San Diego County Orchid Society

Molecular DNA analysis of cultivatable endophytic fungi isolated from the roots of epiphytic orchids in the region of Soconusco, Chiapas, México, for use in orchid restoration and pest control.

Dr. Anne Damon (responsable)

Dr. Aucencia Emeterio Lara (postdoc.)

M. Sc. Fabiola Hernández Ramírez (Ph.D. student. AOS funding)

M. Sc. Trinidad Aguilar Diaz (Ph.D. student)

El Colegio de la Frontera Sur (ECOSUR)

Carretera Antiguo Aeropuerto km 2.5

Tapachula,

Chiapas, México, C.P. 20700

adamon@ecosur.mx

A. Introduction

Epiphytes are a group of plants that grow on other plants (phorophyte or host), usually trees, with no contact with the vascular system of the phorophyte, i.e. they are not parasites, they only use the phorophyte as support for their growth, development and reproduction, and cause no direct damage (Ticktin et al., 2016; Zotz, 2016). The group includes vascular plants such as members of the families Orchidaceae, Bromeliaceae, Piperaceae and Ericaceae, amongst others (Zotz, 2013) and also includes non-vascular plants such as bryophytes (Marchantiophyta, Anthocerotophyta and Bryophyta) (Liepiņa, 2012). The majority are obligate epiphytes, whereas others may be facultative and grow and reproduce on other, natural substrates such a rocks, or man-made structures such as fence posts (Zotz, 2016). Despite numerous adaptations to living, effectively, in the air and disconnected from the soil, epiphytes are often limited in terms of water and nutritional resources, compared to terrestrial plants. To overcome such deficiencies, epiphytes are characterized by morpho-physiological and functional adaptations (Cach-Pérez et al., 2018; Leroy et al., 2013), for example, many orchids have roots that are covered in a spongy layer of cells called the velamen, that allows

rapid absorption of water and nutrients, and have pseudobulbs and leaves with thick, waxy cuticles that store water and nutrients to be used when needed, and also resist desiccation and herbivory (Zhang et al., 2018). In the case of bromeliads, the leaves are covered with trichomes, which also permit storage of water and nutrients, and many species develop a tank-like structure designed to capture and store water, leaf litter, and other organic matter (Cach-Pérez et al., 2018; Leroy et al., 2013). The gametophytes of bryophytes absorb water like a sponge and enter into latency when conditions become too extreme (Tacoronte et al., 2009). These characteristics are well known to have permitted epiphytes to perpetuate in forests and jungles, but little attention has been paid to the microorganisms that also play an important role in the success of these plants.

Endophytic fungi (EF) are microorganisms that live in plant tissues without causing disease (Aly et al., 2011), although the term is somewhat ambiguous, due to the fact that endophytes can be further classified as: neutral, commensalist, mutualist and also parasitic (Aly et al., 2011; Rodriguez et al., 2009), and in some cases may change roles during different stages of the lifecycle, or due to biotic o abiotic signals. Only a minority of these fungi are parasitic and damage their plant hosts, whereas the majority benefit their plant hosts, performing important functions such as the solubilization of phosphate, production of indole-3-acetic acid (IAA), the production of various metabolites and siderophores, and acting in some way to reduce abiotic o biotic stress (Aly et al., 2011; Rodriguez et al., 2009). Endophytic fungi are of vital importance for epiphytic plants in an environment where resources are limited and could be a useful tool for the restoration of populations of orchid species in protected or other safe areas.

For orchids, the EF are found principally in the roots, are termed mycorrhizae forming endophytic fungo (MFEF), and usually belong to the form-genus *Rhizoctonia*, that provide nutrients to the orchid host during different phases of the lifecycle. Various studies indicate that bromeliads also associate with endophytic fungi, they have indeed been isolated from bromeliad tissue, but little is known about the interactions and possible functions in that context. Similarly, in the case of mosses, endophytic fungi have also been isolated from plant tissues, but little is known about how they may interact with the bryophytes. Groups of

epiphytes from different taxonomic groups are often found in close association, which may indicate the sharing of EF, or at least some of the products and services offered by these fungi.

