Orchids keep the ascomycetes outside: a highly diverse group of ascomycetes colonizing the velamen of epiphytic orchids from a tropical mountain rainforest in Southern Ecuador

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Orchid mycorrhizal detection, based only on fungal isolation from roots, is biased due to difficulties in isolating the respective fungi. Previous investigations have shown that mostly ascomycetes, housed in the sheltered compartment of the velamen covering the roots of epiphytic orchids, are isolated on agar plates. Roots of 83 individual epiphytic orchids of Stelis hallii, S. superbiens, S. concinna, Stelis sp. and Pleurothallis lilijae were sampled in the Reserva Biológica San Francisco, Southern Ecuador. The velamen was partly removed and root tissue macerated and transferred to Petri dishes containing corn meal agar, malt extract agar and Melin Norkrans modified medium. DNA was extracted from the cultures and the 5.8S-ITS region and partial nrLSU sequenced. Phylogenetic analysis revealed members of 12 orders of ascomycetes associated with the roots of these orchids, a much higher diversity than previously known. Twelve isolates were related to the Helotiales. Our results have uncovered some of the fungal diversity within the velamen of epiphytic orchids; fungi invading, most likely, from tree bark or humus accumulation close to the roots.

Keywords: ascomycetes; epiphytic orchids; velamen; Helotiales; tropical mountain rain forest

Introduction

Roots of epiphytic orchids are covered by a specialized, multi-seriate epidermis, known as the velamen (Esau 1964). At maturity, the velamen consists of non-living cells, often bearing secondary wall thickening and pores. The principal roles of this tissue are water storage and the reduction of water loss from the cortex (Esau 1964; Benzing et al. 1982). However, the velamen is colonized by a large variety of organisms, especially fungi and bacteria (Benzing et al. 1982; Bayman and Otero 2006). Light and electron microscopy has revealed abundant fungal colonization of the velamen, but only members of the Basidiomycota have, so far, been observed to penetrate the passage cells of the exodermis, forming pelotons in the cortical cells of epiphytic orchids (Kottke and Suárez 2009; Kottke et al. 2010). Ascomycota have not been detected in healthy root cortical cells of tropical, epiphytic orchids, although a representative number of roots, sampled in the tropical mountain rainforest of Southern Ecuador, Northern Andes, and identified by molecular tools.

Materials and methods

Study site and sampling

The study site was located on the eastern slope of the Cordillera El Consuelo in the Northern Andes of southern Ecuador. The area of ~1000 ha belongs to the Reserva Biológica San Francisco and borders the Podocarpus National Park in the north, half way between Loja and Zamora in Zamora-Chinchipe Province (3°58´ S, 79°04´ W). Sampling was carried out along small paths at an altitudinal gradient between 1850 and 2100 m a.s.l. Three roots per plant were collected between August and October 2005 from a total of 83 flowering individuals, 28 of Stelis hallii Lindl., 26 of Stelis superbiens Lindl., 11 of Stelis concinna Lindl., 14 of Pleurothallis lilijae Foldats and four of Stelis sp. All selected plants were epiphytes on trunks or branches of standing trees.

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**Fungal isolation**

Collected roots were transferred to the laboratory on the day of sampling. Three colonized root pieces per plant were surface-sterilized. Roots were rinsed in distilled water with a few drops of liquid soap, immersed in 70% ethanol for 30 s, then immersed in 20% “Ajax Cloro” (household bleach, sodium hypochlorite 5.25%) for 10 min and finally rinsed in sterile distilled water. The velamen was partly removed and discarded. The remaining root tissue with still adhering parts of the velamen was macerated in a 1.5-ml micro-tube containing some drops of sterile distilled water. From each sample, three drops of macerated tissue were placed on plates of corn meal agar (CMA; C1176, Sigma), malt extract agar (MEA; M6907, Sigma) and Melin Norkrans modified medium (MMNC; Suárez et al. 2006). Petri dishes were incubated at 28°C in the dark. The fungal isolates were transferred to potato dextrose agar (PDA) after the colony had reached 1 cm in diameter. Pure cultures are preserved in the Centro de Biología Celular y Molecular of Universidad Técnica Particular de Loja.

**DNA isolation and PCR and sequencing**

Genomic DNA was recovered from fresh fungal cultures using a Plant Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. The nrDNA 5.8S, including the internal transcribed spacers ITS1 and ITS2 and a partial region of nrDNA 28S, were amplified with a set of universal primer combinations ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′; White et al. 1990) and NL4 (5′-GGT CCG TGT TTC AAG ACG G-3′; White et al. 1990) and fungal primer combination ITS1F (5′-GGT CAT TTA GAG GAA GTA A-3′; Gardes and Bruns 1993) and LR5 (5′-TCC TGA GGG AAA CTT CG-3′; Vilgalys and Hester 1990).

