatible en la dispersión en comparación a una orquídea con asociaciones más especialistas (Bonnardeaux *et al.*, 2007). De esta forma es esperable que las orquídeas con rangos de distribución más amplios presenten interacciones más generalistas que las orquídeas más restringidas, debido al papel de las micorrizas en la limitación de la distribución. En este sentido, una de las grandes preguntas en el estudio de las micorrizas de orquídeas es si esta interacción limita la distribución de las orquídeas. Así, se ha estudiado ampliamente el efecto de la especialización micorrícica en la ecología y distribución de las orquídeas, sobre todo en si la especialización puede ser causa de la rareza de las orquídeas o llevarlas a la extinción (Swarts *et al.*, 2010, Phillips *et al.*, 2011, Bailarote *et al.*, 2012, McCormick & Jacquemyn, 2014).

Por otro lado, dentro de la familia Orchidaceae hay una gran variación en el nivel de especialización (Shefferson *et al.*, 2007; Swarts *et al.*, 2010). Hay especies que se asocian

con uno (Bougore *et al.*, 2009; McCormick *et al.*, 2006) o unos pocos micobiontes (Kennedy *et al.*, 2011; Shefferson *et al.* 2005), y otras se asocian con múltiples hongos (Pecoraro *et al.*, 2012; Kartzinel *et al.*, 2013; Pandey *et al.* 2013). Además, los cambios en el nivel de especialización son comunes en la evolución de la familia Orchidaceae (Shefferson *et al.*, 2007). Debido a que la especialización tiene importantes consecuencias en la ecología de las orquídeas, la segunda pregunta importante en el estudio de las micorrizas de orquídeas es qué factores promueven la especialización micorrícica. Los pocos estudios que existen al respecto se han enfocado en la composición de hongos asociados a las orquídeas, demostrando que las especies que habitan suelos similares se asocian a una comunidad de hongos micorrícicos similar (Bunch *et al.*, 2013), también que la composición de hongos micorrícicos puede estar determinada por el estrés asociado a la forma de vida epífita (Martos *et al.*, 2012) o a la disponibilidad de agua (Illyes *et al.*, 2009). Sin embargo, hasta ahora ningún estudio ha evaluado el rol de factores abióticos, como nutrientes del suelo, en el nivel de especialización micorrícica en orquídeas.

La familia Orchidaceae constituye una parte importante de la biodiversidad (Cribb *et al.*, 2003; Chase *et al.*, 2003), y posee una gran cantidad de especies con distribuciones muy restringidas o en alguna categoría de amenaza (Batty *et al.*, 2002, Swarts & Dixon, 2010). Para comprender mejor la ecología de las orquídeas y de esta manera colaborar en su conservación, en esta tesis se buscó responder las dos preguntas anteriormente expuestas ¿Cuál es el efecto de la especialización micorrícica sobre la distribución de las orquídeas? y ¿Qué factores promueven la especialización micorrícica? Estas preguntas fueron abordadas a través del estudio de las micorrizas de las especies chilenas del género *Bipinnula* Comm. Ex Juss.

Dos razones hacen de las especies de *Bipinnula* un buen modelo para estudiar las micorrizas de orquídeas. La primera es que poseen distribuciones geográficas contrastantes (Novoa *et al.*, 2006), *B. fimbriata* (Phil.) I.M. Johnst y *B. plumosa* Lindl. tienen

rangos de distribución amplios, mientras que *B. volckmannii* Kraenzl. y *B. apinnula* Gosewijn, B. tienen rangos muy restringidos, esto permite evaluar la hipótesis que relaciona la diversidad de hongos micorrícicos con la amplitud del rango de distribución de las orquídeas y contestar la primera pregunta. La segunda razón radica en que las especies de *Bipinnula* con mayor distribución abarcan un amplio rango latitudinal (30° a 35°S) implicando una variedad de condiciones ambientales, lo que permite evaluar el rol de los factores abióticos, como los nutrientes del suelo, en la especialización micorrícica y así contestar la segunda pregunta. Adicionalmente, en el sur de Sudamérica existen muy pocos estudios sobre las micorrizas de orquídeas, aún menos en el género *Bipinnula*, por lo tanto, esta tesis contribuirá al conocimiento básico de esta interacción, la identidad y la distribución de los hongos micorrícicos de orquídeas en esta región, que han sido escasamente estudiados hasta ahora.

## Objetivos e hipótesis

### Objetivo general

Evaluar la diversidad de hongos micorrícicos de las especies *Bipinnula volckmannii, B. apinnula, B. fimbriata* y *B. plumosa*, y su relación con el rango de distribución; y evaluar el efecto de los nutrientes del suelo en el nivel de especialización en esta asociación

**Capítulo 1**

- **Pregunta**

¿La distribución de *Bipinnula* está limitada por la diversidad de hongos micorrícicos asociados?

- **Objetivo específico**

Examinar la relación entre la diversidad de hongos micorrícicos y el rango de distribución de las especies, comparando la diversidad micorrícica de *Bipinnula fimbriata* y *Bipinnula plumosa* con la diversidad de *Bipinnula apinnula* y *Bipinnula volckmannii.*

- **Hipótesis**

La distribución de las orquídeas está limitada por la diversidad de hongos micorrícicos, por lo que las especies más restringidas de *Bipinnula* poseerán una diversidad de hongos menor a la encontrada en las especies ampliamente distribuidas.

**Capítulo 2**

- **Pregunta**

¿Qué factores ambientales promueven la especialización micorrícica en *Bipinnula*?

- **Objetivo específico**

Evaluar el efecto de diferentes parámetros del suelo (P, N, MO, K, pH) sobre la diversidad de micorrizas asociada a *Bipinnula fimbriata* y *Bipinnula plumosa.*

- **Hipótesis**

Las orquídeas se asocian a una mayor diversidad de hongos micorrícicos en suelos más pobres en nutrientes, con el objetivo de maximizar la obtención de nutrientes.

## CAPÍTULO 1

**High mycorrhizal specialization in the rare orchids *Bipinnula volckmanni* Kraenzl. and *Bipinnula apinnula* Gosewijn.**


### ABSTRACT

Understanding the causes of rarity is highly relevant to ecology. The requirement of many terrestrial plants to form a symbiosis with mycorrhizal fungi may limit the distribution and abundance of plant species. Orchids rely on mycorrhizal fungi for seed germination and early development, because of the high dependence of orchids on fungi it has been suggested that the degree of mycorrhizal specificity may be associated with rarity. In this study we aim to evaluate if there is a relation between distributional range and mycorrhizal specialization in the genus *Bipinnula* in Chile, comparing the mycorrhizal fungal diversity between widespread and rare species. We evaluated fungal species richness and phylogenetic diversity associated with these species across nine populations in total, by isolating fungi from roots and direct fungal DNA extraction from orchid roots. In the widespread orchids we found a considerable higher fungal diversity than in rare orchids, including taxa from Ceratobasidiaceae and Tulasnellaceae fungal families. In contrast, we observed an extremely specific association in rare orchids, which associated with only one fungal OTU from the Ceratobasidiaceae family. Rare and common *Bipinnula* exhibited significant differences in mycorrhizal diversity, suggesting that mycorrhizal specificity could be contributing to rarity in *Bipinnula*. However, further research including germination experiments are needed to conclude that mycorrhizal associations are limiting the populations of rare *Bipinnula*.

## INTRODUCTION

Understanding the causes of rarity is highly relevant to ecology (Gaston, 1998) and conservation (Fiedler & Ahouse, 1992; Harcourt *et al.*, 2002), as it contributes to predict which species are threatened and to improve conservation efforts (Manne & Pimm, 2001). Rare species are those that have low abundance or small geographical range sizes (Gaston, 1998), therefore this species are more susceptible to extinction (Gaston, 1998; Harris & Pimm, 2007). In addition to abiotic factors, biotic interactions can limit geographic distribution and then contribute to species rarity (Ogura-Tsujita & Yukawa, 2008, Boulangeat *et al.*, 2012), for example seed dispersion can limit plant distribution (Primack & Miao, 1992), and plant rarity can be affected by the interaction with soil biota (Klironomos, 2002; Reinhart *et al.*, 2003).

The Orchidaceae family is an excellent model for investigating the role of biotic interaction in the distribution of species (Bronstein *et al.*, 2013, Selosse *et al.*, 2014). These species present contrasting distribution patterns, some are very widespread and many others are rare, with very restricted geographical range (Batty *et al.*, 2002, Cribb *et al.*, 2003; Phillips *et al.*, 2011). Orchids are characterized by the high dependence on their mutualistic partners, both pollinators and mycorrhizal fungi (Leake and Cameron, 2012; Selosse, 2014) consequently high diversification and contrasting distributional patterns have been suited as a result of the dependence of orchids on mycorrhizas (Otero & Flanagan, 2005; Swarts *et al.*, 2010) and pollinators (Cozzolino & Widmer, 2005).

Orchid seeds are unique, they are very small, produced in large quantities and are lacking of energetic reserves (Arditti & Ghani, 2000), so they depend on mycorrhizal fungi for nutrient provision to early seedling development in nature (McCormick *et al.*, 2006; Smith and Read, 2008). This process is called "symbiotic germination" and it results in the

development of a protocorm, undifferentiated mycoheterotrophic tissue (McKendrick *et al.*, 2002; McCormick *et al.*, 2006) that receives carbon from mycorrhizal symbiosis (Smith, 1967) until the appearance of the first green leaves. At this state the orchid is autotrophic and transfers photosynthetic carbon products to its mycobiont, while the later transfers nitrogen to the plant (Cameron *et al.*, 2006). However, some orchids remain mycoheterotrophic to the adult state obtaining C from mycorrhizal fungi; this nutritional mode has evolved independently many times in the Orchidaceae family (Leake, 1994, Bidartondo *et al.*, 2002).

In plants with mycoheterotrophic life stages like orchids, the major limiting factor for geographic distribution is the presence of their mycorrhizal partner (Ogura-Tsujita & Yukawa, 2008). In addition to find adequate physical condition to establish, orchid seeds have the limitation of encounter a compatible mycorrhizal fungus (Rassmusen, 2002), therefore the requirement to form a symbiotic association with fungi could constrain the distribution and abundance of orchids (Phillips *et al.*, 2011, 2014). Orchids with generalist association will have more chances to find a compatible fungus in the dispersion, than orchids with more specialist association (Bonnardeaux *et al.*, 2007). Then it is expected that specialist orchids tend to have more restricted ranges of distribution than orchids with more generalist association.

Although there is no a general pattern about mycorrhizal fungi constraining orchid population (McCormick & Jacquemyn, 2014), there are studies that have found a relation between mycorrhizal specialization and rarity (Shefferson *et al.*, 2005, Swarts *et al.*, 2010) suggesting that specialist associations can play an important role in causing or contributing to orchids rarity.

*Bipinnula* Comm. Ex Juss (subtribu Chloraeinae, tribu Diuridae) is an endemic genus of the south of South America and all their species are terrestrial and photosynthetic. In Chile, the species are *Bipinnula apinnula* Gosewijn, *B. volckmanni* Kraenzl., *B. fimbriata*

(Phil.) I.M. Johnst, *B. plumosa* Lindl. and B. *taltalensis* I.M. Johnst. These species present contrasting geographic distribution, *B. fimbriata* and *B. plumosa* present wide distributional ranges, while *B. volckmannii* and *B. apinnula* have very restricted distributional ranges (Novoa *et al.*, 2006). These differences allow us to evaluate the hypothesis that proposes a relation between distributional range and mycorrhizal specialization. In this study we aim to evaluate the mycorrhizal fungal diversity associated with both widespread and rare species, to assess if *B. volckmannii* and *B. apinnula* have more specific association than *B. fimbriata* and *B. plumosa* and thus evaluate the role of mycorrhizal fungi in limiting orchid distributional ranges in *Bipinnula*. In particular, we addressed the following questions (1) Which mycorrhizal fungi are associated with *Bipinnula* spp.? (2) How specialist is the mycorrhizal association of these species? (3) Are the rare species of *Bipinnula* associated with similar mycorrhizal fungi than widespread species? (4) Is there a relation between mycorrhizal specialization and distributional range in the genus *Bipinnula*?