GENERAL OBJECTIVE

Molecular DNA analysis of cultivatable endophytic fungi (EF) isolated from the roots of epiphytic orchids, subsequently selected for potential use for *in vitro* symbiotic propagation, symbiotic acclimatization of vitroplants, and pest control protocols. We intend to simplify and adjust protocols for the use of EF in rustic laboratories in rural areas as a contribution towards the conservation of native epiphytic orchids in the biodiverse Soconusco region. To that end, we continue to focus on traditional coffee plantations, and now also cocoa plantations as potential sites for the restoration of decimated epiphytic orchid populations.

SPECIFIC OBJECTIVES

Objective 1

Molecular DNA analysis and characterization of the cultivable EF isolated from *Guarianthe skinneri* (Orchidaceae) in the process of evaluation a) as biocontrol agents against pathogenic fungi and effect in protocorms and vitroplants and b) for their contribution in symbiotic, rustic or in situ propagation and acclimatization protocols; in both cases as a component of the strategy for the conservation of this endangered orchid. Here we include a table with the information on the origin of the epiphytic plant samples from which the EF were isolated. (This is the work carried out by postdoc. Aucencia Emeterio and Ph.D. student Fabiola Hernández).

Table 1. Endophytic fungi samples isolated from the roots of *Guarianthe skinneri*

Plant	**Season**	**Habitat**	**No. isolated strains**	**Strains selected for molecular DNA analysis**
a) *Guarianthe skinneri*	Wet	Botanical Garden 80 m	20	17
b) *Guarianthe skinneri*	Dry		10	3

Objective 2

Molecular DNA analysis and characterization of the cultivable EF isolated from the roots of *Brassia verrucosa* (Orchidaceae).

This is related to the ongoing study (by PH.D. student Trinidad Aguilar) of EF shared by *B. verrucosa*, *Tillandsia guatemalensis* (Bromeliaceae) and moss (Diacranaceae) to be evaluated as potential promoter inoculates for in vitro and in situ germination and development and, in the case of vitroplants, their subsequent acclimatization in suitable sites. In this report we only present the results of the determination of the EF of *B. verrucosa*

Table 2. Endophytic fungi simples isolated from the roots of *Brassia verrucosa* (Orchidaceae)

Plant	Season	Habitat	No. isolated strains	Strains selected for molecular DNA analysis
Brassia verrucosa	Dry and rainy seasons	Coffee plantations and conserved mountain forest at 1,600 m	227	10

Methods

Objective 1: **Molecular DNA analysis and characterization of the cultivable EF isolated from *Guarianthe skinneri* (Orchidaceae)**

The orchid *Guarianthe skinneri* (Bateman) Dressler & Higgins, with attractive mauve flowers, nowadays survives in few natural small populations in forest fragments in Mexico and Central America. This orchid is vulnerable due to illegal extraction and habitat reduction, and it has been placed in the "Threatened" category of the Official Mexican Standard NOM-059-SEMARNAT-2010 (Semarnat, 2010). *Guarianthe skinneri* is grown in the UMA (Environmental Management Unit for endangered species; SEMARNAT, Mexico) of Santa Rita de las Flores (520 masl) (municipality of Mapastepec, Chiapas) (https://es-la.facebook.com/orquisustentable .Santa Rita/). In this scenario, a fungal disease, originally present on a small scale in the wild population, became more frequent within the orchid

galleries, where the plants are grouped in a density greater than that found in nature, and at first, plastic, a black shade net was used, which increased humidity and reduced air flow. The disease causes symptomatic black lesions, causes the death of many of the new shoots and generally reduces growth and vigor and reduces the number of flowers produced. The causative agent of the disease was identified as *Lasiodiplodia theobromae*. In the Jardin Etnobiológico de las Selvas del Soconusco (JESS) (80 masl) orchid collection in Tuzantán, *G. skinneri* is grown without symptoms of the disease. With the purpose of isolating the endophyte communities, roots, leaves and pseudobulbs of wild *G. skinneri* plants were isolated, comparing healthy plants with those infected by *Lasiodiplodia*, and studying the interactions of isolates of these fungi and evaluating their potency as antagonists of the *Lasiodiplodia* pathogenic fungus. Likewise, the effects of the interaction between protocorms and asymbiotic *G. skinneri* vitroplants with endophytic fungi isolated from roots, leaves and pseudobulbs of healthy and infected *G. skinneri* plants were evaluated.

