MOLECULAR EVIDENCE REVEALS FUNGI ASSOCIATED WITHIN THE EPIPHYTIC ORCHID *LAELIA SPECIOSA* (HBK) SCHLTR.

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Abstract: Fungi-orchid relationships have been studied mainly in terrestrial orchids. The present work deals with diversity and biological role of the fungi associated to the epiphytic orchid *Laelia speciosa* through molecular evidence. ITS sequences confirmed fungal presence in roots, capsules, seeds and *in vitro* seedlings. None of the fungi found formed characteristic pelotons, so we do not assume they are mycorrhizal. We identified 18 taxa of fungi belonging to eight known genera (*Alternaria*, *Curvularia*, *Cylindrocarpon*, *Fusarium*, *Myrmecridium*, *Neonectria*, *Penicillium*, and *Tetracladium*) and unknown species of Atractiellales, Helotiales, Hypocreales, Lasiosphaeriaceae, Nectriaceae, Sordariomycetes, and Tricholomataceae. From these, we infer the biology of nine parasites, four saprobes, and two endophytes. One of the fungi, a Helotiales species, was able to colonize all orchid tissues, including seeds and *in vitro* seedlings, so, even if all precautions were taken, “axenic culture” of *L. speciosa* is a difficult task. There is a considerable diversity of fungi within *L. speciosa* organs. Endophytic fungi interact with the whole plant of *L. speciosa* along its life cycle.

Key words: fungi, ITS sequences, *in vitro* culture, orchid seeds.

Resumen: Las relaciones de orquídeas-hongos han sido estudiadas principalmente en orquídeas terrestres. El trabajo aquí presentado trata sobre la diversidad y significado biológico de los hongos asociados a la orquídea epífita endémica *Laelia speciosa*. Se registró la presencia fungal en semillas, protocormos y plántulas durante la germinación y primeros estadios de desarrollo *in vitro* de *L. speciosa* mediante evidencia molecular. Las secuencias de ITS confirmaron la presencia de hongos en raíces, cápsulas, semillas y también en plántulas cultivadas *in vitro*. Ninguno de los hongos encontrados formó pelotones característicos por lo que no se asume que sean hongos micorrízicos. Se identificaron 18 taxa de hongos pertenecientes a ocho géneros (*Alternaria*, *Curvularia*, *Cylindrocarpon*, *Fusarium*, *Myrmecridium*, *Neonectria*, *Penicillium* y *Tetracladium*) y especies desconocidas de Atractiellales, Helotiales, Hypocreales, Lasiosphaeriaceae, Nectriaceae, Sordariomycetes y Tricholomataceae. De éstos, se infirió la biología de nueve parásitos, cuatro saprobios y dos endófitos. Uno de los hongos, perteneciente a una especie de Helotiales coloniza todos los tejidos de la orquídea, incluyendo semillas y plántulas cultivadas *in vitro*; por tanto, aun si todas las precauciones son tomadas, es difícil obtener “cultivos axénicos” de *L. speciosa*. Existe una considerable diversidad de hongos endófitos dentro de los órganos de *L. speciosa* a través de todo su ciclo de vida.

Palabras clave: cultivo *in vitro*, hongos, secuencias de ITS, semillas de orquídea

The fungus-orchid association has been a long-standing issue in the study of orchid development. Orchid seeds, almost devoid of reserves, require the presence of fungi as an energy source for germination and during early developmental stages in nature (Kottke and Suárez, 2009). These symbiotic interactions and the degree of specificity have been demonstrated mainly with fungal isolates and terrestrial orchids *in vitro*, either from roots of orchid species or

from diseased non-host plant species (Clements, 1988; Peterson *et al.*, 1998; Bonnardeaux *et al.*, 2007). Additionally, the improvement of *in vitro* symbiotic germination techniques has been useful for the propagation of orchid species that are difficult to reproduce (Anderson, 1991; Zettler and McInnis, 1994; Tan *et al.*, 1998; Zettler and Hofer, 1998; Ortega-Larrocea, 2008).