## MATERIALS AND METHODS

*Study species*

*Bipinnula fimbriata, B. plumosa, B. apinnula* and *B. volckmannii* are terrestrial orchids endemic to Chile. *Bipinnula fimbriata* and *B. plumosa* are widespread species, *Bipinnula fimbriata* is typically found in lowland (<500 m) coastal areas from 29°S to 35°S (Novoa *et al.*, 2006), preferably on sandy stabilized soils, in sites exposed to sunlight and marine breeze (Elórtegui & Novoa, 2009); while *Bipinnula plumosa* generally occurs above 1000 m.a.s.l. from 31 to 34°S, on south or southwest-facing slopes of the Andean Cordillera and also on coastal hilltops in association with sclerophyllous shrubs. In contrast, *Bipinnula volckmannii* and *B. apinnula* have very restricted distributions, *Bipinnula apinnula* is

distributed above 1400 m.a.s.l. on Andean mountain ranges, from 35° S to 36° S, on slopes of *Nothofagus obliqua* and *Nothofagus glauca* forest. Only three populations are known of this endangered species, with less than 10 individuals each, occupying a total area of 56 $km^2$ (MMA 2010*). *Bipinnula volckmannii* is also a very rare species, being restricted to the Andean mountain ranges above 1500 msnm, between 36° and 37° S (Novoa *et al.*, 2006), and associated to *Nothofagus oblicua* and *Nothofagus domeyi* forest. There are three known population of this species with less than 10 individuals per population, occupying a total area of 39.06 $km^2$, it is also classified as endangered.

*Sampling*

Sampling was conducted during the flowering season between August and December in 2012 and 2013, with a total of 73 plants sampled from three populations of *Bipinnula fimbriata* (SAN, TO, CON) and three of *Bipinnula plumosa* (APO, EM, RC). For these species we collected four roots from 10 orchid individuals from each population. *B. volckmannii* and *B. apinnula* are very rare so is difficult to obtain material from more than one or two populations. We collected 4 roots from 4 - 5 individuals from one population in *B. volckmannii* (SHL) and two in *B. apinnula* (LIR and 7T) (Fig.1).

*Fungal isolation*

Roots were cut in pieces of 3 to 5 cm length, washed under tap water to remove soil and other debris, and sterilized by placing them 1 min in a 10% hypochlorite solution and then 3 min in sterile distilled water three times consecutively. Roots with verified presence of pelotons (orchid mycorrhizal fungi forms pelotons in cortex cells of roots) were cut in section of 3 mm long and plated onto potato dextrosa agar (PDA) containing 0.16 mg/L streptomycin and penicillin, before incubation in a dark room at 18°C. As soon as fungal colonies developed, fresh mycelium from each isolate was subcultured until getting 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) to investigate the presence of fungal species that could not be cultured in vitro.

*DNA extraction, amplification and sequencing*

From both pure fungal cultures and stored root sections, DNA was extracted using a modified cetyltrimethyl ammonium bromide (CTAB) method from Doyle and Doyle (1990). Oligonucleotide primers ITS1 and ITS4 (White *et al.*, 1990) were used for amplification of DNA from fungal isolates. To guarantee the amplification of fungal DNA over plant DNA from root sections, we used the specific primers ITS1F/ITS4, ITS1F/ITS4B (for basidiomycetes) (Gardes & Bruns, 1993), ITS1/ITS4-Tul (for Tulasnellaceae) (Taylor & McCormick, 2008) and CeTh1/CeTh4 (for Ceratobasidiaceae) (Porras-Alfaro & Bayman 2007). For all primers the PCR was carried out in a final volume of 100 uL, containing 10 uL of 10Xbuffer, 6 uL of 50 mM Mg, 2 uL BSA, 2 uL dNTP, 2 uL of each primer, 0.5 Taq polymerase and 4 ul of extracted DNA. PCR was performed using the following temperature profile: 95°C for 5 min, 35°C for 1 min, 72°C for 1 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) in the GenBank database. Sequences that correspond to orchid mycorrhizal fungi (corresponding to the families Ceratobasidiaceae, Tulasnellaceae and Sebacinaceae) were chosen. The sequences were aligned in BioEdit (Hall, 1999) using ClustalW algorithm (Thompson *et al.*, 1994).

*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 approaches implemented in PAUP* version 4.0b10 (Swofford, 2003). For MP, a heuristic search was undertaken using TBR branch swapping. Bootstrap support at nodes for MP and ML was computed for 10,000 replicates of the data. 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 used. Four simultaneous, independent runs were performed for over 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 2,500 (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 (BPP) as node support.

*Mycorrhizal diversity*

Based on the clades resulted from the phylogenetic inferences, sequences were grouped into operational taxonomic units (OTUs). Mycorrhizal diversity was determined by counting the number of fungal OTUs detected in each population. Phylogenetic diversity was calculated with nucleotide diversity (p) and the average number of pairwise nucleotide differences per site ($\pi$; Nei, 1987) which were estimated using DnaSP 5.1 (Librado & Rozas, 2009). To compare mycorrhizal diversity between rare species and widespread species, we performed rarefactions curves, to assess if there were significant differences in the fungal richness.

## RESULTS

We collected a total of 73 plants, all of which present signs of mycorrhizal colonization. From *B. volckmannii* and *B. apinnula* we isolated 14 orchid mycorrhizal fungi, all of which present the same morphological characteristics (Fig. 2) and 16 orchid mycorrhizal fungi sequences were obtained directly from roots extracted DNA. In total we obtained 31 sequences, all of them belonged to Ceratobasidiaceae. Also, we isolated non-orchid mycorrhizal fungi corresponding to *Fusarium* (Hypocreales), *Hormonema* and *Morchella*. All mycorrhizal sequences belong to the same haplotype. Thus, we identified only one OTU in the three populations. BLAST analysis revealed that this haplotype is 98% similar to Unc. Ceratobasidiaceae (GenBank access: JQ972106.1). Therefore the fungal OTU richness and phylogenetic diversity associated with these two species is one and cero, respectively.

Instead, we isolated 38 mycorrhizal fungi from *B. fimbriata* and *B. plumosa*, and we obtained 44 sequences directly from root extracted DNA. The total 82 orchid mycorrhizal sequences obtained belonged to the fungal families Ceratobasidiaceae and Tulasnellaceae. BLAST analysis revealed that within Tulasnellaceae, we identified OTUs closely related to the genus *Tulasnella*, including *Tulasnella calospora* and *T. danica*. While in Ceratobaisdiaceae, we obtained OTUs from *Ceratobasidium* sp., *Rhizoctonia butinii* and Unc. Ceratobasidiaceae. Also, we isolated the non-orchid mycorrhizal fungi *Peziza*, *Pythium*, *Fusarium*, and *Phosmosis*. The OTU richness in populations of these species was between two OTUs (RC) and 6 (EM and SAN). Instead, the population with higher phylogenetic diversity is CON (pi=0.25), while APO had the lowest (pi=0.01).

The phylogenetic inference showed that the haplotype founded in *Bipinnula apinnula* and *B.volckmanii* is also founded in a population of *Bipinnula plumosa* and belongs to a narrow clade of Ceratobasidiaceae integrated by this haplotype and other

Unc. *Ceratobasidiaceae* obtained from *Bipinnula plumosa* (Fig. 3). *Bipinnula fimbriata* and *B. plumosa* (widespread species) have a higher diversity of mycorrhizal fungi, being associated with several taxa of Ceratobasidiaceae and Tullasnellaceae. The rarefaction curves showed that although some populations do not reach saturation, there is a significant difference in mycorrhizal diversity between widespread species and rare species (Fig.4).

## DISCUSSION

Our results showed that *Bipinnula fimbriata* and *B. plumosa* had a considerable higher diversity of mycorrhizal fungi than *Bipinnula apinnula* and *B. volckmannii.* The widespread species are associated with several taxa from Tullasnellaceae and Ceratobasidiaceae families. These results are similar to what Steinfort *et al.,* (2010) found in two population of Bipinnula fimbriata. In contrast, rare species presented an extremely specialist interaction, as they associated exclusively with one species of the Ceratobasidiaceae fungal family. The rarefaction curves showed that *Bipinnula apinnula* and *B. volckmannii* have significant more specialist association than *Bipinnula fimbriata* and *B. plumosa.*

The differences observed suggest that mycorrhizal specialization may be playing an important role limiting orchid distribution in this genus, where orchid rarity may be related to the rarity of symbiotic partners (Phillips *et al.,* 2011). Rarity of *B. apinnula* and *B.volckmannii* may be a consequence of a lower probability to find a mycorrhizal partner after seed dispersion than widespread and generalist species of *Bipinnula.* Similar results have been found in the genus *Caladenia* of Southwest Australia (Swarts *et al.,* 2010) where widespread species have significant higher mycorrhizal diversity than rare species.

The fungus associated with *Bipinnula apinnula* and *B. volckmannii* is also founded in one population of *B. plumosa* (EM). EM is located at a similar altitude approximately 200

km north from populations of *Bipinnula apinnula*, which means that this OTU is at least distributed between these geographical locations. Nevertheless, mycorrhizal fungal communities have a strong spatial segregation (Jacquemyn *et al.*, 2013, Kartzinel *et al.*, 2013) that can limit spatial distribution of orchid species, so maybe this fungus have a widespread distribution at a regional scale, but at locally scale is so segregated that can limit the distribution of *Bipinnula apinnula* and *B. volckmannii*.

The mycorrhizal association of *Bipinnula apinnula* and *B. volckmannii* is one of the most specialists orchid mycorrhizal interactions reported to photosynthetic orchids (McCormick *et al.*, 2004, Shefferson *et al.*, 2005) and it is similar to results found in nonphotosynthetic orchids (Bougoure 2009, Barret *et al.*, 2010, Taylor *et al.*, 2002, McKendrick *et al.*, 2002, Kennedy *et al.*, 2011) which are thought to have more specialist interactions (McCormick *et al.*, 2006). Our results showed that this rare orchids present extremely specialist association. However, this observation could be a result of absolute specificity or a consequence of the fungal availability in habitats where these orchids occurred (i.e. ecological specialization). Therefore, to assess if it is an absolute specialization, it is necessary to study the diversity of mycorrhizal fungi on soil, to assess if *B. apinnula* and *B.volckmannii* are selecting this specific fungus from a pool of mycorrhizal fungi, or this fungi is the only one available. Also, it is necessary to further evaluate if orchid seed exclusively germinate with this fungal species or it is capable to germinate with others fungal species, to ensure the limiting role of mycorrhizal fungi in these orchid species.

Specialist species are more susceptible to disturbances than generalist species (Clavel *et al.*, 2010), as specialization may reduce the accessibility to an alternative partner under unfavorable conditions. For successful conservation strategies and management of orchids it is necessary to identify the associated fungi (Batty *et al.*, 2002), to understand the distribution and ecological requirements of fungal partners, and to take into account the

specialized needs of orchids (Swarts & Dixon, 2010). Therefore, our results have important implications to conservation of *B. apinnula* and *B.volckmannii*, as we showed that this rare orchids present identified the specific fungal partner that associates with these species, a useful information to both *ex-situ* and *in-situ* conservation. We showed that in the genus *Bipinnula* in Chile there is a relation between distributional range and mycorrhizal specialization. This result represents a starting point to potentially diverse lines of research and experimental studies.


## ACKNOWLEDGEMENTS

We thank to all members of the Evolutive Eco-physiology Lab of Pontificia Universidad Católica de Chile, for all their help on fieldwork and data analysis. We thank Paz Montenegro for her help on designing Figure 2. Funding was provided by FONDECYT and by the Institute of Ecology and Biodiversity (grants P05-002 from ICM and PFB-23 from Conicyt).