Faced with the problems cited, we sought to design a rustic, symbiotic, acclimatization protocol for *G. skinneri* vitroplants aiming towards achieving reintroduction under natural conditions. To achieve this, we need to understand the role of endophytic fungi associated with *G. skinneri* in relation to their potential as biocontrol agents for fungal diseases, as well as their participation in other processes needed for survival during acclimatization in natural environments.

Objective 2: **Molecular DNA analysis and characterization of the cultivable EF isolated from the roots of *Brassia verrucosa* (Orchidaceae)**

In the Ejido Benito Juárez el Plan, Cacahoátan, Chiapas, México, at 1,600m on the slopes of the Tacaná Volcano, a variety of epiphytes are to be found growing on arabic coffee bushes and trees, both separately and in apparent groups or communities. The orchid *Brassia verrucosa* Bateman ex Lindl., is frequently found growing alongside the bromeliad *Tillandsia guatemalensis* and the moss belonging to the family Dicranaceae.

This study set out to isolate endophytic fungi from the three different classes of plants that are frequently found living in close association in the experimental site: *Brassia verrucosa* (Orchidaceae), *Tillandsia guatemalensis* (Bromeliaceae) and the moss (family Dicranaceae)

which are found on different types of phorophytes in the coffee plantations on the slopes of the Tacaná Volcano.

The endophytic fungi were evaluated, using standard biochemical tests, for the capacity to solubilize phosphate, produce indoleic acid and degrade tannic acid, in all cases under in vitro conditions. The fungi with capacity for one or more of these functions were referred for further testing and sequencing.

In this report, we only include the results of the sequencing of EF isolated from the roots of the orchid, *Brassia verrucosa*.

Molecular analysis

The samples from both objectives were analyzed using the following procedures.

Total nucleic acids were extracted from selected fungal strains grown for 7 days on PDA, using the method adapted from Karthikeyan et al. (2010), and at the end of the procedure the DNA was resuspended in 50 µl of elution buffer. The extracted nucleic acids were visualized by means of electrophoresis in 0.8% agarose gels, in which 5µl of a total of 50µl of the extraction were used. 1µl of DNA was used to carry out the PCR for two target genes. The PCRs were carried out with a final volume of 20µl, using PCR Master Mix 2X, Promega brand (Cat. No. M7502. Wisconsin, USA), at a final concentration of 1X and 0.2 pM of each primer. For the first, the primers ITS-4 and ITS-5 were used for a region of the 18S ribosomal gene that covers the entire interspace region (ITS), which has the following sequence: ITS-4 (TCCTCCGCTTATTGATATGC), ITS-5 (GGAAGTAAAAGTCGTAACAAGG) (White et al. 1990). PCR for ITS was performed in a BioRad T100® thermal cycler, with initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 1 min, alignment at 55 °C for 1 min, and extension at 72 °C for 1 min; with the final extension of the cycle at 72 °C for 5 min.

The second target was a part of the translation elongation factor 1-alpha gene EF1-α, using the following primers: EF1-983F (GCY CCY GGH CAY CGT GAY TTY AT), and EF1-2218R (ATG ACA CCR ACR GCR ACR GTY TG) (Rehner & Buckley, 2005). The protocol for the amplification of the EF1α gene corresponds to a Touchdown PCR and was as follows:

first initial denaturation cycle at 94 °C for 3 min, 10 denaturation cycles at 94 °C for 30 s, alignment at 68 °C for 30 s, decreasing 1 °C / cycle, with extension 72 °C for 1 min; followed by 30 cycles (denaturation 94 °C for 30 s, alignment 58 °C for 30 s, extension 72 °C for 1 min, and final extension at 72 °C for 5 min.