There is a high diversity of symbiotic fungi associated

Figure 1. *Laelia speciosa* flower.

with green orchid roots (Kottke and Suárez, 2009). Most of the fungi isolated have been assigned to the anamorphic form-genus *Rhizoctonia*, which has been associated with teleomorphic species of the genera *Ceratobasidium*, *Tulasnella*, *Sebacina*, *Thanatephorus*, *Waitea* and others within the subdivision Basidiomycotina. They form characteristic intracellular masses of hyphae, known as pelotons, within root or rhizome cortical tissue of orchids (Rasmussen, 2002). Other fungi of the subdivision Ascomycotina have been also isolated from these organs but their biological role is unknown and may be as important to orchids in nature as those of the Basidiomycotina (Currah *et al.*, 1997).

Studies of *in situ* seed germination on terrestrial orchids allow the identification and enumeration of fungi in the field, that are important for understanding the ecology of orchids and their fungal associates in natural habitats (Rasmussen and Whigham, 1993, 1998; McKendrick *et al.*, 2000; Batty *et al.*, 2001; McCormick *et al.*, 2012). For example, seeds of terrestrial orchids failed to germinate when they are not colonized by fungi in natural habitats (Rasmussen and Whigham, 1993; Batty *et al.*, 2001).

Molecular tools have been a keystone to identify the origin and specificity of endophytic fungi, especially those that are difficult to isolate (Taylor and Bruns, 1997, 1999; Shefferson *et al.*, 2005). DNA sequences of fungi obtained from mycorrhizal roots have shown a highly diverse group of or-

chid mycobionts previously unknown. Sebaciniales Group B, Tulasnellales and Ceratobasidiales have been associated with epiphytic and terrestrial orchids that inhabit grasslands and arbuscular mycorrhizal forests, while Sebaciniales Group A, Thelephorales, Russulales and Tuberales are associated with terrestrial orchids in ectomycorrhizal forests (Smith and Read, 2008; Kottke and Suárez, 2009).

The physiological role and degree of specificity of fungi in epiphytic orchids are poorly understood compared with those of terrestrial species. Endophytic fungi have been commonly isolated from roots and leaves of orchids (Bayman *et al.*, 1997). These endophytes form diverse and heterogeneous assemblages within roots and leaves with no shared species between organs (Tao *et al.*, 2008) or with many species shared between roots and stems, stems and leaves, even between the three organs (Yuan *et al.*, 2009).

During the *in vitro* reproduction of *Laelia speciosa*, an orchid endemic to Mexico, we observed that seeds and protocorms stained with trypan blue were colonized with endophytic fungi. In the present study, we addressed the following questions: (1) how diverse are these endophytes?, (2) what fungi are involved?, and (3) in which organs are they present? To our knowledge, this is the first report of orchid endophytic fungi present on seeds and *in vitro* seedlings.

Materials and methods

Plant and sample localities. *Laelia speciosa* (HBK) Schltr. is an epiphytic orchid endemic to Mexico, widely distributed in the Sierra Madre Oriental, the Sierra Madre Occidental, the Transmexican Volcanic Belt and the southern part of the Mexican Plateau. It grows on oaks, primarily over *Quercus deserticola* Trel. The plants grow in open deciduous forests at 1,900 to 2,500 m in altitude where annual precipitation ranges from 700 to 1,000 mm (Halbinger and Soto, 1997), with severely dry seasons from December to June. Plants are 12–40 cm high, excluding the inflorescence, they have globular or ovoid pseudobulbs which carry one stiff, terminal leaf. They produce an inflorescence that ranges from 15 to 25 cm long, with 1 to 3 flowers which measure 8–16 cm in diameter and range in color from pale to dark pink-lilac to purplish (Figure 1). *Laelia speciosa* is considered subject to special protection under Mexican law (NOM-059-SEMAR-NAT-2010). However, thousands of plants are harvested every year from their habitats to be sold due to the beauty of their flowers. This massive harvesting has caused local extinction, but fortunately, large populations can still be found (Halbinger and Soto, 1997; Ávila-Díaz and Oyama, 2007). Samples were collected in the Olvido population (19° 37' N, 101° 29' W, 2,361 m), and in Indaparapeo (19° 43' N, 101° 55' W, 2,400 m).

