Manila Journal of Science 10 (2017)*, pp. 87-100*

Diversity and Phylogenetic Relationships Among Isolated Root Symbiotic Fungi from *Drynaria quercifolia* L. in La Union, Philippines

Jomar L. Aban[1*,] Roland M. Hipol[2], Teodora D. Balangcod[2], Rosemary M. Gutierrez[2], Racquel C. Barcelo[3], Evelyn E. Oda[3], and Gaudelia A. Reyes[3]

[1] Graduate Programs, Department of Biology, School of Natural Sciences, Saint Louis University, Baguio City 2600, Benguet, Philippines
[2] Department of Biology, College of Science, University of the Philippines, Baguio City 2600, Benguet, Philippines
[3]Department of Biology, School of Natural Sciences, Saint Louis University, Baguio City 2600, Benguet, Philippines

*Corresponding author: jomar_d2@yahoo.com

ABSTRACT

Drynaria quercifolia is an epiphytic fern often exposed to water- and light-stressed environments. One distinct ecophysiological adaptation of epiphytic ferns is their symbiotic relationship with fungi. This is the first study undertaken to explore the phylogenetic relationship, colonization, occurrence rate, and diversity of RSF found in *D. quercifolia*. Two hundred seventy-eight RSF isolates were collected from 300 representative root segments. Genomic DNA of the RSF was extracted, and the ITS (internal transcribed spacer) region of the 18S ribosomal DNA (rDNA) was sequenced. Thirteen species were recorded. Eight of the 13 RSF were identified up to the species level using the Basic Local Alignment Search Tool nucleotide search program (BLASTn) to their closest type match available on the databank of NCBI. However, five RSF were undescribed. The phylogenetic relationship of RSF was determined using Molecular Evolutionary Genetics Analysis (MEGA6), and four distinct monophyletic groups were formed: Sordariomycetes, Eurotiomycetes, Saccharomycetes, and Mucoromycotina. The computed colonization rate (92.67%) implies the abundance of RSF in the roots of *D. quercifolia* where several species of the genus *Trichoderma* were found to occur very frequently. Sites 2 and 5 possess the highest temperature, the highest light intensity, and the lowest substrate moisture content common in a stressful epiphytic habitat. Despite these conditions, the two sites manifested the highest RSF isolate diversity among the five tree-collection sites. Understanding the diversity and the presence of dominating RSF is necessary to determine their principal impact on ecosystem functioning. These principal factors explain their effects on increased plant productivity, nutrient acquisition, and environmental adaptation.

Keywords: biodiversity, epiphytic ferns, fungal symbionts, molecular identification, fungal taxonomy

INTRODUCTION

Twenty nine percent (29%) of fern species are epiphytes making them the second group of vascular plants when it comes to epiphyte diversity (Kress, 1986). Epiphytic ferns grow attached to their host. They absorb water and essential nutrients from their environment but are often nonparasitic (Benzing, 1990; Silvera & Lasso, 2016). *D. quercifolia* is an epiphytic fern with a reduced root system relevant to its hygrophilous epiphytic strategy (Lehmann et al., 2002; Dubuisson et al., 2003). Although economically underrated, *D. quercifolia* is considered ecologically important because it supports large amount of animal life and contribute to the hydrology and nutrient cycling in an ecosystem (Stuntz et al., 2002). Zotz and Hietz (2001) mentioned that one critical abiotic concern in an epiphyte's niche is water stress. Several adaptive mechanisms are exemplified by epiphytes to combat water stress. Benzing et al. (1983), Benzing (1990), Hietz and Briones (1998), and Ng and Hew (2000) added that leaf, stem, and roots structural modifications; morphophysiological adaptations; and the crassulacean acid metabolism (CAM) are just few of their various physioecological alterations for their successful lifestyle in drought and light-stressed environments.

Another distinct ecophysiological adaptation of epiphytic ferns is their symbiotic relationship with fungi. This symbiotic relationship establishes a wide array of metabolic processes and products critical for the positive relationship and survival of the host and the fungal symbiont. Most likely, *D. quercifolia* are able to live in a drought and light-stressed epiphytic environment due to the presence of symbiotic fungi in their roots. In relation to this, it has been theorized that the phototroph–fungi symbiosis enabled the primitive plants to colonize the terrestrial ecosystems. This mutualistic relationship enabled plants to adapt to various ecological stresses including aridness, exposure to extreme radiation, and intense thermal oscillations (Selosse & Le Tacon, 1998). There have been many studies showing that symbiotic fungi can help their host with water scarcity, salinity, and aid in thermal tolerance (Cheplick, 2004; Bayat & Mirlohi, 2009; Rudgers & Swafford, 2009; Hubbard et al., 2012; Hubbard et al., 2014).