PCR conditions were as follows: initial denaturizing at 94°C for 3 min; 35 cycles, each cycle consisting of one step of denaturizing at 94°C for 30 s; annealing, depending on the primer combinations for 45 s and extension at 72°C for 1 min; a final extension at 72°C for 7 min was performed to finish the PCR. A control including PCR mix without DNA template was included in every PCR. Success of the PCR amplifications were tested in 0.7% agarose stained in a solution of 0.5 μg ml⁻¹ ethidium bromide. PCR products were purified using the QIAquick protocol (Qiagen).

ITS-5.8S and partial 28S sequences of nuclear ribosomal RNA genes of our isolates were sequenced using primers ITS1, NL4, NL1 and ITS4. Sequence editing was performed using Sequencher version 4.5 (Gene Codes, Ann Arbor, MI, USA). The sequences obtained in this study are available from GenBank under accession numbers HQ207016–HQ207070.

**Phylogenetic analyses**

BLAST (Altschul et al. 1997) was used against the NCBI nucleotide database (GenBank; http://www.ncbi.nlm.nih.gov/) to detect published sequences of high similarity. A multiple analysis method (Lee 2001) was employed and sequences were aligned using POA software (Lee et al. 2002) and MAFFT v5.667 (Katoh et al. 2005) under the G-INS-i option. A partial nrDNA 28S region of 562 bp was considered for phylogenetic purposes without exclusions. Bayesian analysis, based on Markov chain Monte Carlo (MCMC), was implemented in MrBayes version v3.1.2 (Huelsenbeck and Ronquist 2001), including two runs, each involving four incrementally heated Markov chains over four million generations and using random starting trees. Trees were sampled every 100 generations, resulting in a total of 40,000 trees, from which the last 30,000 were used to compute a 50% majority rule consensus tree. Each dataset was analyzed using the DNA substitution model GTR+I+G (Swoford et al. 1996). Additionally, a maximum likelihood calculation was implemented in RAxML version 7.0.4 software (Stamatakis 2006) using the GTRMIX algorithm. We used *Saccharomyces cerevisiae* (EU649673) as the outgroup. Relationships among the 12 sequences of Helotiales were examined in a further analysis considering the nrDNA region ITS1-5.8S-ITS2.

*Xylaria* sp. (FN392317) was used as the outgroup. Alignments and phylogenetic analyses were performed as described above.

**Results**

Ninety pure cultures were obtained after 249 isolation attempts. Sixty isolates were obtained from *S. superbiens*, 12 from *S. hallii*, five from *Stelis* sp., three from *S. concinna*, and 10 from *P. lilijae*; 56% of isolates grew in CMA, 20% in MEA and 24% in MMNC.

DNA sequences were obtained from 58 of the 90 isolates. Phylogenetic analyses of the partial region of nrDNA 28S placed 55 isolates in 12 different orders of Ascomycota (Figure 1) and three into Basidiomycota (Polyporals, not shown). The most frequent ascomycetes isolates belonged to Hypocreales (16), Helotiales (12) and Xylariales (10) (Table 1). One isolate was located in Boliales, another in Chaetosphaeriales. Two isolates were similar to Capnodiales, four to Pleosporales, two to Botryosphaeriales, and one to Thelebolales. One isolate clustered with Rhytismatales, three isolates with Eurotiales, and two isolates were similar to Chaetothyriales (Table 1).

Members of several ascomycete orders were identified from more than one orchid species (Table 1). Phylogenetic analysis of the Helotiales sequences shows three well-supported groups (Figure 2). Nine isolates form a group allied to *Pezicula* and *Cryptosporiopsis*. The second group with one of our isolates is related to...
Figure 1. (A and B) Phylogenetic placement of ascomycete sequences obtained from the velamen of Stelis hallii, Stelis superbiens, Stelis concinna, Pleurothallis lilijae and Stelis sp. Tree inferred from nuclear rDNA coding for the 5′-terminal domain of the large ribosomal subunit (nucLSU) using a maximum likelihood analysis implemented in RaxML with the GTRMIX algorithm. Numbers on branches designate Bayesian MCMC estimates of posterior probabilities and ML values (only values exceeding 50% are shown). The tree was rooted with Saccharomyces cerevisiae EU649673.
Figure 1. (Continued).
Table 1. Identified isolates from five epiphytic orchid species: Stelis hallii, S. superbis, S. concinna, Stelis sp. and Pleurothallis lilijae. Numbers in parenthesis indicate the number of sampled individuals.