***In vitro* cultivation of *Laelia speciosa* seedlings.** We collected two capsules of *L. speciosa* from El Olvido population.

Capsules had the following characteristics: were closed, without damage, necrosis or senescence, and arose from plants with healthy pedicels. After collecting, capsules were transported to the laboratory in paper bags and immediately disinfected as follows: 15% Extran MA02 (liquid detergent), 5 min; 70% ethanol, 5 min; 3% hydrogen peroxide, 3 min; and 20% sodium hypochlorite (Cloralex, which is a commercial solution, with 5.5% of active chlorine), 15–20 min. The capsules were subsequently rinsed three times with sterile distilled water inside a laminar flow hood, followed by soaking in 96% alcohol and flaming to avoid contamination. Inside a laminar hood, seeds were separated from the maternal tissue, using sterilized dissection needles, bulked, and approximately 5 mg were placed uniformly in a flask containing Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) with 30 g L⁻¹ of sucrose, 7 g L⁻¹ of agar at pH 5.7 (Ávila-Díaz *et al.*, 2009). Seven flasks per capsule were cultured and maintained in a 16 h photoperiod in a controlled environment room at a 25 °C.

Molecular characterization of endophytic fungi. For the purpose of this study, we define fungal endophytes as all resident fungi found within plant tissues independent of their biological role. We sequenced the endophytic fungi from wild plants to assess their taxonomic affinity and their presence in different plant tissues. Two healthy mature plants with capsules from El Olvido and Indaparapeo populations were collected. The whole plants were immediately surface sterilized, as described previously, and stored at -70 °C. We also processed two seedlings grown in vitro from seeds of El Olvido. We extracted DNA by a phenol-chloroform method (Chomczynski and Sacchi, 1987). The ITS1, 5.8S and ITS2 regions were amplified by PCR with the primer pair ITS1F and ITS4, which have shown to be universal for fungi (Gardes and Bruns, 1993), although some reports indicate that they do not amplify the tulasnelloid fungi (Smith and Read, 2008). PCR products were cleaned with Exosap-it (USB) and cloned with Topo-Ta 4 (Invitrogen) according to manufacturer proceedings. Gene inserts were amplified from clones either by direct PCR of positive colonies or by PCR of regrown colonies in LB liquid media. Correct size of inserts was checked by agar electrophoresis and positive products were sequenced in both directions using Big Dye 3.1 chemistry (Applied Biosystems) in an ABI 3100 automatic genetic analyzer. We edited and aligned sequences using Sequencher 4.2.2 (Gene Codes) and eliminated vector sequences using VecScreen (NCBI, 2001). Operational taxonomic units (OTU) were defined at 97% of similarity. We compared our OTU sequences with GenBank using the megaBLAST algorithm (Altschul *et al.*, 1997). We used several criteria to design the taxonomic affinity of sequences. When our sequence matched just one record of at least 97% similarity, we decided both sequences to be conspecific. When our sequence matched more than one record of the

same genus at least at 97% we decided sequences to be congeneric. When our sequence did not match any record of at least at 97% we looked for the nearest taxonomic rank that included the closest sequences. We adopted the nomenclature and taxonomic hierarchy of NCBI. Sequences with high E-values were suspected as chimeras and were analyzed with Chimera Check 2.7 (Cole *et al.*, 2003) and Bellerophon (Huber *et al.*, 2004) software. Sequences indicated as chimeras in both directions were removed from the data set.