Despite the known significance of symbiotic fungi to their host plants, little is known about their taxonomy and diversity. In a conservative estimate, fungal diversity is around 1.5 million species. However, only a fraction of 5% or 75,000 have been identified and characterized (Hawksworth, 2001). This information shows the scarcity of taxonomic studies on symbiotic fungi particularly on epiphytic ferns. This study therefore hopes to contribute to existing knowledge on fungal biodiversity specifically on root symbiotic fungi found in *D. quercifolia*.

MATERIALS AND METHODS

Site Description and Locale

The epiphytic ferns, *D. quercifolia*, were collected at the Don Mariano Marcos Memorial State University–North La Union Campus (DMMMSU-NLUC). The institution is located in the municipality of Bacnotan, in the province of La Union, Philippines. Epiphytic ferns attached in many trees and shrubs are common in the campus. The presence of these epiphytic ferns could be one reason why the environment within the campus is relatively moist and cool as compared to other areas within the municipality. All the activities of the study were conducted from October 2016 to January 2017.

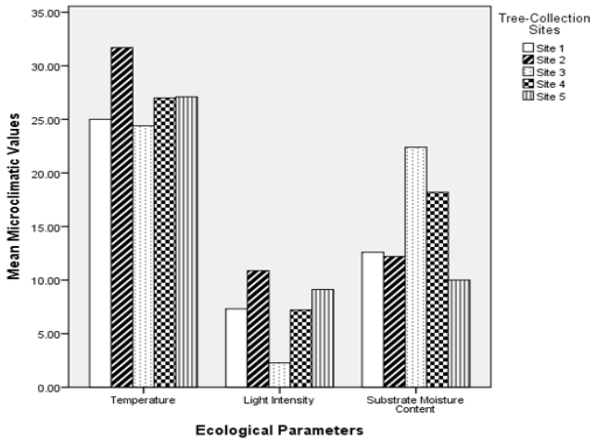

Figure 1. Mean microclimatic values (temperature, light, and substrate moisture content) of the five tree-collection sites.

Figure 1 shows the different microclimatic conditions of the five tree-collection sites. Sites 2 and 5 have the highest temperature (site 2 = 31.7°C, site 5 = 27.10°C), highest light intensity (site 2 = 10,871.30 lux, site 5 = 9,123 lux), and the lowest substrate moisture content (site 2 = 12.20%, site 5 = 10%). On the other hand, site 3 exemplifies the lowest temperature (24.40°C), lowest light intensity (2274.30) and the highest substrate moisture content (22.40%).

Sampling Technique

Five tree-collection sites were chosen using selective-random sampling to capture various microclimates. Some literature claims that spatiotemporal distinctions in fungi-host relationship and diversity are evident when there is niche specification (Gilbert et al., 2007). In addition, of the various microclimatic conditions, atmospheric temperature, light intensity, and substrate moisture content are the most restrictive and important ecological characteristics in an epiphytic habitat (Dubuisson et al., 2009). Thus, these three factors were recorded as ecologically influential factors affecting the species and abundance of RSF on *D. quercifolia*.

Collection of *D. quercifolia*

A total of 10 *D. quercifolia* were collected, two from each of the five tree-collection sites. Using improvised forceps, the entire fern was grasped and was separated slowly from the host tree. The ferns were categorically labeled based on their collection site. The roots were also initially washed with distilled water to remove superficial debris and were transported to the Natural Science Research Unit (NSRU) of Saint Louis University for further processing.

Isolation of RSF

The roots of the 10 epiphytic ferns were thoroughly washed with flowing water to remove unwanted debris adhering superficially to the roots. The roots were cut into 1-cm representative segments. Thirty representative segments were collected for each of the 10 ferns constituting a total of 300 representative segments. The roots were washed thrice with type 1 water and blotted dry with a sterile paper towel. The segments were placed on potato dextrose agar (PDA). Each PDA growth medium contained 10 representative segments, replicated thrice for each of the 10 ferns. Chloramphenicol was added to the PDA growth medium for inhibition of bacteria, and Rose Bengal was added to slow down the development of vigorously growing filamentous fungal species. The plates were grown at 30°C in ambient RT condition for seven days. Each colony of probable root symbiotic fungal isolates that grew from the segments was transferred to PDA slants.