## TABLES

**Table 1.** List of the 17 fungal operational taxonomic units (OTUs) identified from root sections and isolated fungi of four species of *Bipinnula* across nine population. The first letters of the OTU name represent the fungal family to which belong: Cer to Ceratobasidiaceae and Tul to Tullasnelaceae.

| OTU | Closest match in GenBank | *B. fimbriata* | | | *B. plumosa* | | | *B. apinnula* | | *B. volckmannii* |
|---|---|---|---|---|---|---|---|---|---|---|
| | | SAN | TO | CON | APO | EM | RC | LIR | 7T | SHL |
| Cer1 | *Rhizoctonia* sp. WUF-ST-RhT2-9 (JQ859901.1) | 0 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cer2 | *Ceratobasidium* sp. JTO-2010a (GQ850451.1) | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cer3 | Ceratobasidiaceae sp. M327 (HM141014.1) | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| Cer4 | *Rhizoctonia butinii* isolate Bu2c (KF386032.1) | 0 | 0 | 0 | 0 | 5 | 0 | 0 | 0 | 0 |
| Cer5 | *Rhizoctonia butinii* isolate 11026 (KF386035.1) | 0 | 0 | 0 | 12 | 2 | 0 | 0 | 0 | 0 |
| Cer6 | Uncultured fungus clone 126_NA5 (KF297230.1) | 0 | 0 | 0 | 1 | 0 | 5 | 0 | 0 | 0 |
| Cer7 | Uncultured Ceratobasidiaceae isolate 978 (HM141020.1) | 0 | 0 | 0 | 0 | 1 | 0 | 8 | 9 | 14 |
| Cer8 | *Ceratobasidium* sp. JTO-2010a (GQ850421.1) | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| Cer9 | Ceratobasidium sp. L9Rh-col6 (HM117643.1) | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Tul1 | *Tulasnella* sp. 5 MM-2012 isolate (JQ247558.1) | 6 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Tul2 | Uncultured Tulasnellaceae clone FM665.1 (JF691471.1) | 1 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| Tul3 | Uncultured mycorrhizal fungus isolate (DQ790790.1) | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 |
| Tul4 | Uncultured mycorrhiza (Tulasnellaceae) 6009 (AY634123.1) | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Tul5 | Uncultured mycorrhizal fungus isolate 30 (DQ790815.1) | 11 | 2 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| Tul6 | Uncultured Tulasnellaceae clone AP1J07 (JQ994398.1) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Tul7 | *Epulorhiza* sp. CBS 189.90 (DQ278944.1) | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Tul8 | Uncultured Tulasnellaceae clone OTUA7 (JX649080.1) | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Nr. OTUs | 6 | 3 | 4 | 3 | 6 | 2 | 1 | 1 | 1 |
| | Phylogenetic diversity (pi) | 0,08 | 0,08 | 0,25 | 0,02 | 0,17 | 0,17 | 0 | 0 | 0 |

## FIGURES

**Fig. 1** Geographical distribution (approximately) and photographies of *Bipinnula fimbriata* (BF), *Bipinnula plumosa* (BP), *Bipinnula apinnula* (BA) *and Bipinnula volckmannii* (BV). Circles indicate populations sampled, above circles is the population name.

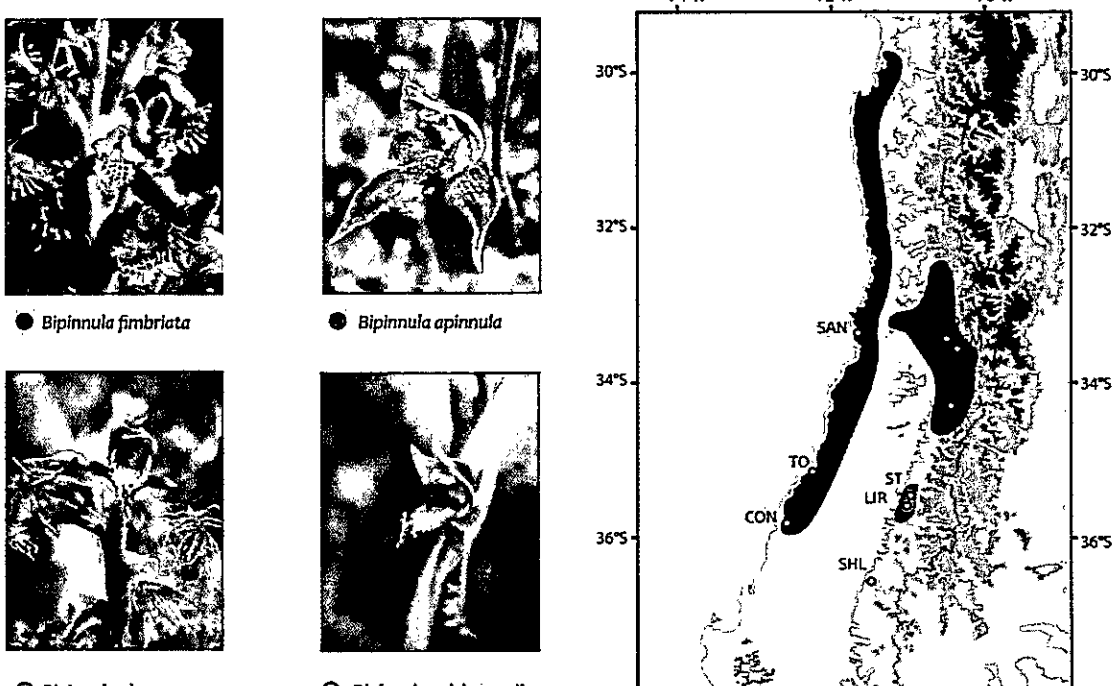


**Fig. 2** Hyphae of Unc. Ceratobasidiaceae, the only haplotype associated with *Bipinnula apinnula* and *Bipinnula volckmanni.* Scale is indicated with black lines of 100 µm

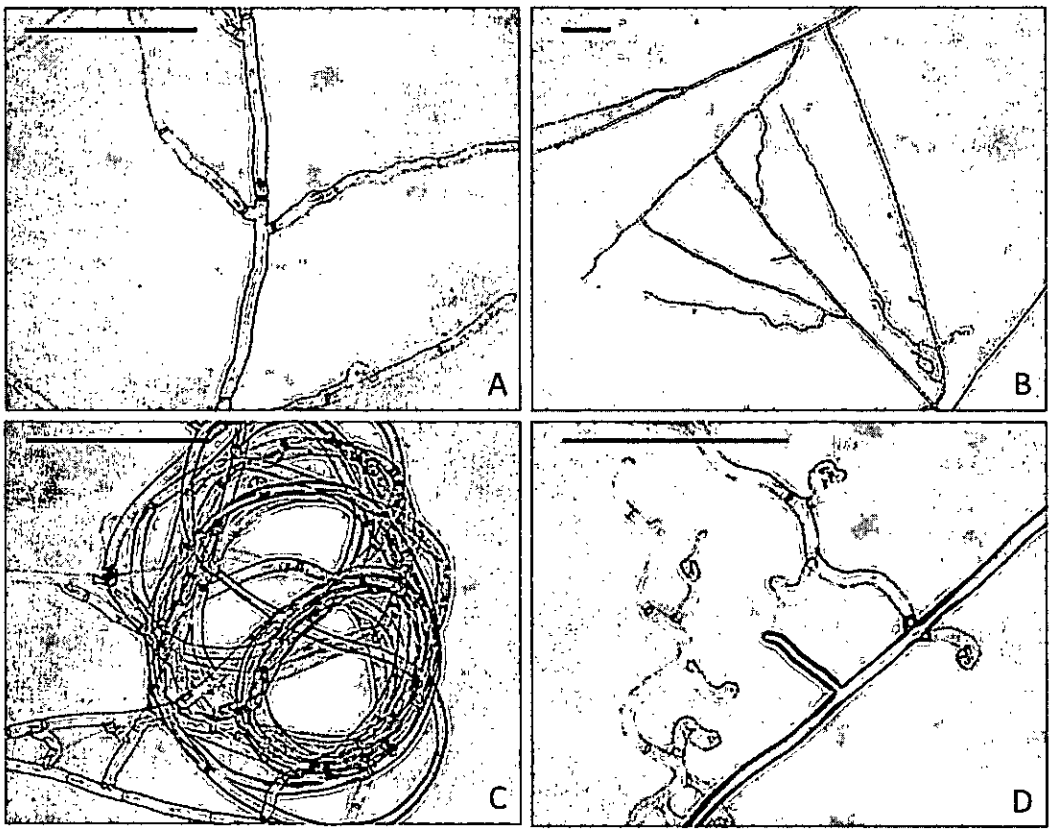
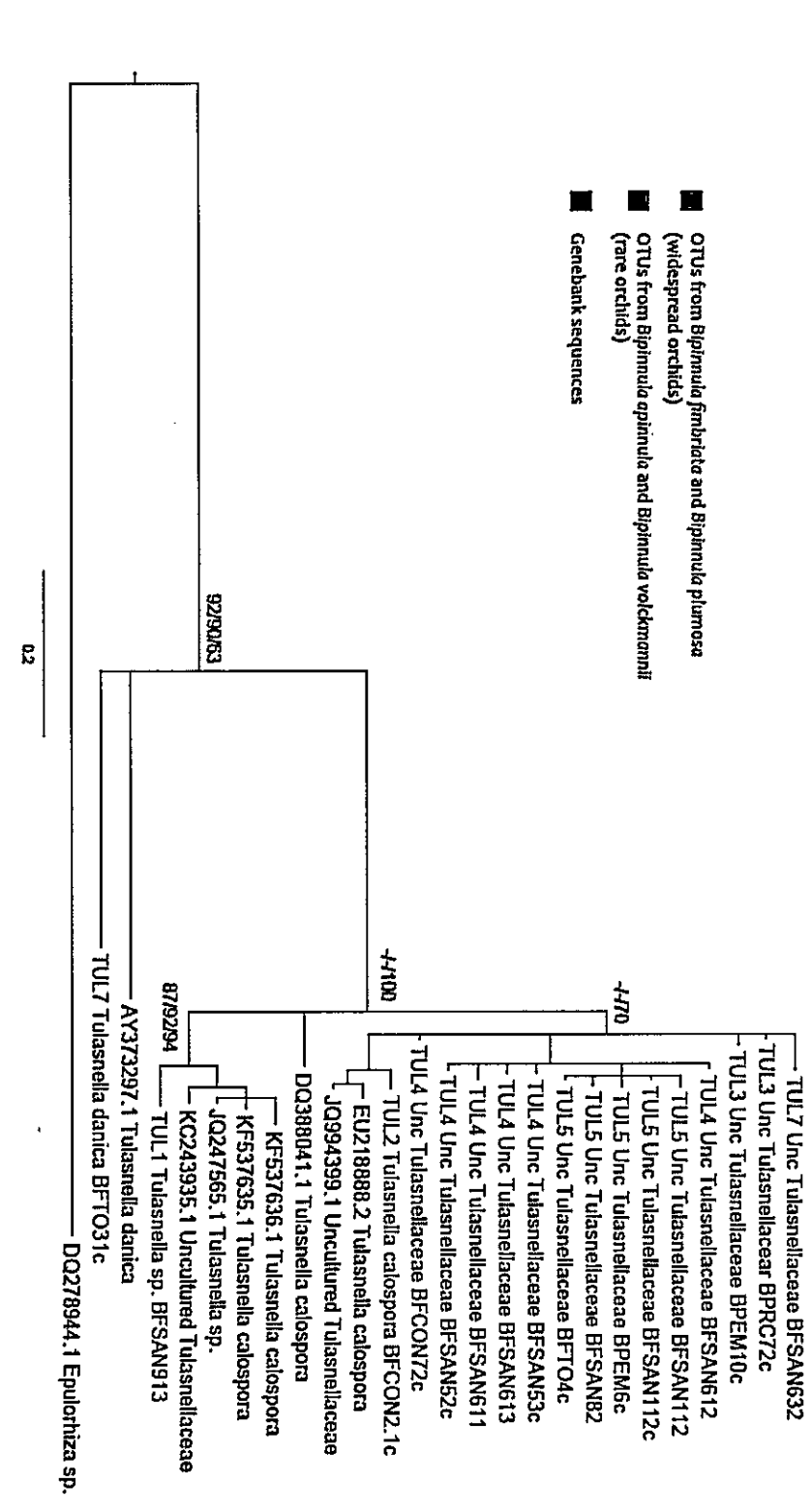


**Fig. 3** Bayesian majority consensus tree based on transcribed spacer (ITS) sequences of Ceratobasidiaceae and Tulasnellaceae fungi. Trees were constructed with operational taxonomic unit (OTU) sequences from (a) Ceratobasidiaceae (b) Tulasnellaceae obtained from *Bipinnula fimbriata* and *Bipinnula plumosa* roots collected from different populations in central Chile. The Ceratobasidiaceae tree (b) was rooted with *Sebacina vermifera* (EU625999.1 genbank). Values on each branch represent Parsimony bootstrap values/ Maximum likelihood bootstrap values /Bayesian posterior probabilities (BPP).

a)


