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Do mycorrhizal fungi drive speciation in *Teagueia* (Orchidaceae) in the upper Pastaza watershed of Ecuador?

Juan Pablo Suárez[1] · **Juan Sebastian Eguiguren**[1] · **Paulo Herrera**[1] · **Lou Jost**[2]

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Abstract The orchid genus *Teagueia* Luer (Orchidaceae, subtribe Pleurothallidinae) presents an extraordinary example of recent local evolutionary radiation. In principle, mutualisms might affect the origin of plant species via an effect on speciation. As orchids depend on mycorrhizal fungi for seed germination and early plantlet development we tested whether certain mycorrhizal fungi are acting as drivers of this radiation in *Teagueia* species. Sampling was carried out near Baños in east Andean Ecuador. Roots were collected from a total of 11 flowering individuals of eight morphospecies (referred to as *Teagueia* spp). The whole ITS1-5.8S- ITS2 nrDNA region and part of the 28S nrDNA were amplified, cloned and sequenced. Molecular phylogeny of the obtained sequences revealed four phylogenetic species of Tulasnellaceae and one of Atractiellales (Pucciniomycotina, Basidiomycota) associated with *Teagueia* spp. Tulasnelloid fungi were detected in all samples. Up to three different phylogenetic species of mycobionts were found associated with one *Teagueia* species. We found that co-occurring *Teagueia* species share mycobionts. All detected mycobionts had wide geographical distribution. Based on the available evidence we conclude that the extraordinary local radiation of *Teagueia* is most likely driven by other factors than by mycorrhizal fungi, but that mycorrhiza may be a key factor for the coexistence of so many closely related orchid species.

✉ Juan Pablo Suárez
jpsuarez@utpl.edu.ec

[1] Departamento de Ciencias Naturales, Universidad Técnica Particular de Loja, San Cayetano Alto s/n C.P., 11 01 608 Loja, Ecuador

[2] Baños, Tungurahua, Ecuador

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1 Introduction

Orchids constitute a large proportion of the vascular plant diversity in Ecuador, with approximately 4000 species (Dodson 2005) of the 17.748 confirmed native vascular plant species (Neill 2012). The Ecuadorian species account for about 16 % of the world's described orchid species (Jørgensen and León-Yánez 1999; Dressler 2005). More than a third of Ecuador's orchid species are endemic to the country. *Teagueia* Luer (Orchidaceae, subtribe Pleurothallidinae) constitutes an extraordinary example of a recent local evolutionary radiation. Jost (2004) reported at least 26 unusual new terrestrial and epiphytic species of *Teagueia* on four neighboring mountains in the upper Pastaza watershed. The species form a monophyletic clade endemic to an area of less than 110 km × 30 km near Baños, Tungurahua, in the east Andes of Ecuador. All 26 species share distinctive floral and vegetative characters, such as a long-repent habit, not found in the six previously described members of *Teagueia*, suggesting that all evolved locally from a recent common ancestor (Jost, 2004). A new species was reported recently in Perú (Chocce et al. 2011) but this belongs to the "traditional" *Teagueia* clade and not to the speciose long-repent clade discovered by Jost (2004).

Orchids can produce thousands of tiny seeds lacking carbohydrate reserves. Colonization of a seed with a suitable mycorrhizal fungus is vital for successful germination, growth and establishment of orchids in nature (Smith and Read 2008). Seed and microsite limitation are the main determinants for orchid recruitment; microsite limitation may result from a lack

of suitable orchid mycobionts (McCormick and Jacquemyn 2014). Besides mutualistic association with mycorrhizal fungus, orchids are also associated with specific pollinators, and both have been proposed as drivers for orchid diversification (Waterman et al. 2011).

Orchids with differences in carbon nutrition have been found to be associated with different groups of fungi. Autotrophic orchids are generally associated with a limited range of Basidiomycota, restricted to members of Tulasnellaceae, Serendipitaceae, Ceratobasidiaceae and Atractiellales (Suárez et al. 2006, 2008; Otero et al. 2002, 2007; Kottke et al. 2010; Weiß et al. 2016). Recently, Tulasnellaceae were recognized as the most frequent and widespread mycobionts of autotrophic orchids (Dearnaley et al. 2012).