PCR results were analyzed on 1% agarose gel. For all strains, the single PCR product of approximately 700 bp and 1100 bp for EF1 were purified using. the GenElute™ PCR Clean-Up Kit (Cat. No. NA1020, Sigma-Aldrich, M.O, USA). All clean products were verified by 1% agarose gel electrophoresis, using 3 of 40µl of reaction to load the gel. The PCR product was purified, according to the manufacturer's instructions, with the Quantum Prep® (ITS) kit and GENCLEAN II® (EF1-α). After electrophoresis, they were visualized with ultraviolet light. The gel was visualized on a ® Kodak Gel Logic 200 Imaging System transilluminator. PCR products were sent to Macrogen, Inc. in Seoul, South Korea for sequencing. The obtained sequences were reviewed and compared with the obtained electropherograms to determine the purity and reliability of the signal. Subsequently, they were analyzed using BLAST algorithms from the National Center of Biotechnology Information (NCBI BLAST) database and the BOLD (Barcode of Life Data) system.

RESULTS

Objective 1

1a) A selection of endophytic fungi isolated from adult plants of *Guarianthe skinneri*, was made for confrontation against *Lasiodiplodia theobromae*, pathogenic fungus causative agent of the black spot in pseudobulbs of *G. skinneri*, and interactions were set up to evaluate the effect of these EF on protocorms and vitroplants of *G. skinneri* under *in vitro* conditions. In total, 17 fungal strains were selected, for showing antagonistic potential against the pathogen, and for being asymptomatic in the interaction with the different stages of development of plants of *G. skinneri*. Fungal strains and fungal identities are shown in Table 3.

1b) We established a rustic protocol for symbiotic acclimatization of vitroplants for the reintroduction of *Guarianthe skinneri* into semi-natural habitat. The vitroplants were inoculated with 3 selected strains of endophytic fungi isolated from mature *G. skinneri* plants and their survival response was evaluated in three phases, after one year.

Table 3. Identification of endophytic fungal strains, isolated from plants of *Guarianthe skinneri*, using the ITS and EF1-α markers (identity/similarity percentage > 90%).

Strains	Marker	Blast Results	Score	% Cover	% Identify	No. Access
TIR2-1.1	ITS	*Trichoderma harzianum* isolate 11-TTR-2	1133	99	100	MT341774.1
	EF1-α	*Trichoderma* aff. *harzianum* MO-2014 strain TRS76	896	99	99.39	KP008903.1
T1R3-1.1	ITS	*Phyllosticta capitalensis* strain LCM 826.01	1170	98	100	MF495391.1
	EF1-α	*Neoscytalidium dimidiatum* strain DSM 109897	806	99	97.07	MN447201.1
T1R3-1.2	ITS	*Trichoderma harzianum* isolate 11-TTR-2	1123	100	99.84	MT341774.1
	EF1-α	*Trichoderma breve* isolate TWS48Abf(b)	861	98	99.79	MN389578.1
T1R3-3.1	ITS	*Pseudopestalotiopsis theae* strain LCM985.01	968	99	98.55	MF495464.1
	EF1-α	*Pseudopestalotiopsis theae* strain MING01	889	97	99.79	MH479105.1
T2R5-5.1	ITS	*Trichoderma lentiforme* strain C. C. Lee AgF2-1	1110	99	99.51	MH796354.1
	EF1-α	*Trichoderma breve* isolate TWS48Abf(b)	865	100	99.58	MN389578.1
T3R1-2.1	ITS	Undetermined				
	EF1-α	*Xylaria acuta isolate* AFTOL-ID 63	723	99	99.93	DQ471048.1
T3R1-5.1	ITS	Fungal sp. ARIZ B314	1107	100	99.51	FJ612983.1
	EF1-α	*Daldinia vernicosa*	710	99	93.67	XM_048008080.1
T3R3-4.1	ITS	*Purpureocillium lilacinum* isolate KoLRI_053291	1098	100	99.83	MZ855443.1
	EF1-α	*Purpureocillium lilacinum* isolate tef NH632	869	100	99.58	LC375363.1
T3R5-5.1	ITS	*Pertusariales* sp. isolate BP0213	1157	99	98.92	MW0459861
	EF1-α	*Hypoxylon fragiforme* isolate AC1102	723	92	96.36	KJ76414.1
T7R2-2.2.1	ITS	Undetermined				
	EF1-α	*Astrocystis mirabilis* culture MFLUCC:11-0636	730	100	93.66	KU940207.1
T7R2-5.1.2	ITS	*Hypocrea lixii* isolate NRCfBA-46	1151	99	99.68	JF923807.1
	EF1-α	*Trichoderma breve* isolate TWS48Abf(b)	880	99	100	MN389578.1
T7R4-2.2	ITS	*Xylaria multiplex* strain DSM 110363	1079	99	99.83	MN833802.1
	EF1-α	*Xylaria* sp. isolate con44a	791	99	96.64	MK202799.1
T7R4-4.2.2	ITS	*Entonaema pallida* isolate 46i	1044	99	99.31	MZ270663.1
	EF1-α	*Xylaria bambusicola*	693	99	92.4	XM_0479784211