OTUs from Bipinnula fimbriata and Bipinnula plumosa (widespread orchids)
OTUs from Bipinnula apinnula and Bipinnula volckmannii (rare orchids)
Genebank sequences
TUL7 Unc Tulasnellaceae BFSAN632
TUL3 Unc Tulasnellacear BPRC72c
TUL3 Unc Tulasnellaceae BPEM10c
TUL4 Unc Tulasnellaceae BFSAN612
TUL5 Unc Tulasnellaceae BFSAN112
TUL5 Unc Tulasnellaceae BFSAN112c
TUL5 Unc Tulasnellaceae BPEM6c
TUL5 Unc Tulasnellaceae BFSAN82
TUL5 Unc Tulasnellaceae BFTO4c
TUL4 Unc Tulasnellaceae BFSAN53c
TUL4 Unc Tulasnellaceae BFSAN613
TUL4 Unc Tulasnellaceae BFSAN611
TUL4 Unc Tulasnellaceae BFSAN52c
TUL4 Unc Tulasnellaceae BFCON72c
TUL2 Tulasnella calospora BFCON2.1c
EU218888.2 Tulasnella calospora
JQ994399.1 Uncultured Tulasnellaceae
DQ388041.1 Tulasnella calospora
KF537636.1 Tulasnella calospora
KF537635.1 Tulasnella calospora
JQ247565.1 Tulasnella sp.
KC243935.1 Uncultured Tulasnellaceae
TUL1 Tulasnella sp. BFSAN913
AY373297.1 Tulasnella danica
TUL7 Tulasnella danica BFTO31c
DQ278944.1 Epulorhiza sp.
-/-/70
-/-/100
87/92/94
92/93/93
0.2

b)

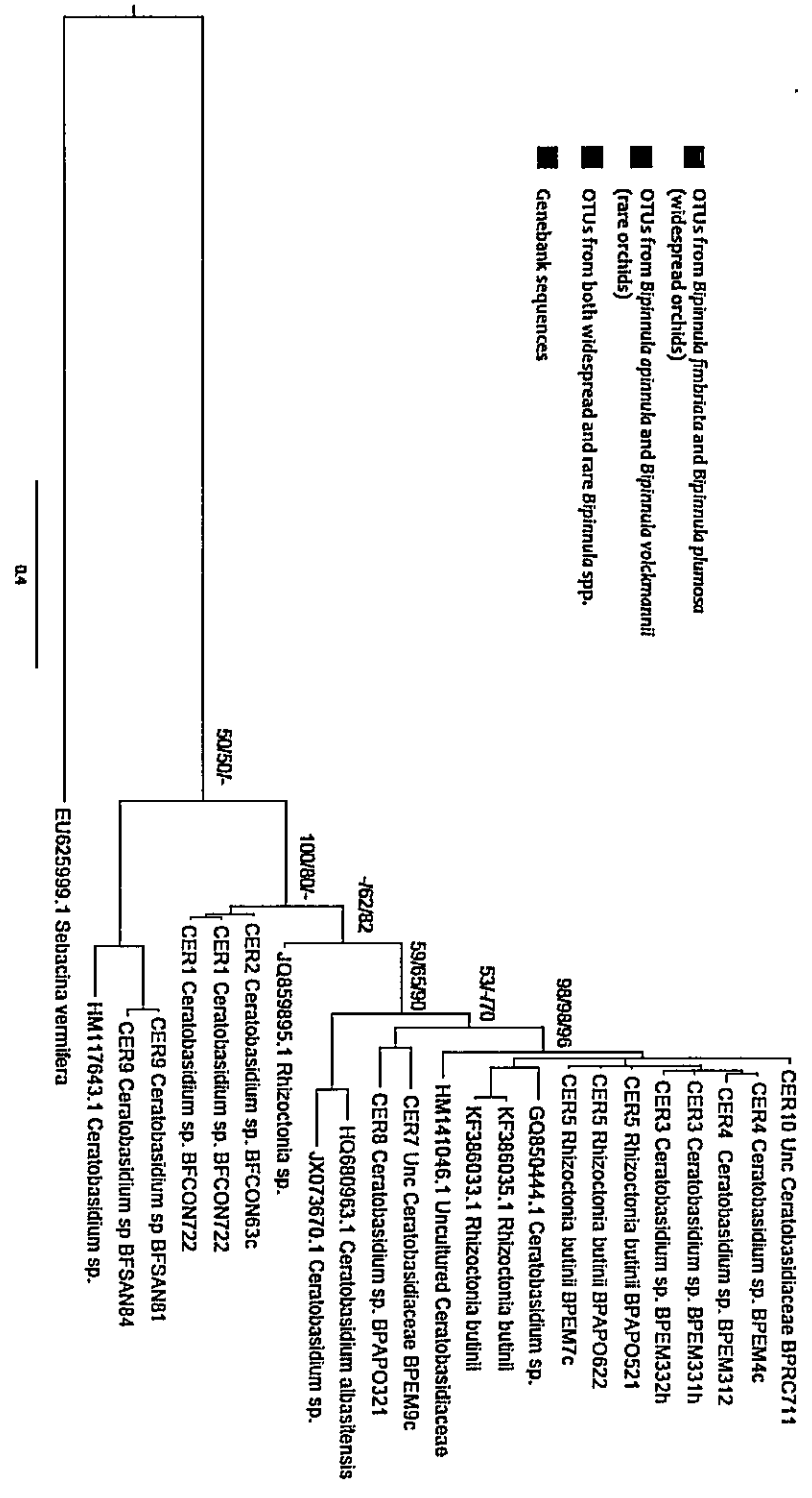
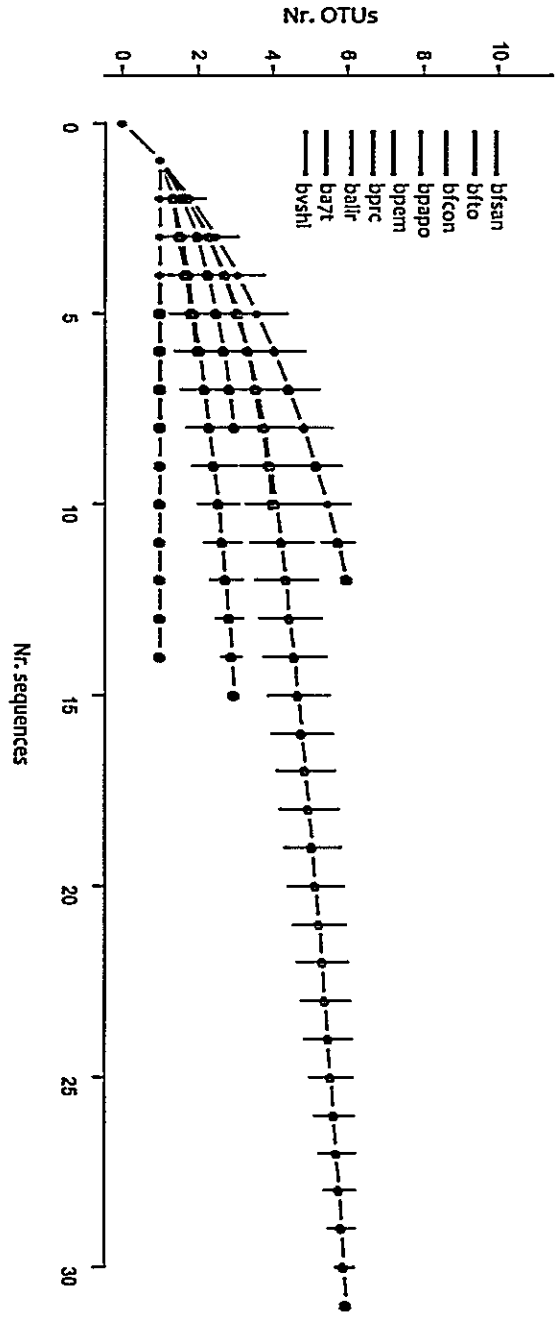

OTUs from Bipinnula fimbriata and Bipinnula plumosa (widespread orchids)
OTUs from Bipinnula apinnula and Bipinnula volckmannii (rare orchids)
OTUs from both widespread and rare Bipinnula spp.
Genebank sequences
CER10 Unc Ceratobasidiaceae BPRC711
CER4 Ceratobasidium sp. BPEM4c
CER4 Ceratobasidium sp. BPEM312
CER3 Ceratobasidium sp. BPEM331h
CER3 Ceratobasidium sp. BPEM332h
CER5 Rhizoctonia butinii BPAPO521
CER5 Rhizoctonia butinii BPAPO622
CER5 Rhizoctonia butinii BPEM7c
98/98/96
GQ850444.1 Ceratobasidium sp.
KF386035.1 Rhizoctonia butinii
KF386033.1 Rhizoctonia butinii
53/-/70
HM141046.1 Uncultured Ceratobasidiaceae
CER7 Unc Ceratobasidiaceae BPEM9c
CER8 Ceratobasidium sp. BPAPO321
59/65/90
HQ680963.1 Ceratobasidium albasitensis
JX073670.1 Ceratobasidium sp.
-/62/82
JQ859895.1 Rhizoctonia sp.
100/80/-
CER2 Ceratobasidium sp. BFCON63c
CER1 Ceratobasidium sp. BFCON722
CER1 Ceratobasidium sp. BFCON722
50/50/-
CER9 Ceratobasidium sp BFSAN81
CER9 Ceratobasidium sp BFSAN84
HM117643.1 Ceratobasidium sp.
EU625999.1 Sebacina vermifera
0.4

**Fig. 4** Rarefaction cumulative operational taxonomic unit (OTU) diversity curves for the nine populations of *Bipinnula fimbriata* (BF), *Bipinnula plumosa* (BP), *Bipinnula apinnula* (BA) *and Bipinnula volckmannii* (BV).



## CAPÍTULO 2

**Soil nutrients affect orchid mycorrhizal specialization: The case of two species of *Bipinnula* (Orchidaceae) from central Chile.**


### ABSTRACT

Mycorrhizal specialization has important consequences for orchid ecology, particularly during seed germination and early development. Variation in mycorrhizal specialization is an evolving trait on orchids. Therefore, understanding the factors that drive specialist mycrorrhizal associations is a very important step to orchid conservation. We evaluated fungal species richness and phylogenetic diversity associated to two terrestrial orchid species, *Bipinnula fimbriata* and *B. plumosa*, across 12 populations from central Chile, by isolating fungi from roots and direct fungal DNA extraction from orchid roots. We related fungal diversity with soil nutrient and climatic differences among the study sites. Mycorrhizal composition was significantly different between the two species. There was high variability in mycorrhizal specialization among population. This variability was largely related to differences in soil N and P availability among sites. Results suggest that the soil environment plays an important role in modulating orchid mycorrhizal associations. Given that mycorrhizal specialization is relevant to orchid ecology and conservation, we propose that this variable should be considered in further orchid-mycorrhiza association research.



Keywords: *Bipinnula* spp., central Chile, Mycorrhizal specialization, orchid mycorrhiza, soil nutrients.

## INTRODUCTION

One of the focal areas in the study of mutualism evolution is the continuum from specialization to generalization (Bronstein, 2009). Ecological specialization is defined as the process of adaptation to a narrow subset of all possible environmental conditions (Poisot *et al.*, 2011). Although there are many disadvantages for species with specialist mutualistic interactions, such as a reduction in the accessibility to an alternative partner under unfavorable conditions, the evolutionary transition from generalization to specialization is common in nature (Bronstein, 2009). There is, however, a lack of studies that assess the factors that drive this phenomenon and its ecological implications (Horton *et al.*, 2013). The environment is one of these driving factors of specialization; particularly predictability, productivity, complexity and habitat quality can promote more specialized ecological associations (Poisot *et al.*, 2011, Thrall *et al.*, 2006). Habitat quality (i.e., the supply of resources that affect growth, survival and reproduction) can affect specialization in two alternative manners: (1) The increment of habitat quality can reduce minimum population constraints on specialization and at the same time increase intra-host competition, both of which favor the evolution of symbiont specificity; or (2) the increment of habitat quality can also increase the availability of alternate hosts, potentially favoring generalism (Thrall *et al.*, 2006).

Mycorrhizas are symbiotic associations between soil fungi and plants (Brundrett, 2002), considered a very widespread mutualism because 92% of land plant families have mycorrhizal associations (Wang & Qiu, 2006). Mycorrhizal symbioses are often generalist, with plants interacting with a broad range of fungal partners (Molina *et al.*, 1992, Smith & Read, 2008). Nevertheless, some plants interact with a very narrow set of mycorrhizal fungi species, and some of these cases are related with habitat quality, measured as differences

in soil nutrient availability., The tropical tree *Pisonia grandis* has a very specialized mycorrhizal association, which has been related to the high of nitrogen content of its habitat, guano-rich soils in a coral island (Chambers *et al.*, 2005, Tedersoo *et al.*, 2012). The genus *Alnus* is associated with a very narrow group of ectomycorrhizaL fungi (Molina *et al.*, 1992) in comparison to the general pattern of ectomycorrhizal associations in the family. Roy *et al.*, (2013) found that mycorrhizal specialization was influenced by soil type, altitude, longitude and region. Differences in mycorrhizal composition among *Alnus* species, for instance, were influenced by soil organic matter, carbon (C), nitrogen (N), phosphorus (P) and potassium (K) contents of soils. At a global scale, Polme *et al.*, (2013) found a positive relation between soil calcium (Ca) content and OTU richness of ectomycorrhiza, i.e. more calcium, less specialization. These results support the idea that in order to maximize nutrient uptake, low soil quality favor generalist mycorrhizal associations, whereas high habitat quality (e.g., soil fertility) enhances competition between fungi leading to specialization and therefore plants that maximize efficiency of nutrient exchange.