Fungal associations are known to vary considerably in specificity. Recent evidence indicates a trade-off between patterns of orchid distribution and mycorrhizal fungi specificity (McCormick and Jacquemyn 2014). Rare orchid species tend to be less selective in their fungal partners (Pandey et al. 2013), even though some evidence suggested a site-dependent fungal association (Kartzinel et al. 2013a). Looking from the perspective of interaction networks, if rare orchid species would interact only with rare mycorrhizal fungi species, they would most likely be prone to extinction (Kottke et al. 2013). In the other hand, widely-distributed orchid species tend to be associated consistently with the same fungal partners, which is the case for both epiphytic (e.g. Otero et al. 2007) and terrestrial orchids (e.g. Roche et al. 2010). Mutualistic interactions have dramatic effects on the evolution and coexistence of species (Waterman et al. 2011).

To test the hypothesis that mycorrhizal fungi may act as speciation drivers of *Teagueia*, we determined the associated mycorrhizal fungi by molecular tools. We expected narrow and distinct fungal associations in case of speciation driving influence. Alternatively, broad sharing of fungi would indicate maintenance of coexisting, closely related orchid species by mycorrhizae.

2 Materials and methods

2.1 Study site and sampling

The study site is located near the town of Baños, Tungurahua province, Ecuador. Samples were collected during 2011 between altitudes of 3100 to 3300 m a.s.l. near the settlement of Viscaya, 10 km west of Mayordomo. Two different paths were sampled, "Viscaya" and "Valencia", separated by about 1.5 km. This tropical montane forest area is exceptionally rich in tree species and epiphytes, particularly orchids.

Roots were collected from a total of 11 flowering individuals, of eight *Teagueia* spp. morphospecies (Table 1). All selected plants were terrestrial growing in humus with a moss layer. Three to five roots per individual plant were kept in ethanol 50 % and stored at −20 °C until molecular analyses. The orchid specimens were assigned to morphospecies by Lou Jost.

2.2 Light microscopy

Light microscopy was used to select material with fungal coils. Transverse sections were cut from the middle part of each root sample by hand using a razor blade. Sections were stained by Methyl blue 0.05 % solution (C. I. 42,780, Merck) in lactic acid for 10 min on microscopic slides. The samples were examined in fresh lactic acid at 100- to 1000-fold magnification (Leitz WETZLAR SM-LUX).

2.3 DNA extraction, PCR and sequencing

A 1–2 cm long piece was cut from each of the three selected colonized roots per plant for one DNA extraction per plant individual. The root pieces were rinsed in sterile water and freed from the velamen. Genomic DNA was recovered using a Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The whole ITS1-5.8S- ITS2 nrDNA region and part of the 28S nrDNA were amplified with the universal primer combination ITS1 (5′-TCC GTA GGT

Table 1 Mycorrhizal fungi found in the eight flowering *Teagueia* morphospecies from Valencia and Viscaya sites. Clades of the phylogenetic trees where inferred from ITS-5.8S nrDNA data for Tulasnellaceae and Atractiellales (Figs. 1 and 2, respectively). *n*, number of orchid individuals collected

Orchid species	Location	*n*	*Tulasnella* clade 1	*Tulasnella* clade 2	*Tulasnella* clade 3	*Tulasnella* clade 4	Atractiellales clade
Teagueia sp1	Viscaya	1	x				
Teagueia sp2	Valencia	2	x		x		
Teagueia sancheziae	Valencia	2	x	x		x	
Teagueia sp3	Viscaya/ Valencia	2		x	x		x
Teagueia sp4	Valencia	1		x	x		
Teagueia sp5	Viscaya	1		x	x		
Teagueia sp6	Valencia	1		x			
Teagueia sp7	Viscaya	1			x	x	x

GAA CCT GCG G-3′; White et al. 1990) and TW14 (5′-GCTATCCTGAGGGAAACTTC-3′; Cullings 1994) using the Phusion High-Fidelity PCR Mastermix (Finnzymes, Espoo, Finland). Success of PCR amplification was tested in 0.7 % agarose stained with GelRed™ Safe Nucleic Acid Gel Stain (Biotium, Hayward, USA).