DNA Extraction

Isolation of genomic DNA followed the procedure of Liu et al. (2014). One milliliter of mycelia broth was contained in a 1500-µl Eppendorf tube with 500 µl of lysis buffer (Tris hydrochloride:

pH 8.0, ethylenediaminetetraacetic acid [EDTA]: pH 8.0, sodium chloride, and 1% $NaC_{12}H_{25}SO_4$). The mycelia in the lysis buffer were disrupted to free the fungal DNA. The mixture was left at ambient room temperature (RT) for 10 minutes and was augmented with 150 µl of KCH_3COO solution (pH 4.8). This solution was made by adding 5-M potassium acetate and glacial acetic acid dissolved in type 1 water. The microtube was vortexed shortly and centrifuged at 10,000 × g for 1 minute. The supernatant was poured to a different 1500-µl microtube and was spun again. The supernatant (500 µl) containing the DNA samples was moved to another 1500-µl microtube with the addition of sodium acetate solution (0.3M, pH 5.2, 1/10 volume). Two volumes of cold ethanol (100%) were added and mixed well. DNA was precipitated at 20°C for 20 minutes followed by thawing and centrifugation of the sample. The supernatant was gradually drawn off. One milliliter of 70% ethanol was added, mixed, and spun as previously described. The supernatant was decanted again. The DNA samples were exposed to air drying and were resuspended in a buffer (TE: 50 µl). The DNA suspension was stored at 4°C. The isolated fungal DNA was tested for purity in 1% agarose gel.

DNA Amplification

The 18S rDNA–ITS region of the root symbiotic fungal isolates were sequenced. ITS-1 (5'TCCGTAGGTGAACCTGCGG-3') forward primer and ITS-4 (TCC TCCGCTTATTGATATGC') reverse primer were used in the PCR. The mixture (50 µl) contained the following: 25 µl of the PCR Master Mix (*Vivantis*), 1 µl each of the primers, 22 µl of PCR water, and 1 µl of the RSF isolates. The thermal cycler was run with a (1) preparatory denaturation phase, (2) amplification phase, and (3) final extension phase. In the predenaturation phase, the PRC mixture containing the isolated DNA was subjected to 95°C temperature for 5 minutes. This was followed by the amplification phase where the PCR mixture is subjected to 35 cycles of 95°C temperature for 30 seconds, 60°C temperature for 1 minute, and 72°C for 1 minute. In the final extension phase, the PCR mixture was subjected to 72°C for 6 minutes. For the detection of amplification products with an approximately 550bp length, 1.5% *Vivantis* (MB grade) agarose gel was used. The amplicons were sent to the 1st Base Sequencing Facility in Malaysia for gel extraction purification followed by sequencing.

Basic Local Alignment Sequence Tool Nucleotide (BLASTn) Sequence Homology

To identify the root symbiotic fungal isolates, the BLASTn was utilized to detect the homologous 18s rDNA partial ITS region of the isolates compared to sequence databases in BLASTn. Intraspecific similarity or species level identification using BLASTn sequence similarity comparison is set to a threshold level of 97% (Kwasna et al., 2008). In this study, the sequences of isolates having a homology greater than 97% to sequence databases were considered similar species. The sequencing strategy for identification from the ITS region by Romanelli et al. (2010) was adapted in the study where a cutoff criteria of 95%–97% identity for genus-level identification was utilized. Sequence similarities below 95% were treated as undescribed species (Sanchez-Marquez et al., 2008).

Colony Morphology Documentation

The colonies of root symbiotic fungal isolates were documented using a stereomicroscope (Swift SM100). The following colony morphological features of RSF isolates were recorded: colony form (CF), colony elevation (CE), colony margin (CM), colony surface (CS), colony opacity (CO), and colony color (CC).

Phylogenetic Circumscription and Analysis

The phylogenetic circumscription of the RSF was investigated through the Tamura et al. (2011) MEGA v6 by maximum likelihood (ML) phylogenetic test, and tree reconstruction was performed. To test the reliability of the ML tree, bootstrap method was used as a test of phylogeny using 1000 bootstrap replications.