Orchids associate with a relatively narrow group of mycorrhizal fungi in comparison to other vascular plant families (Smith & Read, 2008), but variation in specificity among orchid species is high (Shefferson *et al.*, 2007, Swarts *et al.*, 2010). There are species associated with only one (Bougoure *et al.*, 2009, Mccormick *et al.*, 2006) or a few mycobionts (Kennedy *et al.*, 2011, Shefferson *et al.*, 2005), and other species that form associations with multiple fungal partners (Pecoraro *et al.*, 2012, Kartzinel *et al.*, 2013, Pandey *et al.*, 2013). Since orchid seeds are very tiny and lack energy reserves (Arditti & Ghani, 2000), they rely entirely on mycorrhizal fungi for germination and early development (Rasmussen, 2002). The need to form symbiotic associations with fungi could constrain the distribution and abundance of orchid species (Phillips *et al.*, 2011; 2014), especially fungus-specialist species, and therefore is important to assess which factors drive specialization in orchid-mycorrhiza associations.

The role of the environment in the evolution of specialization in orchid mycorrhiza associations remains largely unexplored; however, few studies of the subject suggest that the environment can modulate the diversity of mycorrhizal associations. For example, orchids associate with a different mycorrhizal diversity depending on site conditions, including environmental stress associated with epyphitism (Martos *et al.*, 2012) and differences in water supply (Illye´s *et al.*, 2009), and it has been shown that mycorrhizal fungi are more similar between orchids that occur in similar soils (Bunch *et al.*, 2013). Further, McCormick *et al.* (2006) demonstrated that *Goodyera pubescens* switches fungal partner when growing under stressful environmental conditions. Although the association remains specialized, this result reveals that site conditions have an effect on the identity of fungal partners involved in mycorrhizal associations. Moreover, mycorrhizal specificity appears to be an evolving trait in orchids (Sheferson *et al.*, 2007) suggesting that site conditions can drive specialization.

*Bipinnula* Comm. Ex Juss (subtribu Chloraeinae, tribu Diuridae) is an orchid genus endemic to South America, with all their species terrestrial and photosynthetic. The genus is distributed in Brasil, Uruguay and Argentina, with a separate group of species endemic to Chile (Cisternas *et al.*, 2012, Novoa *et al.*, 2006). The Chilean endemic species are *Bipinnula apinnula* Gosewijn, *B. volckmanni* Kraenzl., *B. fimbriata* (Phil.) I.M. Johnst, *B. plumosa* Lindl., and *B. taltalensis* I.M. Johnst. Research on orchid mycorrhizal fungi has not attracted much attention from scientists in southern South America (Fracchia *et al.*, 2014) and Chile (Steinfort *et al.*, 2010; Pereira *et al.*, 2014). Therefore, we lack information on the fungal species associated with Chilean orchids and do not know how specialized are these associations.

In this work we report the mycorrhizal associations of *B. fimbriata* and *B. plumosa*. These two orchid species are co-distributed along a latitudinal range from 30 to 35°S; the first species is found on the coast (<500 m above sea level, m asl) while the second one

occurs in the Andean foothills between 1000 and 1700 m asl, covering a broad soil nutrient gradient. We assessed fungal species richness and fungal phylogenetic diversity in mycorrhizal associations of *B. fimbriata* and *B. plumosa.* We sampled 12 populations of these two species, and used this system to examine the relationship between mycorrhizal specialization and soil nutrient contents. In particular, we addressed the following questions: 1) Are there differences in mycorrhizal diversity and composition between these two orchid species? 2) Are there differences in mycorrhizal specificity across populations of both species? 3) Is the observed variability in mycorrhizal specialization linked to site-related differences in soil nutrients and climatic factors? 4) Is mycorrhizal composition more similar between sites that are closer with respect to environmental conditions?

## MATERIALS AND METHODS

*Species studied*

*Bipinnula fimbriata* (Phil.) I.M. Johnst, and *Bipinnula plumosa* Lindl. are both terrestrial orchids endemic to Chile. *Bipinnula fimbriata* is a relatively more frequent species, distributed in lowland (<500 m) coastal areas from 29°S to 35°S (Novoa *et al.*, 2006), preferably on sandy stabilized soils, in sites exposed to the sun and marine breeze (Elortegui & Novoa, 2009). It often forms large and dense populations associated with sclerophyllous coastal shrubs and perennial herbs, such as *Oxalis* sp., *Carpobrotus aequilaterus, Bahia ambrosoides* and *Puya* spp. (Steinfort *et al.*, 2010). In turn, *Bipinnula plumosa* generally occurs above 1000 m from 31 to 34°S, on south or southwest-facing slopes of the Andean Cordillera and also on coastal hilltops. Plants grow in association with sclerophyllous shrubs of *Quillaja saponaria, Kageneckia oblonga* and *Colliguaja odorifera.* Populations of *Bipinnula plumosa* are generally sparse or patchy, with groups of 10 to 30

individuals.

*Sampling*

Sampling was conducted during the flowering season (August to December) in 2012 and 2013. We sampled seven populations of *Bipinnula fimbriata* (FJ, LV, ZP, CC, SAN, TO, CON) and five of *Bipinnula plumosa* (LA, LD, APO, EM, RC) for a total of 115 individuals, encompassing almost the entire distributional range of both species (Table 1, supplementary material). We collected four roots from 10 orchid individuals from each population (except for two populations of *Bipinnula plumosa* which had only 8 and 7 individuals). Collected roots were individually labeled and taken to laboratory for further analysis. We also collected a mixed soil sample from each of the 12 populations; each sample was analyzed for pH in water, percentage of organic matter (MO%), nitrate content (N-NO3 mg/kg), Olsen P (mg/Kg), exchangeable K (mg/kg), and available K (cmol/kg). Analyses were performed in the Soils Laboratory of Universidad de Concepción, Chillán, Chile.

*Fungal isolation*

Roots were cut into pieces 3 to 5-cm long, washed under tap water to remove soil and dirt, and sterilized following this protocol: the sample was placed 1 min in a 10% hypochlorite solution and then 3 min in sterile distilled water three times consecutively. Orchid mycorrhizal fungi form pelotons in cortex cells of roots. The groups of pelotons can be noted in the washed root surfaces as dots ranging from light yellow to dark brown (Appendix 3, Supplementary Information). For all root pieces we assessed the level of colonization by mycorrhizal fungi, which were quantified as number of dots (produced by presence of pelotons) and the fraction of the surface covered with dots. Roots with verified presence of pelotons were cut in sections of 3 mm longitude and placed on petri dishes

with potato dextrosa agar (PDA) containing 0.16 mg/L streptomycin and penicillin, which were then placed in a dark room at 18°C. When fungal colonies developed, fungal tips from each isolate were subcultured 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) to investigate the presence of fungal species that could not be cultured in vitro.

*DNA extraction, amplification and sequencing*

For both pure fungi culture and stored root sections, DNA was extracted using a modified cetyltrimethyl ammonium bromide (CTAB) method from Doyle & Doyle (1990). Oligonucleotide primers ITS1 and ITS4 (White *et al.*, 1990) were used for amplification of DNA 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 & Bruns, 1993), ITS1/ITS4-Tul for Tulasnellaceae (Taylor & McCormick, 2008) and CeTh1/CeTh4 for Ceratobasidiaceae (Porras-Alfaro & Bayman, 2007). For all primers the PCR was carried out in a final volume of 100 uL, containing 10 uL of 10Xbuffer, 6 uL of 50 mM Mg, 2 uL BSA, 2 uL dNTP, 2 uL of each primer, 0.5 Taq polymerase and 4 ul of extracted DNA. PCR analysis was performed using the following temperature profile: 95°C for 5 min, 35°C for 1 min, 72°C for 1 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) in the GenBank database. Sequences that corresponded to orchid mycorrhizal fungi (families Ceratobasidiaceae, Tulasnellaceae and Sebacinaceae,

N=166) were chosen. The sequences were aligned in BioEdit (Hall, 1999) using ClustalW algorithm (Thompson *et al.*, 1994), a separate alignment was performed for each fungal family to further phylogenetic analyses.

*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 approaches implemented in PAUP* version 4.0b10 (Swofford, 2003). For MP, a heuristic search was undertaken using TBR branch swapping. Bootstrap support at nodes for MP and ML was computed for 10,000 replicates of the data. 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 used. Four simultaneous, independent runs were performed for over 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 2,500 (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 (BPP) as node support.

*Mycorrhizal diversity*

Based on the clades resulting from the phylogenetic inferences, sequences were grouped into operational taxonomic units (OTUs) at 99% sequence similarity. Mycorrhizal diversity was determined by counting the number of fungal OTUs detected in each orchid population. Phylogenetic diversity was calculated with nucleotide diversity (p) and the average number of pairwise nucleotide differences per site ($\pi$; Nei, 1987), which were

estimated using DnaSP 5.1 (Librado & Rozas, 2009). To evaluate differences in mycorrhizal phylogenetic diversity between the two *Bipinnula* species, we performed a multivariate analysis of variance (MANOVA) in Arlequin (Excoffier & Lischer, 2010).

*Environmental conditions and mycorrhizal diversity*

We analyzed the effect of soil nutrients and climate on mycorrhizal OTU richness, phylogenetic diversity and mycorrhizal colonization, through general linear models (GLIM) in R (R Development Core Team 2008). We also explored the effect of soil nutrients and climatic differences on mychorrhizal diversity using non-linear regression models conducted independently for each factor. Bioclimatic variables of each site were obtained from the global database Worldclim (Hijmans *et al.,* 2005) at 1 $km^2$ resolution. Then, to visualize differences among populations we performed a principal component analysis and the two first PCA vectors were used as climatic variables for further analysis.

Finally we tested whether populations of *B. fimbriata* and *B. plumosa* growing in similar abiotic conditions showed similar michorrhizal assemblages. To estimate mychorrizal similarity among each pair of orchid populations we used two indices: (1) Proportional Similarity Index (PS) (Schoener, 1968) that estimates the similarity between the frequency distributions of OTUs, and (2) Nei's Fst (obtained from DNAsp) that considers genetic distance among OTUs. We made pairwise comparisons among all pairs of populations and we constructed two 12 x 12 mychorrizal similarity matrices ($M_{Mich\text{-}Fst}$, $MS_{Mich\text{-}PS}$). We also used the worldclim bioclimatic variables and soil fertility data (pH, MO%,N-NO3 mg/kg, Olsen P, exchangeable and available ) to construct a climatic ($M_{CLIM}$) and a soil (Msoil) similarity matrix. Then, we estimated correlations betweeen michorrhizal ($M_{Mich\text{-}Fst}$, $MS_{Mich\text{-}PS}$) and abiotic ($M_{CLIM}$ and $M_{SOIL}$) matrices using the Mantel's test in PAST software run for 10,000 iterations (Hammer *et al.*, 2001)

## RESULTS

All of the 115 plants sampled presented signs of mycorrhizal colonization, but with different levels of intensity. We isolated a total of 149 fungi, from which 88 corresponded to orchid mycorrhizal fungi. We also obtained 78 DNA sequences from root sections, producing a total of 166 orchid mycorrhizal fungi sequences. All sequences belonged to the fungal families Tullasnelaceae (112 sequences) and Ceratobasidiaceae (54 sequences). Based on the clades that resulted from the phylogenetic trees, we identified 16 OTUs for Tulasnellaceae and 13 OTUs for Ceratobasidiaceae. Within Tulasnellaceae, we identified OTUs closely related to the genus *Tulasnella*, including *Tulasnella calospora*, *T. danica* and *T. asymmetrica* (Fig. 1a), but the majority of OTUs were related to uncultured Tulasnellaceae fungi. In Ceratobaisdiaceae, we obtained OTUs of the genus *Ceratobasidium* (*Ceratobasidium sp.* and *Ceratobasidium albasitensis*) and *Rhizoctonia* (*Rhizoctonia sp.* and *Rhizoctonia butinii*) (Fig. 1b). The non-orchid mycorrhizal fungi detected were mainly Ascomycetes of the genera *Peziza* (Pezizales), *Phosmosis* (Diaporthales), *Hypocrea* (Hypocreales) and *Fusarium* (Hypocreales). We also found *Neonectria* (Hypocreales), *Leptodontidium* (Helotiales), *Piromyces* (Neocallimastigales), *Cylindrocarpon (Hypocreales), Acremoniula,* and *Pythium.*

Orchid species did not differed in the level of specialization, as the number of OTUs and phylogenetic diversity did not differ significantly between orchid species. Instead, there were important differences in mycorrhizal composition between species. Tulasnellaceae was more frequent (84.7 %) in *B. fimbriata* while Ceratobasidiaceae was more frequent (75%) in *B. plumosa.* Accordingly, AMOVA showed that most of the mycorrhizal genetic variance was explained by differences between orchid species (44.3%; $p<0.001$) (Table 1).