PCR products were cloned with the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) according to manufacturer's protocol. Twelve colonies per individual were selected for PCR amplification using modified M13F and M13R primers (Krüger et al. 2009). Success of PCR was tested in 1 % agarose stained with GelRed™ Safe Nucleic Acid Gel Stain. Eight colonies per orchid individual were grown in liquid LB Broth, MILLER (Difco) and the plasmid DNA purified with S.N.A.P. miniprep kit (Invitrogen) according to manufacturer's instructions. Clones were sequenced at Macrogen (Seoul, Korea) using universal primers M13F and M13R.

2.4 Sequence editing, identity estimation and phylogenetic analysis

Sequences were edited and consensuses were generated using Sequencher 4.6 software (Gene Codes, Ann Arbor, MI, USA). BLAST (Altschul et al. 1997) against the NCBI nucleotide database (GenBank; http://www.ncbi.nlm.nih.gov/) was used to find published sequences with high similarity in the ITS-5.8S region. These sequences were aligned using MAFFT v. 5.667 (Katoh et al. 2005) under the G-INS-i option. Two datasets were created, one comprising sequences closest to members of Tulasnellaceae and the other with sequences closest to members of Atractiellales. Phylogenetic reconstructions were performed for both datasets using a Maximum Likelihood analysis (ML) in MEGA 5 software (Tamura et al. 2011) under the General Time Reversible DNA substitution model with 1000 bootstrap replicates, conserving all sites of the alignment. A pairwise analysis was performed in MEGA to determine the similarity between sequences of mycobionts within each clade and between clades. From the pairwise similarity scores sequences were treated as belonging to the same phylogenetic species following the thresholds defined by Cruz et al. (2014) and Kottke et al. (2010).

3 Results

3.1 Light microscopy observations

Fungal pelotons were present in nearly all cross-sections of roots sampled. Pelotons were distributed throughout the cortex. Vital, blue staining and collapsed, slightly yellow coloured pelotons were visible in the same cells suggesting that cells became re-infected several times (not shown).

3.2 Molecular identification of mycobionts associated to *Teagueia* spp.

The total number of investigated roots was 30, with two to four roots collected from each of the 11 orchid individuals of eight *Teagueia* morphospecies. High quality sequences were obtained only for 43 of the total number of 88 sequenced clones. After elimination of identical clones from the same individual only 17 sequences were considered for further analysis. As a result of the BLAST search, 15 sequences were found to belong to members of Tulasnellaceae and two sequences belonged to members of Atractiellales. Tulasnelloid fungi were detected in all studied samples (Table 1).

The phylogenetic analysis of ITS-5.8S sequences of members of Tulasnellaceae, including similar sequences available in GenBank, showed sequences in four clades (Fig. 1). Pairwise sequences analysis within each clade showed differences varying from less than 4 % to more than 4 % between clades. Therefore, we treated sequences as belonging to the same phylogenetic species following the threshold of 4 % to delimitate phylogenetic species in Tulasnellaceae as defined by Cruz et al. (2014). Mycobionts of the same phylogenetic species were shared among orchid species. Clade 1 comprised three sequences of one phylogenetic species of *Tulasnella* from three different *Teagueia* morphospecies. Clades 2 and 3 comprised five sequences of one phylogenetic species of *Tulasnella* from five different *Teagueia* morphospecies; while clade 4 comprised two sequences of one phylogenetic species of *Tulasnella* from two different *Teagueia* morphospecies (Fig. 1). The phylogenetic analysis of ITS-5.8S sequences of members of Atractiellales, including similar sequences available in GenBank, clustered the two sequences obtained in this study in one clade (Fig. 2). Two *Teagueia* morphospecies shared identical sequences of Atractiellales mycobionts.

Tulasnella belonging to different phylogenetic species were detected in mycorrhizas from the same plant for several samples (Table 1). There is no evidence of site preference in *Tulasnella*, as all phylogenetic species of *Tulasnella* are present in both Viscaya and Valencia locations. But Atractiellales species were detected only in the Viscaya location.

4 Discussion

Our finding of constant colonization of roots supports the view that the role of the fungi may be crucial also for adult orchids in nature (Dearnaley et al. 2012). The most important benefit for the orchid may be to retain the fungus in order to assure further seed germination (see Kartzinel et al. 2013b).