Colonization, Occurrence, and Biodiversity Rates Estimation

The colonization and occurrence rates were computed using the formulas introduced by Alias et al. (2010). The percentage of colonization was computed using the formula

$$\% \text{ Colonization} = \text{NSCz} / \text{NSCt} \times 100$$

where NSCz is the number of segments colonized and NSCt is the number of segments collected.

The frequencies of fungal occurrence were classified as very frequent (above 10%), common (5%–9%), and rare (<5%).The percentage of taxon occurrence was computed using the formula

$$\% \text{ Taxon Occurrence} = \text{NTCt} / \text{NSCtot} \times 100$$

where NTCt is the taxon collected and NSCtot is the total segments collected.

The Paleontological Statistics (PaSt) software version 2.17c adapted by Hammer et al. (2001) was used to calculate the biodiversity indices of the root symbiotic fungal isolates. The Shannon's (H) index, Simpson's (D/1-D) index, and Equitability (J) index were computed for the different fungal isolates. The Shannon's and Simpson's indices were calculated to determine species richness. On the other hand, the Equitability index was calculated to measure species evenness as it ascertains the number of individuals distributed into the determined species. The Bray–Curtis index was computed to measure the abundance of root symbiotic fungal isolates based on the measure of similarity.

RESULTS AND DISCUSSION

Molecular and Morphological Identification of RSF Isolates

Thirteen RSF morpho-species were recorded. Genomic DNA of these RSF isolates were extracted using the protocol of Liu et al. (2014), and the partial ITS of the 18S ribosomal DNA of the RSF isolates were sequenced. The BLASTn was used to find regions of homology using the amplified ITS region as query sequences to the closest type match available on the NCBI databank.

Table 1 shows that eight of the 13 RSF isolates were identified up to the species level since the sequences of these isolates obtained the highest total score (highest sections of similarity between the query and the hit compared to the entire list of type match hits in the BLASTn database), high query cover, and an identity threshold >97% (Kwasna et al., 2008). These include (1) $F_1P_1RSF_1$—*Trichoderma crassum*, (2) $F_1P_3RSF_3$—*Meyerozyma guilliermondii*, (3) $F_2P_3RSF_5$—*Trichoderma yunnanense*, (4) $F_3P_3RSF_8$—*Trichoderma simmonsii*, (5) $F_3P_2RSF_{10}$—*Aspergillus brunneoviolaceus*, (6) $F_4P_2RSF_{13}$—*Trichoderma hispanicum*, (7) $F_4P_1RSF_{15}$—*Trichoderma lixii*, and (8) $F_9P_3RSF_{18}$—*Trichoderma scalesiae*. Five of the RSF isolates have sequence similarity below 95% and hence are classified as undescribed species: $F_3P_2RSF_9$, $F_4P_2RSF_{14}$, $F_5P_1RSF_{16}$, $F_8P_3RSF_{17}$, and $F_9P_2RSF_{21}$. From these five undescribed species, four were designated as undescribed Mucoromycotina ($F_4P_2RSF_{14}$, $F_5P_1RSF_{16}$, $F_8P_3RSF_{17}$, and $F_9P_2RSF_{21}$) based

Table 1. Molecular Comparison and Identification Using BLASTn Algorithm in NCBI of the Closest TYPE Match