The majority of plants (52%) were infected by a single OTU. The remaining plants were infected by 2–4 fungal OTUs, belonging in most cases to a single family (92%). At the population level, 25% of populations had only Tulasnellaceae fungi, 8.3% had only Ceratobasidiaceae fungi and 66.7% had both. There was a high variability in mycorrhizal specialization among populations. The number of OTUs found ranged between 2 in FJ population (*B. fimbriata*) and RC population (*B. plumosa*) and 8 in CC population (*B. fimbriata*) (Fig.2). Phylogenetic diversity showed different results than OTUs richness, with the populations LA (*B. plumosa*) and LV (*B. fimbriata*) being the most diverse ($\pi$=122, pi=0.279 and $\pi$=118, pi= 0.276 respectively), while FJ and CC populations (*B. fimbriata*) showed the lowest phylogenetic diversity ($\pi$=1.3, pi=0.002 and $\pi$=8.4, pi=0.016 respectively).

*Environmental factors and mycorrhizal diversity*

We analyzed soil pH, % organic matter, nitrate content (N-NO3, mg/kg), Olsen phosphorus (mg/kg), and available potassium (mg/kg). Nearly all the variation in soil parameters was unrelated to mycorrhizal diversity; few parameters showed significant associations with fungal diversity, mainly soil N (nitrate) and P (Olsen phosphorus). The general linear model showed that N had a significant and negative effect on phylogenetic diversity ($R^2$=0.39, p=0.02; Table 2), and we also detected a significant quadratic relation between the number of OTUs and P ($R^2$=0.62, p=0.01) (Fig. 3), meaning that at lower P values there was a positive relation, while at higher P values the relation was negative. Mycorrhizal colonization had no significant correlation with soil parameters. Orchid populations growing at sites with similar soils and climatic conditions did not show similar michorrhizal assemblages. Mantel tests between michorrizal similarity matrices ($M_{Mich\text{-}Fst}$, $MS_{Mich\text{-}PS}$) and both soil ($M_{SOIL}$) and climatic ($M_{CLIM}$) similarity matrices were not significant for any pairwise combination of matrixes compared.

## DISCUSSION

We did not find differences in mycorrhizal OTU richness and phylogenetic diversity of mycorrhizal fungi between the two terrestrial orchid species *B. fimbriata* and *B. plumosa*. However, mycorrhizal composition (OTU composition) was significantly different between species. Further, we found a high variability in mycorrhizal specialization across orchid populations. Differences in specialization were clearly related to environmental variables, particularly soil N and P availability. Similarity in mycorrhizal composition did not increase for sites characterized by closer soil and climatic parameters.

In the populations sampled, we identified a total of 29 OTUs, which represent a high mycorrhizal diversity compared with similar studies of orchid mycorrhizal diversity in one species (Pandey *et al.*, 2013, Kartzinel *et al.*, 2013) or several species within same genus (Jacquemyn *et al.*, 2012). We detected mycorrhizal fungi from the families Tulasnellaceae and Ceratobasidiaceae, which agree with previous evidence that demonstrate that these two fungal families are common mycorrhizal partners in the Orchidaceae family (Smith & Read, 2008; Dearnaley, 2012). However, we did not find in *Bipinnula* representatives of the fungal family Sebacinaceae, which is often isolated from orchid roots (McKendrick *et al.*, 2002; Pandey *et al.*, 2013; Kartzinel *et al.*, 2013). This observation is consistent with the limited number of studies of Chilean orchid mycorrhizas, which have only reported fungi of two mycorrhizal families (Steinfort *et al.*, 2010; Pereira *et al.*, 2014). We also found a high number of additional fungal taxa, which have been previously found in association with orchid roots in other regions (Stark *et al.*, 2009, Yuan *et al.*, 2011).

AMOVA analysis showed differences in mycorrhizal composition between orchid species (Table 1). As we explained before, *Bipinnula fimbriata* and *B. plumosa* have non-overlapping geographic distributions. *B. fimbriata* is distributed on coastal areas while *B.*

*plumosa* occupies the foothills of the Andes. We observed a predominance of Tulasnellaceae in *B. fimbriata* populations and a predominance of Ceratobasidiaceae in *B. plumosa*. The only population of *B. plumosa* that had a predominance of Tulasnellaceae was LD, which is located on the coastal range highlands, and is the *B. plumosa* population found in closer proximity to populations of *B. fimbriata*. These results suggest that probably there could be a marked altitudinal difference in the distribution of orchid mycorrhizal fungi in Chile.

We detected a high variability in mycorrhizal specialization among populations of the same orchid species, a pattern that has been seldom studied in orchid- mycorrhizal associations (Taylor *et al.*, 2002, Jacquemyn *et al.*, 2012). We provide evidence that this variability in specialization could be related to differences in environmental variables among sites. Other studies that have explored specialization across sites in orchid-mycorrhizal associations had found no significant relations with site conditions (Kartzinel *et al.*, 2013, Pandey *et al.*, 2013). In contrast, we observed a strong effect of soil nutrients on mycorrhizal specialization, which has also been observed for other types of non-orchid mycorrhizas (Tedersoo *et al.*, 2012, Roy *et al.*, 2013, Polme *et al.*, 2013). We did not observe a relation between mycorrhizal colonization and soil parameters; although there is evidence of a decrease in mycorrhizal colonization with increasing soil P contents in non-orchid mycorrhizas (Blechem & Alexander, 2012) and with increasing soil N availability in some orchid mycorrhizas (Beyrle *et al.*, 1995). There is evidence for similar orchid mycorrhizal compositions when orchids grow in similar soil conditions (Bunch *et al.*, 2013), but in this study we found no correlation among these variables.

We found a significant quadratic relation between soil P content and mycorrhizal diversity ($R^2$ =0.623, p=0.012, Fig 3). The mycorrhizal association increase plant nutrient uptake (P and N) by increasing the absorbing area, mobilizing nutrient sources or by excretion of chelating compounds or ectoenzymes (Marschner & Dell 1994), in turn fungus

gets carbon products of photosynthesis from plants (Pfeffer *et al.*, 1994). N and P are essentials nutrients that limit plants and fungal growth (Treseder & Allen 2002). Depending on nutrients availability, plants have developed different acquisition strategies related to mycorrhizal associations, in order to get the nutrients necessaries (Lambers *et al.*, 2008). At very low P availability, fungi and plants are limited, so associating with multiple fungi may increase nutrient uptake by orchids (Jacquemyn *et al.*, 2012). As nutrients increase but are still low, orchids will interact with more mycorrhizal fungi to increase the nutrient uptake, and mycorrhizal fungi will receive carbon from orchids (Cameron *et al.*, 2006), therefore as P increases the association will become more generalist. When P availability reaches a value that is not limiting for plants growth, orchids will not need to be associated with several mycorrhizal fungi to increase nutrient uptake. Considering that associating with fungi means a carbon-cost to orchids (Cameron *et al.*, 2006), they will associate with less mycorrhizal fungi, so as P increases, fungal diversity decrease thus the mycorrhizal association becomes more specialist. This results support the idea that under conditions of high environmental quality, mycorrhizal specialization is favored, while in low quality environments generalization is favored (Thrall *et al.*, 2006).

All the points at the right side of the curve (Negative correlation) in Fig. 3 (number of OUT vs P Olsen) belongs to *Bipinnula plumosa*, while nearly all points representing *Bipinnula fimbriata* are found to the left (positive correlation). *Bipinnula plumosa* occurs in the Andean foothills where due to volcanic activity there is higher soil P availability for plant growth (IGM 1984). Given that P is not limiting in Andean habitats, the relationship between P and specialization is positive. In contrast, the coastal species *Bipinnula fimbriata* may grow in soils characterized by limiting P availability and therefore with an increase of P the mycorrhizal association becomes more generalist.

The relation between number of mycorrhizal OTUs and soil nutrients was only significant for P Olsen, but there was no relationship with N availability. Instead,

mycorrhizal phylogenetic diversity (nucleotide diversity and π) was negatively related to soil N availability (pi, $R^2$ =0.389, p=0.029; π $R^2$ =0.3631, p=0.02) for the different populations. This result suggests that different fungal lineages are more likely to have access to different nutrient sources, thus under low N availability orchid association with multiple fungi may enhance nutrient uptake (Jacquemyn *et al.*, 2012, Waterman *et al.*, 2011). In the latter case, there is a positive relation between specialization and nutrient availability. Accordingly, N may not limit plant growth in this system, so that orchids have more specialist associations as N increases, thereby enhancing nutrient exchange efficiency.

In conclusion, in this work we report a high diversity of mycorrhizal fungi associated with each *Bipinnula* species and a different mycorrhizal composition between species, suggesting distinct altitudinal distribution patterns for orchid mycorrhizal fungi in Chile. We found a high variability in the levels of mycorrhizal specialization among populations, which seems to be related to differences in environmental conditions among sites where orchids grow. Mycorrhizal specialization has important consequences for orchid ecology (Dearnaley, 2012), and can contribute to orchid rarity and conservation status (Swarts & Dixon, 2010, Batty *et al.*, 2002). Our work contributes to understanding the causes of mycorrhizal specialization and therefore it provides clues to orchid conservation. Based on our results, we suggest that it is critical to incorporate site-related environmental variables into orchid mycorrhizal research to detect whether the pattern of association with specialization can be generalized.

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## TABLES

**Table 1.** General lineal model. Effects of environmental variables on phylogenetic diversity of mycorrhizas associated with *Bipinnula fimbriata* and *B. plumosa*. Asterisc indicates significant values ($p<0.05$)

| Factor | Estimated | Std. Error | t value | Pr(>\|t\|) |
|---|---|---|---|---|
| Intercept | -4,43E-01 | 2,75E-01 | -1,615 | 0,2047 |
| PC1 (bioclim) | -5,34E-05 | 9,42E-05 | -0,567 | 0,6102 |
| PC2 (bioclim) | 1,11E-03 | 4,51E-04 | 2,458 | 0,091 |
| pH | 1,19E-01 | 4,90E-02 | 2,429 | 0,0934 |
| Organic matter | 2,65E-02 | 1,29E-02 | 2,056 | 0,132 |
| LogN | -4,17E-01 | 8,75E-02 | -4,761 | 0.0176 * |
| P Olsen (mg/kg) | 7,33E-03 | 4,49E-03 | 1,631 | 0,2014 |
| K interchangeable | -9,01E+00 | 9,55E+00 | -0,943 | 0,4153 |
| K available | 2,28E-02 | 2,46E-02 | 0,924 | 0,4238 |

**Table 2.** AMOVA calculated for mycorrhizal diversity of *Bipinnula fimbriata* and *Bipinnula plumosa*. All variance components (Va, Vb, and Vc were significant ($p<0.001$). Fixation indexes $F_{SC}$ (0.36262), $F_{ST}$ (0.64478) and $F_{CT}$ (0.44268) were all significant ($p<0.001$).