Molecular phylogeny of mycobiont sequences revealed that *Teagueia* morphospecies are associated with members of Tulasnellaceae and Atractiellales. Other orchids that are autotrophic in the adult stage have been found to form

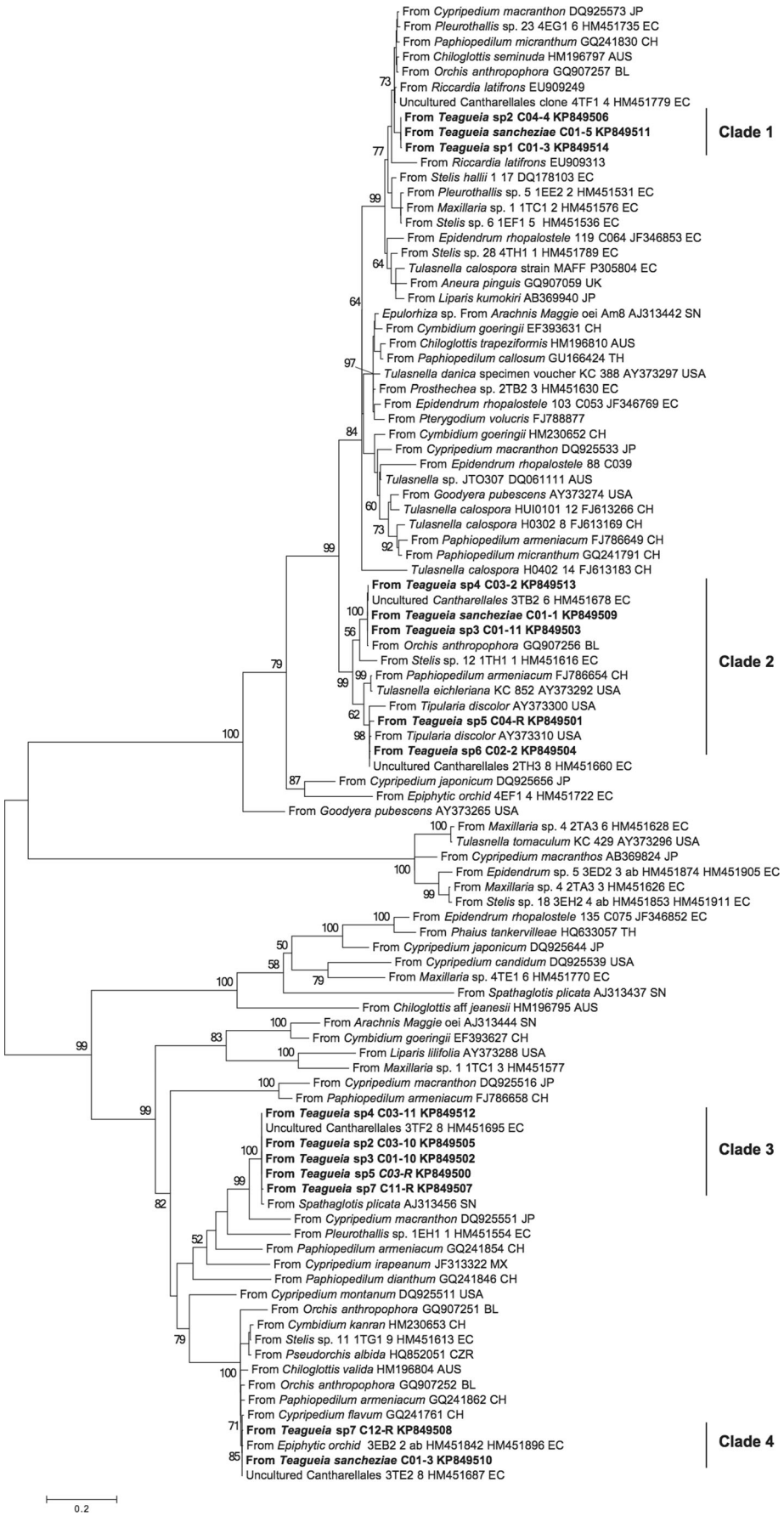

Fig. 1 Maximum Likelihood tree of members of Tulasnellaceae, associated with 11 flowering individuals of eight *Teagueia* morphospecies. Tree was inferred from ITS-5.8S nrDNA data. Four clades (1–4) are indicated as distinguished by distance calculations and congruent with phylogenetic analyzes. The values that support the nodes correspond to Maximum Likelihood