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High diversity of root-associated fungi isolated from three epiphytic orchids in southern Ecuador

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ABSTRACT

Knowledge of fungal root-associates is essential for effective conservation of tropical epiphytic orchids. We investigated the diversity of root-associated fungi of *Cyrtochilum myanthum*, *Scaphyglottis punctulata* and *Stelis superbiens* from a tropical mountain rainforest in southern Ecuador, using a culture dependent approach. We identified 115 fungal isolates, corresponding to 49 fungal OTUs, based on sequences of the nrDNA ITS and partial 28S region. Members of Ascomycota were unambiguously dominant (37 OTUs), including *Trichoderma* sp. as the most frequent taxon. Members of Basidiomycota (Agaricales and Polyporales) and Mucoromycota (Umbelopsidales and Mortierellales) were also identified. Four potential mycorrhizal OTUs of Tulasnellaceae and Ceratobasidiaceae were isolated from *C. myanthum* and *S. superbiens*. Fungal community composition was examined using Sørensen and Jaccard indices of similarity. Alfa diversity was significantly different between *C. myanthum* and *S. superbiens*. No difference in beta diversity of the fungal communities between the 3 orchid species and the collecting sites was detected. The study revealed a high diversity of fungi associated with orchid roots. Our results contribute to a better understanding of specific relationships between epiphytic orchids and their root-associated fungi.

1. Introduction

Roots of tropical epiphytic orchids harbor diverse fungal taxa, including mutualistic mycorrhiza and considerably diverse non-mycorrhizal fungal root-associates (Bayman & Otero, 2006; Herrera, Suárez, & Kottke, 2010; Ma, Kang, Nontachaiyapoom, Wen, & Hyde, 2015). Investigations of tropical epiphytic orchids were mainly focused on orchid mycorrhizal fungi (Suárez, Weiß, Abele, Oberwinkler, & Kottke, 2008, 2009, 2016, 2006; Kottke et al., 2013, 2010; Otero, Flanagan, Herre, Ackerman, & Bayman, 2007, 2002; Xing, Gai, Liu, Hart, & Guo, 2014; Yokoya et al., 2015; Zettler, Corey, Jacks, Gruender, & Lopez, 2013). They form typical peloton-like structures in root cortical cells (Smith & Read, 2008). The common mycorrhizal fungi of epiphytic orchids belong to Tulasnellaceae, Ceratobasidiaceae and Serendipitaceae (Cevallos, Sánchez-Rodríguez, Decock, Declerck, & Suárez, 2017; Suárez & Kottke, 2016; Weiß, Waller, Zuccaro, & Selosse, 2016) but Kottke et al. (2010) reported Atractiellomycetes as forming mycorrhiza with some Neotropical orchids.

Besides mycorrhizal fungi, the roots of epiphytic orchids are colonized by diverse non-mycorrhizal fungal species (Bayman & Otero, 2006; Herrera et al., 2010; Martos et al., 2012; Oliveira et al., 2013). They are represented in much greater numbers than mycorrhizal fungi (Bayman & Otero, 2006; Jumpponen, 2002). Ma et al. (2015) reported that orchid non-mycorrhizal fungal root-associates contain over 110 genera, of which roughly 76 belong to Ascomycota. They are referred to as “core group fungi” because their frequency of occurrence is $\geq$ 10% (Sudheep & Sridhar, 2012). The function of the root-associated fungi is not exactly known. They are mostly found in velamen (Herrera et al., 2010; Suárez et al., 2009, 2006), without causing any symptoms of disease (Bayman

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& Otero, 2006). They may promote the growth of orchids by mobilizing soil nutrients in the rhizosphere (Newsham, 2011; Zhao et al., 2014). Influence on amounts or changes of secondary metabolites was also indicated (Zhang, Lv, Zhao, & Guo, 2013). In general, they are thought to be a resource for bioactive compounds that protect the host from soil pathogens (Ma et al., 2015).

Considering the total richness of the orchid family, only 200 genera (less than 30%) have been studied for their endophytic fungal diversity (Ma et al., 2015). The tropical epiphytic orchids constitute over 80% of Orchidaceae (Givnish et al., 2015). In Ecuador, the orchid family is the richest plant group, currently containing 4537 scientifically accepted species (http://www.tropicos.org/Project/CE). The highest species diversity of orchids in Ecuador (>2650 species) occurs in "premontane to montane" forest regions at elevations of 300–3000 m above sea level (a.s.l.) (Dodson & Escobar, 1993). In Neotropical montane rain forests, the epiphytic orchids are unambiguously dominant (Dodson, 1999), representing ca. 50% of all epiphytic species (Küper, Kreft, Nieder, Köster, & Barthlott, 2004). The study of orchids in Ecuador is among conservation priorities, especially because of the 2 biodiversity hotspots identified in this country (Myers, Mittermeier, Mittermeier, da Fonseca, & Kent, 2000). Well founded knowledge on their root-inhabiting fungi is therefore of crucial interest.