T8R1-3.1	ITS	*Fusarium proliferatum* strain TH12-5	1002	100	100	MT560218.1
	EF1-α	*Fusarium musae* strain CBS:624.87	872	99	99.17	MT010991.1
T9R4-4.1	ITS	Fungal endophyte culture-collection STRI:ICBG-Panama:TK1262	989	91	99.81	KF436186.1
	EF1-α	*Xylaria bambusicola*	638	99	90.78	XM_0479784211
T9R5-5.1.4	ITS	Fungal sp. voucher Robert L. Gilbertson Mycological Herbarium 1480	1044	97	99.48	KT895611
	EF1-α	*Xylaria* sp. isolate con44a	734	99	94.53	MK202799.1
L01	ITS	*Lasiodiplodia theobromae* strain C.C. Lee AgF3-39	1005	100	100	MH793582.1
	EF1-α	Select seq *Lasiodiplodia theobromae* isolate Caceres MT	603	90	98.53	KP642037.1

Ib. cont.

Fungal identity

From the amplifications using the ITS-4 and ITS-5 primers, 665bp of sequence fragments were obtained per strain 1; 697bp for strain 2, and 560bp for strain 3. For the amplifications of the EF1 primer, 838bp of sequence fragments were obtained for strain 1, 1103bp for strain 2 and 831bp for strain 3. (Table 4)-

The ITS sequences obtained for strain 1 showed the highest percentage of identity with the genus *Nigrospora* with 97.8% according to the NCBI database (Accession Number NR_174838.1) and 99.8 in BOLD (Accession Number FJ527872.1); in the same way with the first EF1 with 96.3% in the NCBI (Accession Number MK336057.1).

For strain 2, the ITS sequences obtained showed the highest percentage of identity with the genus *Coprinellus* with 91.7% according to the NCBI database (Accession Number NR_172438.1) and 98.1% with BOLD (Accession Number HQ248226.1); in the same way with the first EF1 with 92.5% in the NCBI (Accession Number KJ732822.1).

For strain 3, the ITS sequences obtained showed the highest percentage of identity with the *Fusarium* genus with 99.2% according to NCBI (Accession Number NR_111889.1) and 100% with BOLD (Accession Number KC254038.1); in the same way with the first EF1 with 93.4 % in the NCBI (Accessions Numbers XM_031232653.1, MH828026.1).

Table 4. Identity of endophytic fungal strains, isolated from the roots of mature plants of *Guarianthe skinneri*, according to the National Center of Biotechnology Information (NCBI BLAST) and BOLD (Barcode Of Life Data) system, using the ITS and EF1-α markers (identity/similarity percentage > 91%).