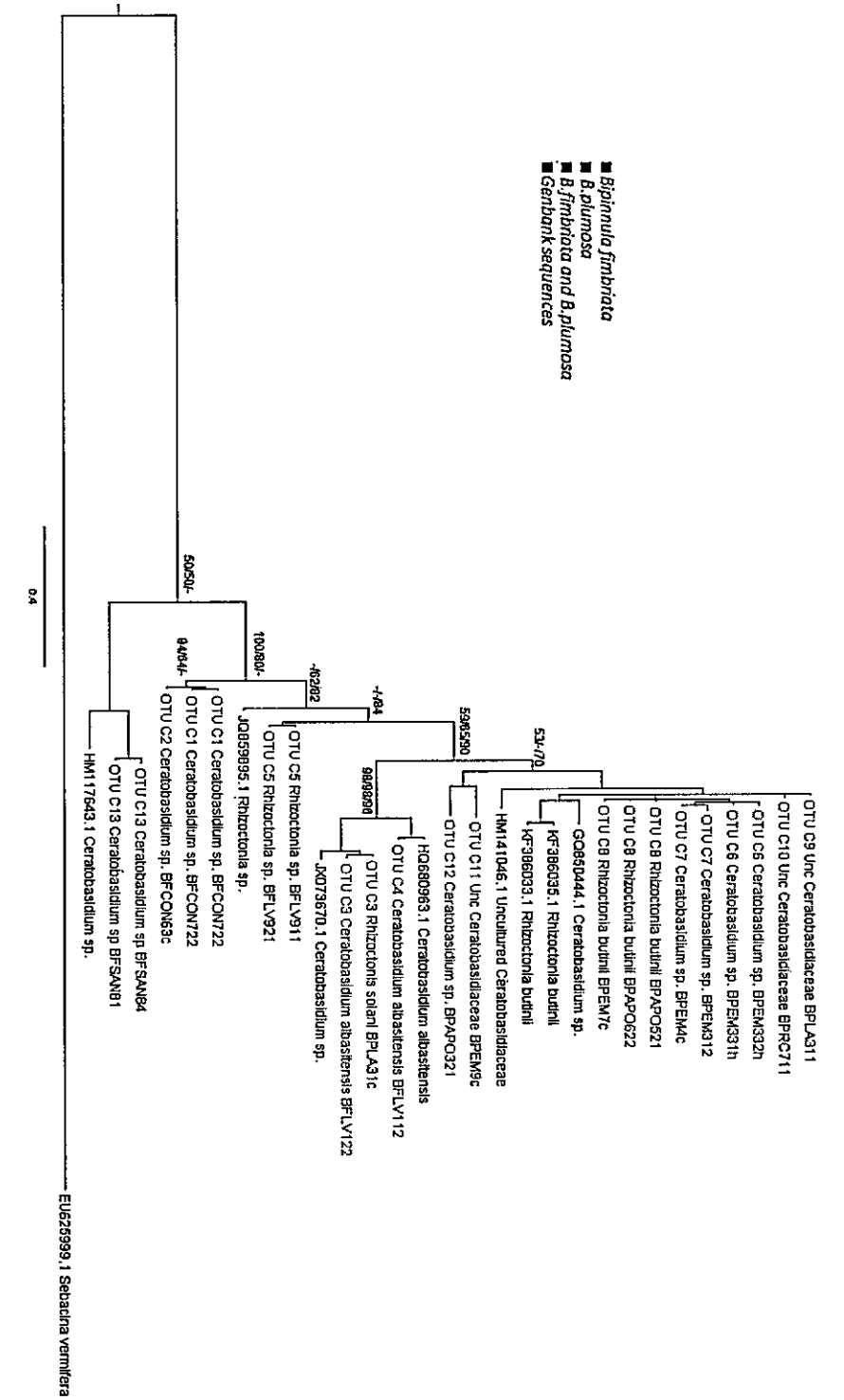
| Source of variation | d.f. | Sum of squares | Variance components | Percentaje of Variation |
|---|---|---|---|---|
| Among *Bipinnula* species | 1 | 4264.977 | 56.17982 | 44.27 |
| Among populations within species | 10 | 3739.153 | 25.6476 | 20.21 |
| Within population | 153 | 6897.355 | 45.08075 | 35.52 |
| Total | 164 | 14901.485 | 126.90817 | |

## FIGURES

**Fig. 1** Bayesian majority consensus tree based on transcribed spacer (ITS) sequences of Ceratobasidiaceae and Tulasnellaceae fungi. Trees were constructed with operational taxonomic unit (OTU) sequences from (a) Ceratobasidiaceae (b) Tulasnellaceae obtained from *Bipinnula fimbriata* and *Bipinnula plumosa* roots collected from different populations in central Chile. The Ceratobasidiaceae tree (b) was rooted with *Sebacina vermifera* (EU625999.1 genbank). Values on each branch represent Parsimony bootstrap values/ Maximum likelihood bootstrap values /Bayesian posterior probabilities (BPP).

**Fig. 1**

**a)**


Bipinnula fimbriata
B.plumosa
B.fimbriata and B.plumosa
Genbank sequences
OTU C9 Unc Ceratobasidiaceae BFLA311
OTU C10 Unc Ceratobasidiaceae BPRC711
OTU C6 Ceratobasidium sp. BPEM332h
OTU C6 Ceratobasidium sp. BPEM331h
OTU C7 Ceratobasidium sp. BPEM312
OTU C7 Ceratobasidium sp. BPEM4c
OTU C8 Rhizoctonia butinii BPAPO521
OTU C8 Rhizoctonia butinii BPAPO622
OTU C8 Rhizoctonia butinii BPEM7c
GQ850444.1 Ceratobasidium sp.
KF386035.1 Rhizoctonia butinii
KF386033.1 Rhizoctonia butinii
HM141046.1 Uncultured Ceratobasidiaceae
OTU C11 Unc Ceratobasidiaceae BPEM9c
OTU C12 Ceratobasidium sp. BPAPO321
HQ680963.1 Ceratobasidium albasitensis
OTU C4 Ceratobasidium albasitensis BFLV112
OTU C3 Rhizoctonis solani BPLA31c
JX073670.1 Ceratobasidium albasitensis BFLV122
OTU C5 Rhizoctonia sp. BFLV911
OTU C5 Rhizoctonia sp. BFLV921
JQ859895.1 Rhizoctonia sp.
OTU C1 Ceratobasidium sp. BFCON722
OTU C1 Ceratobasidium sp. BFCON722
OTU C2 Ceratobasidium sp. BFCON63c
OTU C13 Ceratobasidium sp BFSAN84
OTU C13 Ceratobasidium sp BFSAN81
HM117643.1 Ceratobasidium sp.
EU525999.1 Sebacina vermifera
0.4
50/50
100/80
94/84
-/62
-/64
59/59
99/89
53/70

**b)**

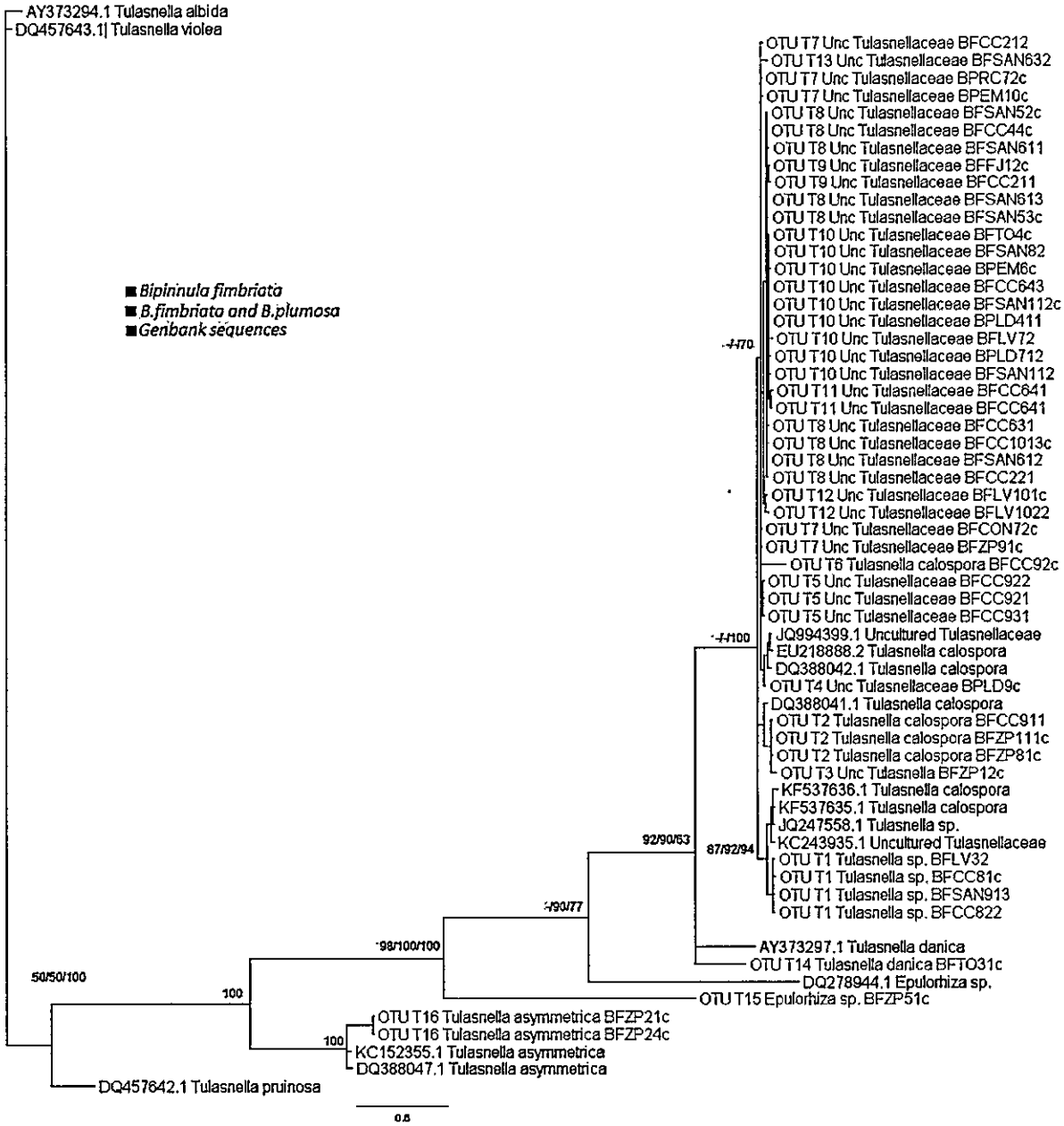

AY373294.1 Tulasnella albida
DQ457643.1| Tulasnella violea
Bipinnula fimbriata
B.fimbriata and B.plumosa
Genbank sequences
OTU T7 Unc Tulasnellaceae BFCC212
OTU T13 Unc Tulasnellaceae BFSAN632
OTU T7 Unc Tulasnellaceae BPRC72c
OTU T7 Unc Tulasnellaceae BPEM10c
OTU T8 Unc Tulasnellaceae BFSAN52c
OTU T8 Unc Tulasnellaceae BFCC44c
OTU T8 Unc Tulasnellaceae BFSAN611
OTU T9 Unc Tulasnellaceae BFFJ12c
OTU T9 Unc Tulasnellaceae BFCC211
OTU T8 Unc Tulasnellaceae BFSAN613
OTU T8 Unc Tulasnellaceae BFSAN53c
OTU T10 Unc Tulasnellaceae BFTO4c
OTU T10 Unc Tulasnellaceae BFSAN82
OTU T10 Unc Tulasnellaceae BPEM6c
OTU T10 Unc Tulasnellaceae BFCC643
OTU T10 Unc Tulasnellaceae BFSAN112c
OTU T10 Unc Tulasnellaceae BPLD411
OTU T10 Unc Tulasnellaceae BFLV72
OTU T10 Unc Tulasnellaceae BPLD712
OTU T10 Unc Tulasnellaceae BFSAN112
OTU T11 Unc Tulasnellaceae BFCC641
OTU T11 Unc Tulasnellaceae BFCC641
OTU T8 Unc Tulasnellaceae BFCC631
OTU T8 Unc Tulasnellaceae BFCC1013c
OTU T8 Unc Tulasnellaceae BFSAN612
OTU T8 Unc Tulasnellaceae BFCC221
OTU T12 Unc Tulasnellaceae BFLV101c
OTU T12 Unc Tulasnellaceae BFLV1022
OTU T7 Unc Tulasnellaceae BFCON72c
OTU T7 Unc Tulasnellaceae BFZP91c
OTU T6 Tulasnella calospora BFCC92c
OTU T5 Unc Tulasnellaceae BFCC922
OTU T5 Unc Tulasnellaceae BFCC921
OTU T5 Unc Tulasnellaceae BFCC931
JQ994399.1 Uncultured Tulasnellaceae
EU218888.2 Tulasnella calospora
DQ388042.1 Tulasnella calospora
OTU T4 Unc Tulasnellaceae BPLD9c
DQ388041.1 Tulasnella calospora
OTU T2 Tulasnella calospora BFCC911
OTU T2 Tulasnella calospora BFZP111c
OTU T2 Tulasnella calospora BFZP81c
OTU T3 Unc Tulasnella BFZP12c
KF537636.1 Tulasnella calospora
KF537635.1 Tulasnella calospora
JQ247558.1 Tulasnella sp.
KC243935.1 Uncultured Tulasnellaceae
OTU T1 Tulasnella sp. BFLV32
OTU T1 Tulasnella sp. BFCC81c
OTU T1 Tulasnella sp. BFSAN913
OTU T1 Tulasnella sp. BFCC822
AY373297.1 Tulasnella danica
OTU T14 Tulasnella danica BFTO31c
DQ278944.1 Epulorhiza sp.
OTU T15 Epulorhiza sp. BFZP51c
OTU T16 Tulasnella asymmetrica BFZP21c
OTU T16 Tulasnella asymmetrica BFZP24c
KC152355.1 Tulasnella asymmetrica
DQ388047.1 Tulasnella asymmetrica
DQ457642.1 Tulasnella pruinosa
-/-/70
-/-/100
92/90/63
87/92/94
-/90/77
98/100/100
50/50/100
100
100
0.5

**Fig. 2** Geographical distribution of mycorrhizal operational taxonomic units (OTU, calculated from constructed phylogenies of Tulasnellaceae and Ceratobasidiaceae) associated with different populations of *Bipinnula fimbriata* (BF) and *Bipinnula plumosa* (BP) in central Chile. a) Pictures of BF (black triangle) and BP (blue triangle). b) Geographical map of the sampling locations, with pie charts displaying the frequency of occurrence of each OTU in each population. Above pie charts is the population name; names in black are populations of BF and names in blue are populations of BP. Yellow to purple colors represent OTUs that belong to the Tulasnellaceae family, while blue to green colors belong to Ceratobasidiaceae. The organization of colors is consistent with the clades observed in the phylogenetic inferences for both mycorrhizal families.