bootstrap. Only bootstrap values up to 50 % are shown. The tree was midpoint rooted. *AUS* Australia, *BL* Belgium, *CH* China, *CZR* Czech Republic, *JP* Japan, *SN* Singapore, *TH* Thailand, *UK* United Kingdom, *USA* United States of America

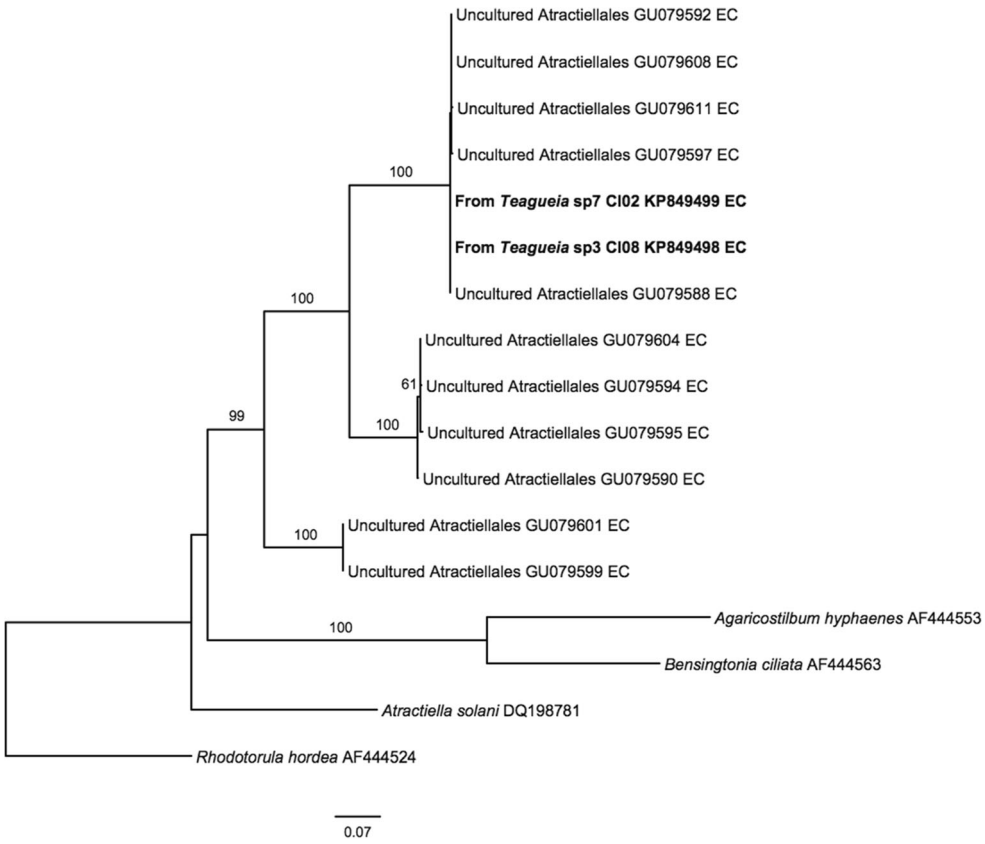

Fig. 2 Maximum Likelihood tree of members of Atractiellales, associated with 2 flowering individuals of *Teagueia* spp. inferred from ITS-5.8S nrDNA data. The values that support the nodes correspond to Maximum Likelihood bootstrap. Only bootstrap values greater than 50 % are shown. The tree was outgroup rooted with *Rhodotorula hordea* AF444524