We examined, by culture dependent method, the diversity of root-associated fungi of the 3 epiphytic orchid species *Cyrtochilum myanthum* (Lindl.) Kraenzl., *Scaphyglottis punctulata* (Rchb.f.) C. Schweinf. and *Stelis superbiens* Lindl. occurring abundantly in 2 sampling sites located in southern Ecuador. The aims of the study were to (i) isolate and genetically identify fungal taxa associated with roots of selected orchid species; (ii) determine species richness (= alfa diversity) of fungal communities of examined sampling sites and orchid species; (iii) compare species richness among sites and hosts; (iv) determine the degree of differentiation (= beta diversity) in structure of fungal communities of studied sites and orchid species. To the best of our knowledge, this is the first study on fungal root-associates of *S. punctulata* in South America.

2. Materials and methods

2.1. Collecting sites

Two collecting sites were established. The first collecting site is located in the Reserva Biológica San Francisco (RBSF) (3°58′S, 79°04′W; Suárez et al., 2006), where elevation ranges from 1850 to 2083 m a.s.l. The second collecting site is situated in the area of the natural reserve Arcoiris (AI) (3°98′S, 79°09′W) at an elevation from 2095 to 2144 m a.s.l. Both sites are located in a tropical mountain rainforest in the province Zamora-Chinchipe at the edge of the Podocarpus National Park in southern Ecuador. The collecting sites are parts of the premontane forest region in the Tropical Andes biodiversity hotspot (Myers et al., 2000).

2.2. Sampling

Sampling was carried out from Mar to Nov 2014. Three or 4 roots per plant were collected from a total of 110 adult individuals of *Cyrtochilum myanthum* (28 individuals), *Scaphyglottis punctulata* (29 individuals) and *Stelis superbiens* (53 individuals; Table 1). The samples were collected from flowering or post-flowering individuals. The 3 orchid species occurred abundantly in the study area, growing on tree trunks or branches at ca. 2 m above ground.

Roots in direct contact to the substrate (tree bark) were gently lifted and cut off by a sterile scalpel blade avoiding cross-contamination. Samples were placed into plastic bags and transported to laboratories at the Universidad Técnica Particular de Loja (UTPL) on the same day.

2.3. Fungal isolation

Fungal isolation from root cells was performed according to standard protocols (Zettler, Sharma, & Rasmussen, 2003, 2013) on the day of collection or the following day. The presence of fungal pelotons was briefly assessed under a light microscope, and selected root pieces (2 or 3 per sampled root) were rinsed gently under tap water to remove surface debris. Velamen was not removed. Under sterile conditions in a laminar flow bench, the root pieces were surface-sterilized for 1 min in a solution of 5% (v/v) absolute ethanol, 5% (v/v) Clorox bleach in 90 mL of sterile distilled-deionized (DI) water. This was followed by two 1 min rinses in sterile DI water. The surface-sterilized root pieces were chopped in a drop of sterile DI water in 9 cm diam Petri plates to release pelotons from cortical cells. Fungal Isolation Medium (FIM, Mitchell, 1989), with added streptomycin sulfate salt (0.13 g/L) to reduce bacterial growth, was poured onto the released pelotons. The next day, pelotons that produced actively growing hyphae visible under a light microscope, were isolated and transferred into Petri plates with potato dextrose agar (PDA). After 3–7 d of incubation at 27 °C in the dark, hyphal tips emerging from agar cubes with pelotons were transferred to fresh PDA. Fungal cultures were maintained at ambient temperature in the light until the colony attained a sufficient size to facilitate a mycelial sample for DNA extraction.

All fungal isolates obtained in this study have been deposited in the fungal culture collection of UTPL. Potential mycorrhizal fungi were cryopreserved for future use.

2.4. DNA extraction, PCR and cloning

The DNA was extracted from fresh tissue of pure fungal cultures using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The ITS1-5.8S-ITS2 region, including a portion of the 28S nrDNA gene, was PCR amplified using the universal primer pair ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′, White, Bruns, Lee, & Taylor, 1990) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′, White et al., 1990). In cases where the initial DNA concentration was higher than 25–30 ng/μL, a 1:10 dilution was carried out prior to PCR. The PCR was performed on a DNA Engine Peltier thermal cycler (BIO-RAD, PTC-200, Foster City, CA, USA) with a program consisting of initial denaturation at 94 °C for 5 min, 36 cycles of repeated reactions (denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 2 min) and final elongation of 10 min at 72 °C. Success of amplification was visualized on 1% (w/v) agarose gels stained by 5% (v/v) GelRed (Biotium, Hayward, USA). PCR products were purified using PureLink™ Quick PCR Purification Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Purified PCR products were sequenced in both directions by Macrogen (Seoul, Korea) using primers ITS1 and NL4.

The amplicons of several problematic isolates were cloned. Cloning was performed by Macrogen (Seoul, Korea) using TOPcloner™ TA Kit (Enzynomics, Daejeon, Korea). Transformed *Escherichia coli* cells were grown on LB agar medium with 100 mg/L ampicillin. Five colonies were randomly chosen and their DNA was purified using BioFact™ Plasmid Prep Solution Kit (BioFact, Daejeon, Korea). Subsequent sequencing was performed using primers M13F (5′-GTAAAACGACGGCCAGT-3′) and M13R (5′-GCGGATAACAATTTCACACAGG-3′). The sequences representing each delimited fungal OTU are available on GenBank under accession numbers KU955369–KU955417.

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