Results

Identity of endophytic fungi. We sequenced more than 150 clones with positive inserts in both directions. Around 50% of sequences belonged to *Laelia speciosa*, although the primers employed are specific to fungi, they have slight amplification of plant DNA particularly if it has high concentration (Gardes and Bruns, 1993). We obtained 71 sequences of fungi from *L. speciosa* tissues. At 97% similarity, these sequences were grouped in 18 OTUs. Contig sequences from each OTU were deposited into GenBank under accession numbers FJ708598 to FJ708615 (Table 1). Species belonged to eight known genera (*Alternaria*, *Curvularia*, *Cylindrocarpon*, *Fusarium*, *Myrmecridium*, *Neonectria*, *Penicillium*, and *Tetracladium*) and unknown species of Atractiellales, Helotiales, Hypocreales, Lasiosphaeriaceae, Nectriaceae, Sordariomycetes, and Tricholomataceae. Fifteen fungi were Ascomycota and three were Basidiomycota. We could not identify eight of the taxa beyond family because they had no similar sequences in Genbank (Table 1). Nine OTUs were found in roots and capsules. Only one fungal species belonging to the Helotiales was present in all tissues and all plants. One species of Tricholomataceae was present in roots and seedlings, *Alternaria longissima* in capsules and seeds, and the remaining fungi were present only in one tissue type (Table 2).

Discussion

Identity of endophytic fungi within Laelia speciosa tissues. There is a considerable diversity of endophytic fungi within *L. speciosa* tissues. Although several studies have studied the endophytic diversity in orchids, they are limited because they are based on sequencing fungal isolates. Our results are comparable with studies of Abadie *et al.* (2006) and Tao *et al.* (2008) because they are also based on cloned PCR products directly amplified from DNA tissue extractions.

From the 18 taxa found in *Laelia speciosa*, based on the phylogenetic similarity of our samples to known taxa, we infer the biology of nine parasites, four saprobes, and two endophytes. Some of the species found here have been previously described as parasitizing monocot roots as *Curvularia affinis* (Feldman *et al.*, 2008). *Fusarium solani* is a widely distributed parasite that has been reported as an endophyte

Table 1. *Laelia speciosa* endophytic fungi sequences best matches in GenBank. ¹Accession number of sequences produced in this research. ²Accession number of sequences already uploaded in GenBank.

Accession number ¹	Taxa	% identity	% coverage	E value	Closest GenBank match	Accession number ²
FJ708614	<i>Alternaria longissima</i>	99	100	0	Uncultured ascomycete	EF154350
		99	100	0	<i>Alternaria longissima</i>	DQ865104
FJ708615	<i>Alternaria</i> sp.	100	88	0	Uncultured ascomycete	EU358786
		98	88	3E-179	<i>Alternaria longissima</i>	DQ865104
FJ708602	<i>Atractiellales</i> sp.	92	76	1E-133	<i>Atractiella solani</i>	DQ198781
		91	72	5E-122	<i>Atractiellales</i> sp.	DQ198780
FJ708613	Basidiomycete	99	58	2E-141	<i>Cryptococcus albidus</i>	EU839451
		99	58	2E-141	<i>Cryptococcus adeliensis</i>	AY733078
		99	58	2E-141	<i>Cryptococcus kuetzingii</i>	AF145327
FJ708612	<i>Curvularia affinis</i>	100	100	0	Dothideomycete sp.	EU680530
		100	98	0	<i>Curvularia affinis</i>	AF071335
FJ708611	<i>Cylindrocarpon pauciseptatum</i>	99	95	0	<i>Cylindrocarpon</i> sp.	DQ682573
		98	88	0	<i>Cylindrocarpon pauciseptatum</i>	EF607090
FJ708610	<i>Fusarium solani</i>	100	100	0	<i>Fusarium</i> sp.	EU750688
		99	99	0	<i>Fusarium solani</i>	FJ478114
FJ708609	<i>Helotiales</i> sp.	98	100	0	Uncultured fungus	AJ875364
		98	98	0	Uncultured helotiales	DQ182424
		98	74	0	Uncultured <i>Phialophora</i>	AY578280
FJ708608	<i>Hypocreales</i> sp.	99	95	0	uUncultured root fungus	EU144855
		97	100	0	<i>Gibberella moniliformis</i>	EU717682
		97	100	0	<i>Fusarium oxysporum</i>	AY667488
FJ708607	<i>Lasiosphaeriaceae</i> sp.	95	93	0	<i>Schizothecium fimbriatum</i>	AY999115
		95	94	0	<i>Podospora curvispora</i>	AF443850
FJ708606	<i>Myrmecridium schulzeri</i>	99	99	0	<i>Myrmecridium schulzeri</i>	EU041777
FJ708605	Nectriaceae	91	99	0	<i>Nectria mauritica</i>	AY138847
		90	99	5E-177	<i>Rubrinectria</i> sp.	AY554218
		89	99	2E-171	<i>Penicillium</i> sp.	AF434795
FJ708604	<i>Neonectria</i> sp.	99	87	0	<i>Neonectria macrodidyma</i>	AM419076
		99	87	0	<i>Cylindrocarpon</i> sp.	AB369421
		99	87	0	<i>Neonectria radiculicola</i>	AJ875336
FJ708603	<i>Penicillium</i> sp.	99	100	0	<i>Penicillium</i> sp.	FJ176474
		99	100	0	<i>Penicillium commune</i>	EU833215
		99	100	0	<i>Penicillium citrinum</i>	EU833214
FJ708601	<i>Sordariomycete</i> sp.	98	100	0	Uncultured fungus	AJ875386
		86	64	2E-107	Nectriaceae sp.	EF060504
		86	64	2E-106	<i>Lasiosphaeria lanuginosa</i>	EF060504
FJ708599	<i>Tetracladium</i> sp.1	99	96	0	<i>Tetracladium marchalianum</i>	AY204620
		99	100	0	<i>Tetracladium breve</i>	EU883418
FJ708600	<i>Tetracladium</i> sp.2	98	100	0	<i>Tetracladium setigerum</i>	FJ000374
		98	100	0	<i>Tetracladium palmatum</i>	FJ000372
FJ708598	Tricholomataceae	88	52	5E-99	<i>Gymnopus contrarius</i>	DQ486708
		91	43	3E-96	<i>Crinipellis zonata</i>	AY916692
		84	62	1E-94	<i>Laccaria</i> cf. <i>bicolor</i>	DQ658853