| Orders of ascomycetes | Stelis superbis (26) | Stelis hallii (28) | Stelis concinna (11) | Pleurothallis lilijae (14) | Stelis sp. (4) | Total isolates (n) |
|-----------------------|----------------------|-------------------|---------------------|-------------------------|---------------|-------------------|
| Xylariales            | 6                    | 3                 | 1                   |                         |               | 10                |
| Boliniales            | –                    | –                 | 1                   |                         |               | 1                 |
| Chaetosphaeriales     | 1                    | –                 | –                   | 2                       | 2             | 1                 |
| Hypocreales           | 12                   | –                 | –                   | 2                       | 2             | 16                |
| Capnodiales           | 1                    | 1                 | –                   | –                       | –             | 2                 |
| Pleosporales          | 4                    | –                 | –                   | –                       | –             | 4                 |
| Botryosporales        | 2                    | –                 | –                   | –                       | –             | 2                 |
| Chaetothyriales       | –                    | –                 | –                   | 2                       | –             | 2                 |
| Eurotiales            | 1                    | –                 | –                   | 2                       | –             | 3                 |
| Thelebolales          | 1                    | –                 | –                   | 3                       | –             | 1                 |
| Helotiales            | 7                    | 1                 | –                   | –                       | 1             | 12                |
| Rhytismatales         | 1                    | –                 | –                   | –                       | –             | 1                 |
| Total (n)             | 35                   | 5                 | 3                   | 7                       | 5             | 55                |

Discussion

Analysis of the 28S nrDNA gene revealed a broad systematic coverage of the ascomycete orders associated with the roots of the five pleurothallid orchids. Previously, the most common ascomycetes isolated from the roots of epiphytic orchids have been circumscribed to only six orders: Xylariales, Phyllachorales, Diaporthales, Hypocreales, Botryosphaeriales and Pleosporales (Richardson and Currah 1995; Bayman et al. 1997; Tremblay et al. 1998; Yuan et al. 2009). We have recorded members related to 12 orders: Xylariales, Boliniales, Chaetosphaeriales, Hypocreales, Capnodiales, Pleosporales, Botryosphaeriales, Chaetothyriales, Eurotiales, Thelebolales, Helotiales, and Rhytismatales. As sampling efforts increase and identification is improved by molecular techniques, the diversity of fungi in orchid roots may be much higher than previously thought (Kageyama et al. 2008), which may account for the highly diverse epiphytic orchids with large velamen in the tropical mountain rainforests of the Northern Andes.

The Order Helotiales includes an ecologically diverse group of plant pathogens, wood, debris and soil saprobes, plant endophytes, and mutualistic ericoid mycorrhizal (ERM) and ectomycorrhizal (ECM) fungi (Vrålstad et al. 2002; Haug et al. 2004). Our isolates belonging to the Helotiales were isolated from four of the five orchid species and were not closely related to the mycorrhiza-forming Helotiales. This result is of special interest because we found mycorrhiza-forming Helotiales in this rainforest associated with G. emarginata (Haug et al. 2004) and Ericaceae (Setaro 2009, unpublished data) and speculated on the potential symbiosis with orchids. The result, however, substantiates our TEM observations that only Basidiomycota from the Tulasnellales, Sebacinales, Ceratobasidiales (Heterobasidiomycetes) and the recently detected Atractiellales (Pucciniomycotina) are present in cortical tissue and form mycorrhizae with the respective epiphytic orchids (Suárez et al. 2006, 2008; Kottke et al. 2010). TEM observations also excluded the isolated Polyporales as a mycorrhizal fungi of the investigated orchids. As orchid mycobionts, members of the Homobasidiomycetes appeared to be restricted to partial or fully mycoheterotrophic orchids of the northern hemisphere and East Asia (for references, see Yukava et al. 2009; Kottke and Suárez 2009; Selosse et al. 2010).

We only sampled roots in close contact with the bark of standing trees where mosses or debris had accumulated, because mycorrhizae are only formed under these circumstances. In our opinion, saprobic ascomycetes associated with the velamen may indirectly improve nutrient access of the orchids by decomposing the substrate outside the roots from where they most likely invaded the velamen. Experiments to substantiate this assumption could be of interest for the orchid cultivation.

A large diversity of ascomycetes was recorded, indicating that moderate surface sterilization and partial removal of the velamen did not eliminate all fungi from the root surface. This fact should be kept in mind when searching for orchid mycobionts via isolation from surface sterilized tissue. Many fungi colonize only the nonliving cells of the velamen and should neither be considered endophytes, as these, by definition, colonize healthy tissue.
Figure 2. Phylogenetic placement of Helotiales fungal isolate sequences from the velamen of Stelis hallii, Stelis superbiens, Stelis concinna, Pleurothallis lilijae and Stelis sp. Tree inferred from the ITS-5.8S region using a maximum likelihood analysis implemented in RaxML with the GTRMIX algorithm. Numbers on branches designate Bayesian MCMC estimates of posterior probabilities and ML values (only values exceeding 50% are shown). The tree was rooted with Xylaria sp. FN392317.
without causing disease (Arnold et al. 2000), nor mycorrhizal fungi. The absence of true orchid mycobionts from our isolation trials may also indicate that other unculturable fungi are present in the tissue.

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