Strains PCR	Data Base	Result	Identity/ Similarity (%)	Accesion Number
Strain 1				
ITS	NCBI Blast	*Nigrospora macarangae MFLU 18-2518 ITS region; from TYPE material*	97.78	NR_174838.1
	BOLD Systems	*Nigrospora sphaerica*	99.81	FJ527872.1
EF1-α	NCBI Blast	*Nigrospora sp. ZZ-2018a strain LC12441 translation elongation factor 1-alpha (TEF1a) gene, partial cds*	96.29	MK336057.1
Strain 2				
ITS	NCBI Blast	*Coprinellus magnoliae MFLUCC 18-0942 ITS region; from TYPE material*	91.70	NR_172438.1
	BOLD Systems	*Coprinellus sp. M-15*	98.1	HQ248226.1
EF1-α	NCBI Blast	*Coprinellus sp. 4 EL-2013 isolate 10216 translation elongation factor 1-alpha gene, partial cds*	92.46	KJ732822.1
Strain 3				
ITS	NCBI Blast	*Fusarium fujikuroi CBS 221.76 ITS region; from TYPE material*	99.2	NR_111889.1
	BOLD Systems	*Fusarium proliferatum*	100	KC254038.1
EF1-α	NCBI Blast	*Fusarium proliferatum ET1 translation elongation factor 1 alpha (FPRO_09277), partial mRNA.*	93.40	XM_031232653.1
		Fusarium fujikuroi isolate TX75 translation elongation factor 1-alpha (EF-1 alpha) gene, partial cds.	93.40	MH828026.1

Objective 2

Please note that, although we isolated EF from the orchid, a bromeliad and a moss, we used the San Diego funding for sequencing the selected EF of the orchid only.

The 10 EF were selected from the fungi isolated from the roots of *Brassia verrucosa*, for their ability to solubilize inorganic phosphate, the production of indole acetic acid and/or the degradation of tannic acid. Table 5 shows the selected strains with their labels and species names according to data from NCBI BLAST and BOLDSYSTEN.

The strain R1-BV-R-C18 with the ITS marker presented a fit of 99.22% similarity to *Colletotrichum vietnamense* CBS 125478, and with the EF1, 97.46% similarity to *Colletotrichum* sp. 3386.

The strain R1-BV-R-C14 with the ITS marker, gave 99.86% similarity to *Aspergillus foetidus* CBS 121.28 and with the EF1 primers, 85.76% related to *Aspergillus puulaauensis* MK2.

The R1-BV-R-C12 strain with the ITS marker, amplification could not be achieved was obtained and therefore, no result was obtained, but with the EF1 primers it presented 98.70% similarity to *Camarops ustulinoides* isolate AFTOL-ID 72.

The R1-BV-R-C13 strain with the ITS marker, when analyzed in the databases, presented 95.55% similarity to *Diaporthe humulicola* UAMH 12076 and with the EF1 primers it presented 96.88% similarity to *Diaporthe batatas.*

The R1-BV-R-C26 strain with the ITS primers was 98.74% related to *Aspergillus versicolor* ATCC 9577 and with the ITS primers no response was obtained.

For R2-BV-R-C11 strain with the ITS primers, 100% similarity was obtained *Colletotrichum cigar* ICMP 18539 and with the EF1 primers 99.36% related to *Colletotrichum* sp. 7767.

The strain R2-BV-R-C5 with the ITS primers, gave 100% similarity to *Colletotrichum kahawae* ICMP 17816 and with the primers EF1 98.56% related to *Colletotrichum chrysophilum*.

Regarding the strain R2-BV-R-C8 with the ITS primers, 100% coincidence with *Colletotrichum cigar* ICMP 18539 was obtained, and with the Primers EF1 98.74% similarity to *Colletotrichum chrysophilum*.

The R2-BV-R-C6 strain with the ITS primers was 100% similar to *Colletotrichum cigar* ICMP 18539, and with the EF1 primers 99.36% similar to *Colletotrichum* sp. 7767.