te in roots of the orchid *Dendrobium nobile* (Yuan *et al.*, 2009). In case of species of the genus *Tetracladium*, usually they have been reported as aquatic hyphomycetes, however, they have been also found within plant tissues by cloning and sequencing (Selosse *et al.*, 2008). Recently, sequences

of 98% similar or higher with our *Helotiales* species have been found from multiple locations around the world. These fungi seem to be always endophytes on a range of host organisms including other fungi (Zhang *et al.*, 2010) and non-orchid plants (Neubert *et al.*, 2006; Brevik *et al.*, 2010). They

Table 2. Presence of endophytic fungi in different *Laelia speciosa* tissues and developmental stages. Two plants and *in vitro* seedlings from different populations were sampled. N°C: Number of clones; R: Roots; C: Capsules; S: Seeds, Iv S: *In vitro* Seedlings. 1 means present in one sample and 2 means present in two samples.

Species	N°C	R	C	S	Iv S	Habit
<i>Alternaria longissima</i>	2		1	1		plant parasite
<i>Alternaria</i> sp.	1		1			plant parasite
Atractiellales sp.	2	1				
Basidiomycete	2	1				
<i>Curvularia affinis</i>	2	1				parasite on Poaceae
<i>Cylindrocarpon pauciseptatum</i>	6	1				plant parasite
<i>Fusarium solani</i>	8	1				plant parasite
Helotiales sp.	17	2	2	2	2	plant endophyte
Hypocreales sp.	3		1			plant parasite
Lasiosphaeriaceae sp.	2		1			saprobe
<i>Myrmecridium schulzeri</i>	3		1			generalist parasite in plants and humans
Nectriaceae	2	1				plant parasite
<i>Neonectria</i> sp.	2		1			plant parasite
<i>Penicillium</i> sp.	2				1	common saprobe and facultative parasite
Sordariomycete sp.	5		1			
<i>Tetracladium</i> sp.1	5	1				fresh water saprobe
<i>Tetracladium</i> sp.2	3		2			fresh water saprobe
Tricholomataceae	2	1			1	mycorrhizal or saprobic

are mostly found in orchid roots of *Cephalanthera longifolia* (Abadie *et al.*, 2006), *C. damasonium* (GU327459, Malinova *et al.*, unpublished), and *Cypripedium* spp. (Shefferson *et al.*, 2005). Although, these sequences have been individually reported as occasionally occurring root endophytes, the combined data suggest that this clade of Helotiales is a group of fungi with an unknown ecological role, particularly in orchids (Herrera *et al.*, 2010).