mycorrhizae with Tulasnellaceae, Serendipitaceae, and Ceratobasidiaceae (see Kottke and Suárez 2009), all hosted in subphyla Agaricomycotina of Basidiomycota. Members of Atractiellales forming orchid mycorrhiza were reported for the first time by Kottke et al. (2010). Atractiellales belongs to the subphyla Pucciniomycotina, which was known to comprise mainly parasites and to a lesser extent presumed saprophytes (Aime et al. 2006). Our finding is, to the best of our knowledge, the second evidence (in addition to Ávila-Díaz et al. 2013) of mycorrhizal fungi in this subphylum. The phylogenetic position of the mycobiont among potential saprophytes may indicate physiological flexibility from saprophytism to mutualism, which is required for orchid mycobionts (Rasmussen and Rasmussen 2009). There are convincing molecular arguments that mycorrhiza forming fungi derived from saprotrophs multiple times (Hibbett et al. 2007) by loss of decay genes, but Tulasnellaceae preserved both capabilities (Kohler et al. 2015). The capacity of these fungi to survive as saprophytes could explain their broad availability in the habitat (Cruz et al. 2011, 2014). As was expected by Kottke et al. (2010) the ecological amplitude of Atractiellomycetes appeared as broad as Tulasnellaceae mycobionts, at least in tropical montane forests.

The phylogenetic analysis of ITS-5.8S sequences of members of Tulasnellaceae showed sequences in four clades (Fig. 1). Clade 1 (73 % ML bootstrap support) comprised three sequences of one phylogenetic species of *Tulasnella* from three different *Teagueia* spp. Sequences of mycobionts from *Cypripedium* sp. DQ925573 located in Japan, *Paphiopedilum micranthum* GQ241830 from China, *Chiloglottis seminuda* HM196797 from Australia and sequences (labeled "EC", Fig. 1) from a previous study carried out in Reserva Biológica San Francisco (RBSF) in Zamora Chinchipe (Ecuador) are closely related. Clade 2 (99 % ML bootstrap support) comprised five sequences of one phylogenetic species of *Tulasnella* from five different *Teagueia* spp. and sequences of mycobionts from *Orchis anthropophora* GQ907256 from Belgium and *Tipularia discolor* AY373300 from United States that are closely related, or even identical as in the case of GQ907256. Sequences from the previous study in RBSF are also closely related to this clade. Clade 3 (100 % ML bootstrap support) comprised five sequences from five different *Teagueia* spp., *Spathaglotis plicata* AJ313456 from Singapore, and the tulasnelloid mycobiont HM451695 from the previous study in RBSF. Clade 4 (100 % ML bootstrap support) comprised two sequences from two different *Teagueia* spp. and sequences from *Cymbidium kanran* HM230653 from China, *Pseudorchis albida* HQ852051 from Czech Republic, *Chiloglottis valida* HM196804 from Australia, and others, as well as sequences from the previous study in RBSF. Furthermore, Atractiellomycetes that form mycorrhizae were found in one clade (100 % ML bootstrap

support) associated to two *Teagueia* spp. (Fig. 2; *Teagueia* sp7, *Teagueia* sp3). The obtained Atractiellales sequences were identical to "phylotype I″ as found by Kottke et al. (2010) from the previous study in RBSF, Zamora, Ecuador.

Up to three different phylogenetic species of mycobionts are associated with one *Teagueia* sp. (Tulasnellaceae and Atractiellales), and mycobionts of the same phylogenetic species were shared among several *Teagueia* species, suggesting an ample sharing potential of mycobionts among *Teagueia* sp. There is no evidence of site preference in *Tulasnella*; all phylogenetic species are present in both the Viscaya and Valencia locations. But in the case of Atractiellales, sequences were detected only in the Viscaya location. This result is consistent with a recent investigation that reveals high taxon diversity and broad sharing of mycobionts among orchid genera and species irrespective of environmental conditions or epiphytic and terrestrial habitats (Kottke et al. 2013). For instance, *Tulasnella calospora* was proposed by Hadley (1970) as a universal orchid symbiont considering the capacity to establish in vitro symbiotic associations with a broad range of orchid species. Other *Tulasnella* sp. have been detected as mycobionts of a large range of orchid species, and this genus is now considered as the most frequent mycobiont of orchids (Dearnaley et al. 2012). In contrast, with limited distribution of mycobionts, more seeds are needed to increase probability of encountering sites with appropriate fungi, resulting in a concerted seed and fungal limitation (McCormick and Jacquemyn 2014). Seed and fungal limitation are responsible for small orchid population sizes in which genetic drift leads to population differentiation (Otero and Flanagan 2006).