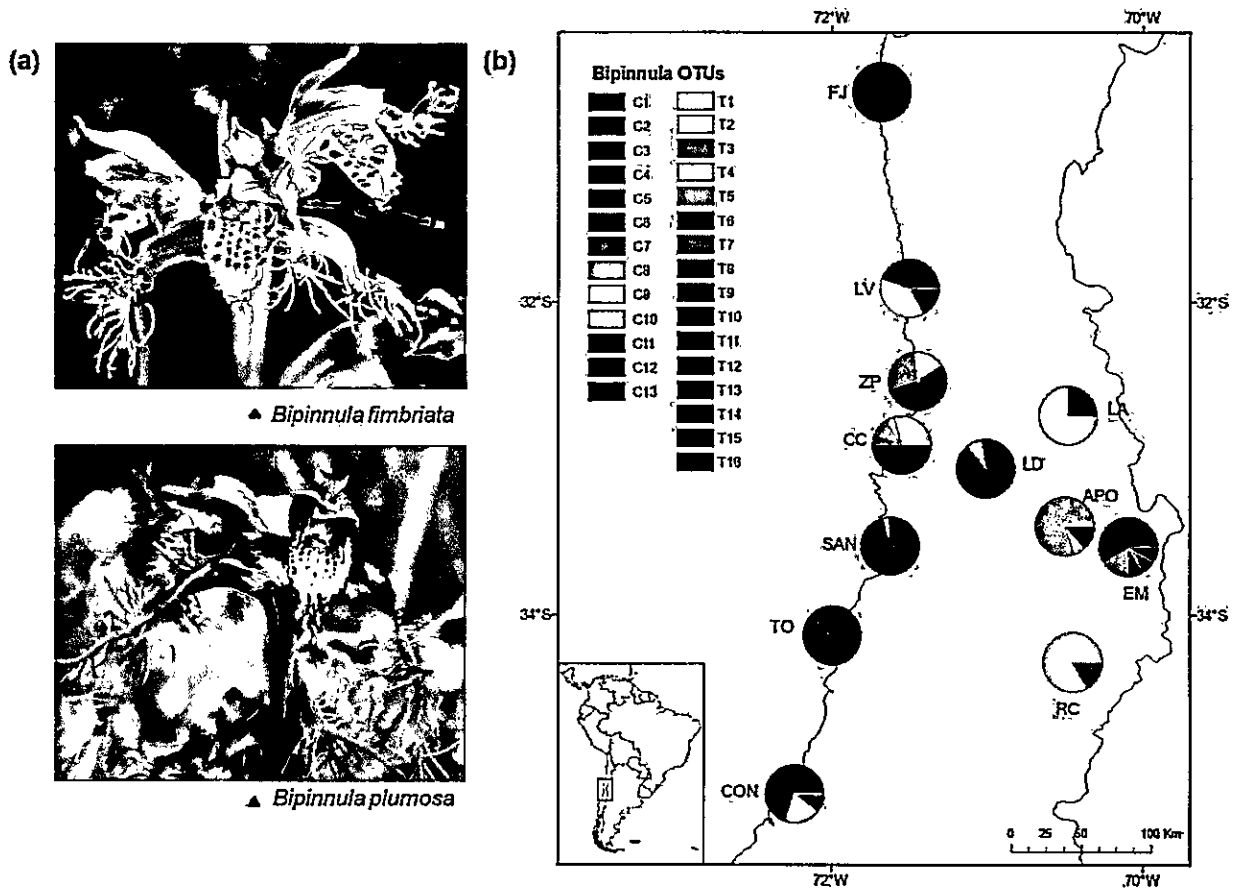
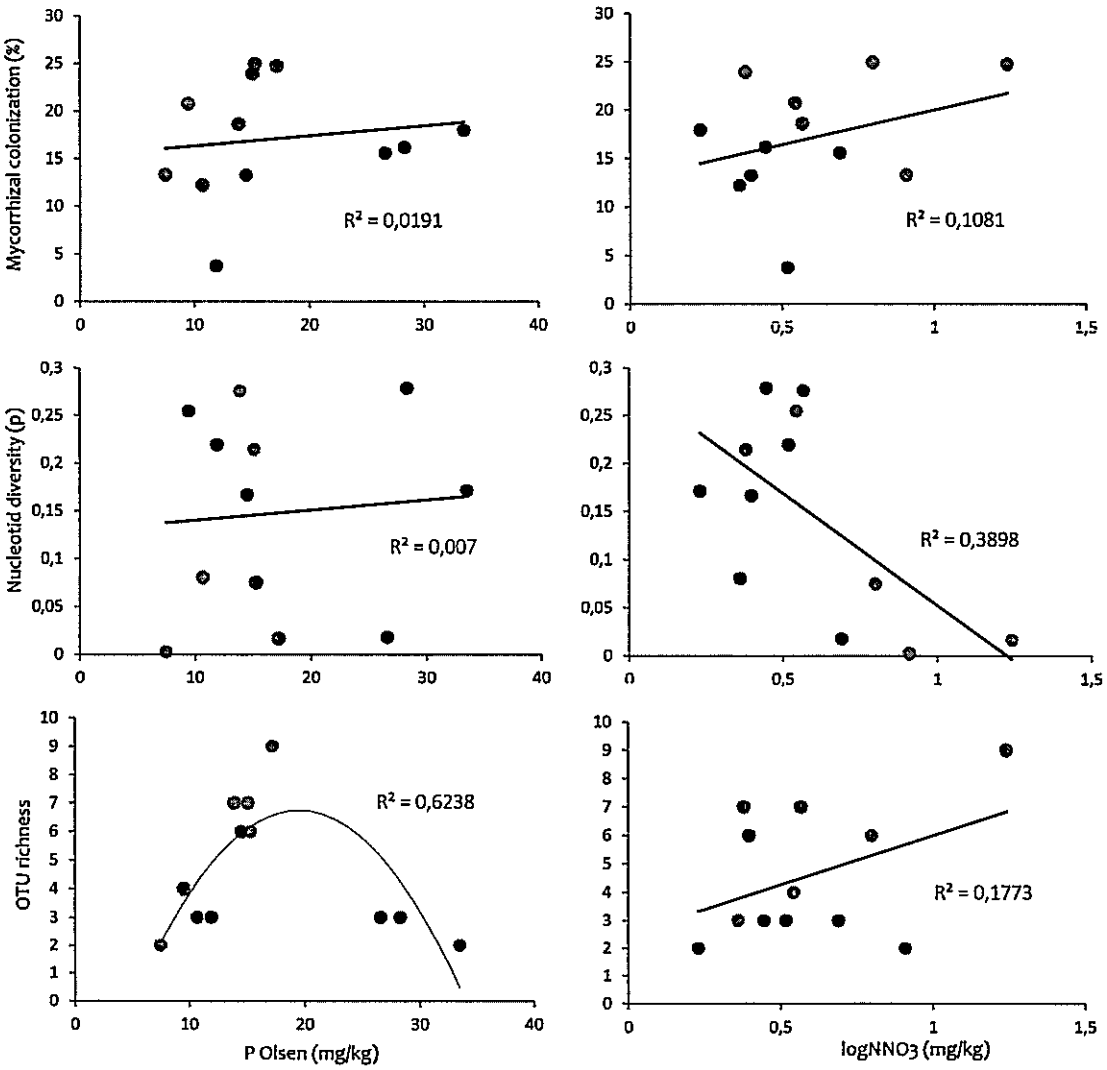


**Fig 3.** Correlations between soil N and P content across population sites and mycorrhizal OTU richness, mycorrhizal phylogenetic diversity and mycorrhizal colonization. Grey dots represent mycorrhizal diversity for populations of *Bipinnula fimbriata*, and black dots represent fungal diversity for populations of *Bipinnula plumosa*. Asterisks show significant correlations ($p<0.05$).



## CONCLUSIÓN GENERAL

Existen muy pocos estudios acerca de la ecología de las micorrizas de orquídeas en el sur de Sudamérica, por lo que hay poco conocimiento acerca de qué hongos interactúan con estas especies de orquídeas, los patrones de distribución de estos hongos o qué tan especialista es la interacción. Por lo tanto, los resultados de esta tesis contribuyen de manera significativa al entendimiento de las micorrizas de orquídeas en esta región.

En el primer capítulo se abordó la siguiente pregunta ¿La distribución de *Bipinnula* está limitada por la diversidad de hongos micorrícicos? Los resultados mostraron una diversidad de hongos micorrícicos significativamente mayor asociados a las especies comunes (*B. fimbriata* y *B. plumosa*) respecto a las raras (*B. volckmanni* y *B. apinnula*), tal como se esperaba. Estos resultados soportan la hipótesis de que las micorrizas limitan a las poblaciones de orquídeas y sugieren que la rareza en *B. volckmanni* y *B. apinnula* podría estar dada por la especialización micorrícica. Sin embargo para poder confirmarlo es necesario hacer experimentos de germinación *in vitro* e *in situ*, por lo que estos resultados representan un punto de partida para estudios experimentales futuros. Adicionalmente, la información obtenida tiene importantes implicancias para la conservación de las especies de *Bipinnula*, sobre todo de las raras, ya que al presentar una asociación micorrícica tan especialista, son aún más vulnerables a perturbaciones de su hábitat.

En el segundo capítulo, en cambio, se estudió qué factores ambientales promueven la especialización micorrícica en las dos especies comunes de *Bipinnula*. Para este capítulo se esperaba encontrar que en suelos con menor disponibilidad de nutrientes las orquídeas se asociaran a una mayor diversidad de hongos micorrícicos, con el fin de maximizar la obtención de nutrientes. Se encontró una alta diversidad de hongos micorrícicos asociados a ambas especies y una gran variabilidad en el nivel de especialización entre las

poblaciones muestreadas. Los resultados muestran que esta variabilidad está afectada por la disponibilidad de nitrógeno y fósforo en el suelo. Para el caso del nitrógeno, se observó que a mayor disponibilidad de nutrientes disminuye la diversidad de hongos micorrícicos asociados a *Bipinnula*, tal como se esperaba. Para el fósforo se observa la misma relación en niveles altos de disponibilidad, sin embargo cuando la disponibilidad de fósforo es muy baja, a medida en que aumenta el fósforo aumenta la diversidad de hongos micorrícicos. Esto sugiere que las *Bipinnula* cambian la diversidad de hongos con los que se asocian en función de la disponibilidad, probablemente como una estrategia para maximizar la obtención de nutrientes. Estos resultados son muy novedosos en el estudio de las micorrizas de orquídeas, ya que nunca antes se había explorado la relación entre variables ambientales y el nivel de especialización. Además se encontraron diferencias significativas en la composición de hongos entre las dos especies y al estar éstas geográficamente separadas, muestra un posible patrón de distribución altitudinal de los hongos micorrícicos de orquídeas en Chile.

Las micorrizas tienen un rol clave en la ecología de las orquídeas (Dearnaley, 2012) ya que pueden limitar su distribución y contribuir en su rareza y amenaza (Swarts & Dixon, 2009, Batty *et al.*, 2002). Por lo tanto, nueva información acerca de esta interacción tan determinante para las orquídeas es muy valiosa para el diseño de estrategias de conservación, sobre todo considerando que dos de las especies estudiadas son extremadamente escasas.

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