Finally, the R1-BV-R-C15 strain with the ITS primers gave 84.94% similarity to *Xylaria ellisii* DAOMC 252031, and with the EF1 primers a similarity of 92.23% was obtained with *Xylaria acuta* isolate AFTOL-ID 63.

Table 5. Identification of endophytic fungi isolated from the roots of *Brassia verrucosa* (Orchidaceae) with the molecular markers ITS and EF1.

Cepa	PCR	Data Base	Resultado Blast	Score	% Cobertura	% Identidad	No. Acceso
R1-BV-R-C18	ITS	*NCBI BLAST*	*Colletotrichum vietnamense* CBS125478	959	92	99.25	NR_132058.1
	EF1	*NCBI BLAST*	*Colletotrichum* sp. 3386	806	97	97.46	GU994509.1
R1-BV-R-C14	ITS	*NCBI BLAST*	*Aspergillus foetidus* CBS 121.28	1096	99	99.86	NR_163668.1
	EF1	*NCBI BLAST*	*Aspergillus puulaauensis* MK2	339	94	85.76	AP024446.1
R1-BV-R-C12	ITS						
	EF1	*NCBI BLAST*	*Camarops ustulinoides* isolate AFTOL-ID 72	817	95	98.70	DQ471050.1
R1-BV-R-C13	ITS	*NCBI BLAST*	*Diaporthe humulicola* UAMH 12076	924	100	95.55	172855.1
	EF1	*NCBI BLAST*	*Diaporthe batatas*	804	99	96.88	XM_044789722.1
R1-BV-R-C26	ITS	*NCBI BLAST*	*Aspergillus versicolor* ATCC 9577	987	97	98.74	NR_131277.1
	EF1						

R2-BV-R-C11	ITS	*NCBI BLAST*	*Colletotrichum cigarro* ICMP 18539	1066	100	100	NR_120138.1
	EF1	*NCBI BLAST*	*Colletotrichum* sp. 7767	846	96	99.36	GU994490.1
R2-BV-R-C5	ITS	*NCBI BLAST*	*Colletotrichum kahawae* ICMP 17816	1057	99	100	NR_144787.1
	EF1	*NCBI BLAST*	*Colletotrichum chrysophilum*	857	99	98.56	XM_053174744.1
R2-BV-R-C8	ITS	*NCBI BLAST*	Colletotrichum cigarro ICMP 18539	1051	98	100	NR_120138.1
	EF1	*NCBI BLAST*	*Colletotrichum chrysophilum*	874	99	98.74	XM_053174744.1
R2-BV-R-C6	ITS	*NCBI BLAST*	*Colletotrichum cigarro* ICMP 18539	1057	99	100	NR_120138.1
	EF1	*NCBI BLAST*	*Colletotrichum* sp. 7767	854	96	99.36	GU994490.1
R1-BV-R-C15	ITS	*NCBI BLAST*	*Xylaria ellisii* DAOMC 252031	520	99	84.94	NR_172972.1
	EF1	*NCBI BLAST*	*Xylaria acuta* isolate AFTOL-ID 63	675	99	92.23	DQ471048.2

Final Products

- Sequences, and total or partial identification, were obtained for 30 fungal strains isolated from the roots of *Guarianthe skinneri* (17 and 3) and *Brassia verrucosa* (10).

Objective 1a.

- Chapter V (of Ph. D. thesis). "Effect of endophytic fungi on protocorms and vitroplants of *Guarianthe skinneri* and its potential for biological control.
- PhD thesis (to be finalized 2023) entitled: "Interaction between endophytic fungi and *Lasiodiplodia theobromae*, pathogenic fungus of *Guarianthe skinneri* (Bateman) Dressler & Higgins, in Soconusco, Chiapas."

Objective 1b.

- Paper submitted to the Journal for Nature Conservation on June 25th, 2023. (Proof of submission to scientific journal and DNA results are attached).
  Title: "Symbiotic acclimatization and reintroduction of *Guarianthe skinneri*, a threatened, native orchid of cultural value in southern Mexico.