Endophytic fungi interact with the whole plant of *Laelia speciosa* along its life cycle. Roots contained the richest diversity of endophytic fungi, particularly among parasitic lineages, but also with some saprobic and endophytic ones. Roots are a common substrate for fungal colonization and many studies have together shown around 450 different fungal taxa on orchid roots (Currah *et al.*, 1997). Contrary to the common belief on the sterility of orchid capsules (McKendrick *et al.*, 2000) we found 50% of fungal species inside the capsule tissue. Given that we found in seeds just two fungal species, it seems that the capsule does not provide a sterile environment, although it can be an effective barrier against fungal pathogens. The two plants of *L. speciosa* examined from different populations carried in their seeds, one Helotiales species and one *Alternaria* species. The first belongs to an order with common endophytic fungi, which in many cases have been interpreted as mycorrhizal (Jumpponen, 2001; Tedersoo *et al.*, 2009, 2010). The second is an aggressive parasite and supports our observations of a 24% of dead seeds within capsules (Ávila-Díaz, 2007). Seedlings cultivated *in vitro* harbored three fungal taxa (the Helotiales species, a Tricholomataceae species and a *Penicillium* spe-

cies). The first two were found also in roots of *L. speciosa*. The Helotiales species was found in all tissues (i.e. roots, capsules, seeds) and *in vitro* seedlings in both samples from different populations (Table 2).

Although a number of clones may bias abundance estimates because DNA fragments are ligated into a vector plasmid with possibly differential efficiencies (van Elsas and Boersma, 2011), it is sometimes used as a rough indirect measure of fungal DNA concentration in plant tissues and environmental samples (Tao *et al.*, 2008; Fitzsimons and Miller, 2010). The Helotiales species was the OTU with the highest clone abundance in the two mature plants and in both *in vitro* seedlings; it was sequenced from the 23.9% of clones (Table 2). Its DNA was particularly recovered from root and seed samples. This evidence indicates that this species of fungus is abundant in *Laelia speciosa* tissues.

It has been assumed that orchid seeds are free of endophytic fungi (Warcup, 1985). Seeds of epiphytic orchids are able to germinate in simple media that contain minerals and C sources under the assumption that fungi are not present (Arditti *et al.*, 1990). The results of this study demonstrate that *Laelia speciosa* carries endophytic fungi within its seeds. Our data further demonstrate that fungi are also present in healthy *in vitro* seedlings grown from seeds from the same population. Endophytic fungi have also been found in seeds of several plant groups including Fabaceae (Braun *et al.*, 2003), Pinaceae (Ganley and Newcombe, 2006), and Poaceae (Latch *et al.*, 1987; Ernst *et al.*, 2003; Schardl *et al.*, 2004).

Data of endophytic fungi in seeds and seedlings of *Laelia speciosa* would contradict the theory of “axenic” *in vitro*

culture of this orchid and could have significant implications for its international trade, *in vitro* propagation, management practices and restoration projects. To further understand the biological importance of this fungal association it will be necessary to explore its physiology, ecology, and adaptive traits as well as its occurrence in other epiphytic orchids.

Acknowledgements

We thank to A. C. Cortés-Palomec for comments to preliminary drafts of this manuscript, A. Valencia and H. Ferreira for their technical assistance and M. García and C. Garibay for their assistance in molecular procedures. We are grateful to two anonymous reviewers who suggested improvements to the manuscript. KO was supported by Fondo Mexicano para la Conservación de la Naturaleza grant FMCN, A 1-99/130 and UNAM grant SDEI-PTID-02. IA-D was supported by Coordinación de la Investigación Científica, Universidad Michoacana de San Nicolás de Hidalgo. RGO was funded by Fondo Mixto CONACYT-Michoacán grant Mich-2009-C05-112966.

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Received: September 30th, 2012

Accepted: March 21st, 2013