The investigated mycorrhizal associations occurred at the study site in orchids frequently growing very close to each other. We therefore expected to find differences in mycobiont preferences among *Teagueia* spp. as indicator of niche differentiation (Waterman et al. 2011, Jacquemyn et al. 2014). However, instead of that, we found that co-occurring orchid species share mycobionts. Even more, all detected mycobionts from *Teagueia* spp. display a worldwide geographical distribution. Considering the limited number of samples collected in the present study we expect that the number of phylogenetic species will increase significantly when a higher number of orchid specimen will be examined. However, the finding of "cosmopolitan" mycobionts associated to *Teagueia* spp. was an unexpected result. In principle, mutualisms might affect either the origin of plant species, via an effect on speciation, or the maintenance of diversity, via an effect on community assembly and species coexistence (Kottke et al. 2013). Our findings contradict the view that different fungal partners are needed for orchid species co-occurrence (Waterman et al. 2011, McCormick and Jacquemyn 2014) and supports the view of Kottke et al. (2013) that sharing of mycobionts will promote co-existence of closely related species and maintenance of diversity as observed in the tropical montane rain forest of Southern Ecuador.

Teagueia is an extraordinary example of a recent evolutionary speciation (Jost 2004). Mutualisms can be important for structuring plant communities by habitat filtering, in which only species with a certain trait (or interaction) are able to persist in a particular environment (Keddy 1992). This will lead to communities in which species are more similar than expected, compared to the regional species pool. The variety of colours, sizes, and shapes among sympatric *Teagueia* species (Jost 2004) strongly suggests that they each use a different set of pollinators. It is likely that these innovations in floral traits are accompanied by a pollinator switch, as indicated by the structural differences between the lips of the long-repent species and those of the "normal" species (Jost 2004). As a consequence of the expected role in reproduction and, potentially, reproductive isolation, pollinator interactions differ frequently between recently diverged species (Waterman et al. 2011). However, as with mycobionts, winged insects are typically amply distributed. Therefore, pollinator specialization, like mycobiont specialization, probably will not by itself explain the observed narrow local geographic endemism of these *Teagueia* species, with no long-repent *Teagueia* species shared between the equally-diverse *Teagueia* communities of north side of the Rio Pastaza and of the south side of the Rio Pastaza. Orchid seeds are highly vagile, so dispersal limitation also seems an unlikely explanation.

Molecular phylogenetic analyses of the family Orchidaceae are consistent in respect to a basal position of Cypripedioideae and Orchidoideae compared to Epidendroideae (e.g. Cameron et al. 1999). Switches from terrestrial to epiphytic habit or back were found to be major driving forces in radiation and specialization of orchids (Cameron 2005), and beside pollinator relationships, mycorrhizal interactions were recognized as crucial for orchid evolution (Taylor et al. 2003). Preferences for fungal partners have been demonstrated in other epiphytic orchids (Otero et al. 2002, 2004; Riofrío et al. 2013); however, more species need to be sampled to arrive at convincing conclusions about coevolution between orchids and their mycobionts. In this regard it is striking that Atractiellales, known to belong to a basal fungal lineage, is capable of forming mycorrhizal symbiosis with a fairly recent clade of orchid species such as *Teagueia*.

In contrast to previous expectations (Jost 2004), it seems to be that *Teagueia* spp. distribution is not limited by the presence of specific fungi. However, we have not eliminated the possibility that more specialized mycobiont relations are important during the crucial early embryonic stage of *Teagueia* seed germination. Mycorrhiza and pollinator relationships operate in parallel, both fostering speciation and maintenance of diversity. Our results shown up to three different phylogenetic species of mycobionts shared by one *Teagueia* sp. suggesting

a high potential for sharing mycobionts among *Teagueia* spp., even considering the limited number of samples in our study. All detected mycobionts had wide geographical distribution. Based on the available evidence we conclude that the extraordinary sympatric speciation of *Teagueia* is most likely partly driven by pollinator switches (see Pauw et al. 2013), but considering the observed broad sharing of mycobionts, mycorrhiza may be a key factor for coexistence of closely related *Teagueia* species.

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