Objective 2.
Paper o be submitted to the Journal Frontiers in Fungal Biology, edition Fungi-Plant Interactions.

- Title: "Characterization of endophytic fungi isolated for the epiphytic plants: *Brassia verrucosa* (Orchidaceae), *Tillandsia guatemalensis* (Bromeliaceae) and a moss (family Dicranaceae), growing closely associated on phorophytes in a coffee plantation in southern Mexico"

Part of an ongoing PhD thesis.

Bibliography

Aly, A. H., Debbab, A., & Proksch, P. (2011). Fungal endophytes: unique plant inhabitants with great promises. *Applied Microbiology and Biotechnology 2011 90:6*, *90*(6), 1829–1845. https://doi.org/10.1007/s00253-011-3270-y

Cach-Pérez, M. J., Andrade, J. L., & Reyes-García, C. (2018). Morphophysiological Plasticity in Epiphytic Bromeliads Across a Precipitation Gradient in the Yucatan Peninsula, Mexico. *Tropical Conservation Science*, *11*, 1–10. https://doi.org/10.1177/1940082918781926

Karthikeyan, V., Patharajan, S., Palani, P., Spadaro, D., Gullinoi, M. ., & Garibald, A. (2010). Modified Simple Protocol for Efficient Fungal DNA Extraction Highly Suitable forC PR Based Molecular Methods. *Global Journal of Molecular Sciences*, *5*(1), 37–42.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, *16*(2), 111–120. https://doi.org/10.1007/BF01731581

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular biology and evolution*, *33*(7), 1870–1874. https://doi.org/10.1093/molbev/msw054

Leroy, C., Carrias, J., Corbara, B., Pélozuelo, L., Dézerald, O., Brouard, O., Dejean, A., & Céréghino, R. (2013). Mutualistic ants contribute to tank-bromeliad nutrition. *Annals of Botany*, *112*, 919–926. https://doi.org/10.1093/aob/mct147

Liepiņa, L. (2012). Occurrence of fungal structures in bryophytes of the boreo-nemoral zone. *Environmental and Experimental Biology*, *10*, 35–40.

Rehner, S. ., & Buckley, E. (2005). A Beauveria phylogeny inferred from nuclear ITS and EF1-α sequences: Evidence for cryptic diversification and links to Cordyceps teleomorphs. *Mycologia*, *97*(1), 84–98. https://doi.org/10.1080/15572536.2006.11832842

Rodriguez, R., White, J., Arnold, A., & Redman, R. (2009). Fungal endophytes: diversity and functional roles. *New Phytologist*, *182*(2), 314–330. https://doi.org/10.1111/j.1469-8137.2009.02773.x

Tacoronte, B., León, V., Olivo, A., & Vielma, M. (2009). Crecimiento in vitro de musgos del bosque nublado andino de Venezuela. *Revista Forestal Latinoamericana*, *24*(2), 69–89.

Ticktin, T., Mondragón, D., & Gaoue, O. G. (2016). Host genus and rainfall drive the population dynamics of a vascular epiphyte. *Ecosphere*, *7*(11), 1–13. https://doi.org/10.1002/ecs2.1580

White, T., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. En *Innis M, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic, New York* (pp. 315–322).

Zhang, S., Yang, Y., Li, J., Qin, J., Zhang, W., Huang, W., & Hu, H. (2018). Physiological diversity of orchids. *Plant Diversity*, *40*(4), 196–208. https://doi.org/10.1016/j.pld.2018.06.003

Zotz, G. (2013). The systematic distribution of vascular epiphytes - a critical update. *Botanical Journal of the Linnean Society*, *171*, 453–481. https://doi.org/10.1111/boj.12010

Zotz, G. (2016). Plants on plants – the biology of vascular epiphytes. En *Springer International Publishing* (pp. 1–283). https://doi.org/10.1007/978-3-319-39237-0