RSF Isolate Code	Closest TYPE Match	TS	QC	Ident.	Accession[a]	RSF Isolate Proposed I.D.	RSF Isolate Accession[a]
$F_1P_1RSF_1$	*T. crassum*	1005	96%	99%	NR_134370.1	*T. crassum*	KY474515
$F_1P_3RSF_3$	*M. guilliermondii*	946	94%	98%	NR_111247.1	*M. guilliermondii*	KY474516
$F_2P_3RSF_5$	*T. yunnanense*	940	96%	99%	NR_134419.1	*T. yunnanense*	KY474517
$F_3P_3RSF_8$	*T. simmonsii*	1003	94%	99%	NR_137297.1	*T. simmonsii*	KY474518
$F_3P_2RSF_9$	*T. lixii*	810	93%	93%	NR_131264.1	Undescribed Sordariomycetes	KY474519
$F_3P_2RSF_{10}$	*A. brunneoviolaceus*	946	96%	99%	NR_138279.1	*A. brunneoviolaceus*	KY474520
$F_4P_2RSF_{13}$	*T. hispanicum*	962	96%	98%	NR_138451.1	*T. hispanicum*	KY474521
$F_4P_2RSF_{14}$	*M. fusiformis*	686	81%	89%	NR_111660.1	Undescribed Mucoromycotina	KY474522
$F_4P_1RSF_{15}$	*T. lixii*	987	94%	99%	NR_131264.1	*Trichoderma lixii*	KY474523
$F_5P_1RSF_{16}$	*M. fusiformis*	686	87%	89%	NR_111660.1	Undescribed Mucoromycotina	KY474524
$F_8P_3RSF_{17}$	*M. fusiformis*	686	87%	89%	NR_111660.1	Undescribed Mucoromycotina	KY474525
$F_9P_3RSF_{18}$	*T. scalesiae*	962	93%	99%	NR_144876.1	*T. scalesiae*	KY474526
$F_9P_2RSF_{21}$	*M. fusiformis*	686	87%	89%	NR_111660.1	Undescribed Mucoromycotina	KY474527

[a]National Center for Biotechnology Information (NCBI). TS = total score, QC = query cover, Ident. = Identification %

Table 2. Colony Morphological Characteristics

RSF Isolates	CF	CE	CM	CS	CO	CC
$F_1P_1RSF_1$—*T. crassum*	Filamentous cottony	Flat	Filiform	Dull and rough	Translucent	Greenish yellow
$F_1P_3RSF_3$—*M. guilliermondii*	Irregular	Flat	Entire	Glistening	Opaque	Yellowish white
$F_2P_3RSF_5$—*T. yunnanense*	Irregular - Circular	Raised	Entire	Dull and rough	Opaque	Grayish green
$F_3P_3RSF_8$—*T. simmonsii*	Wavy circular	Flat	Undulate	Concentric	Opaque	Dirty white with cream edge
$F_3P_2RSF_9$—Undesc. Sordariomycetes	Filamentous	Raised	Filiform	Rough	Translucent	White
$F_3P_2RSF_{10}$—*A. Brunneoviolaceus*	Wavy circular	Rugose	Undulate	Wrinkled	Opaque	Dark brown with white margin
$F_4P_2RSF_{13}$—*T. hispanicum*	Filamentous	Crateriform	Filiform	Dull and rough	Translucent	White
$F_4P_2RSF_{14}$—Undesc. Mucoromycotina	Circular	Flat	Filiform	Dull	Translucent	Grayish white
$F_4P_1RSF_{15}$—*T. lixii*	Wavy circular	Flat	Undulate	Concentric	Opaque	Dark brown with white margin
$F_5P_1RSF_{16}$—Undesc. Mucoromycotina	Filamentous	Convex	Filiform	Concentric	Opaque	White buff
$F_8P_3RSF_{17}$—Undesc. Mucoromycotina	Filamentous	Convex	Filiform	Buff	Opaque	Compact white buff
$F_9P_3RSF_{18}$—*T. scalesiae*	Wavy circular	Rugose	Undulate	Wrinkled	Opaque	Grayish brown with yellowish margin
$F_9P_2RSF_{21}$—Undescribed Mucoromycotina	Filamentous circular	Convex	Filiform	Buff	Opaque	Distinct yellow

CF = colony form, CE = colony elevation, CM = colony margin, CS = colony surface, CO = colony opacity, CC = colony color.

on their closest type match (*Mucor fusiformis*, Ident = 89%, KY474519), while one species was assigned as undescribed Sordariomycetes ($F_3P_2RSF_9$) based on its closest type match (*Trichoderma lixii*, Ident = 93%, KY474519).

The colony characteristics of the different RSF isolates found in *D. quercifolia* are summarized in Table 1.3 and depicted in Figure 1.3. *T. crassum* ($F_1P_1RSF_1$) is characterized by a filamentous cottony appearance with a flat elevation and a distinct greenish yellow colony color. This corresponds to the colony description of type *T. crassum* given by Chaverri et al. (2003). *T. yunnanense* (isolate $F_2P_3RSF_5$) shows the characteristics described by Yu et al. (2007), which includes the formation of irregular circular masses that are white at first but soon turn gray-green. The isolated *T. simmonsii* ($F_3P_3RSF_8$) is characterized by a wavy circular formation with concentric colony surface and a dirty white color and creamy-colored edges similar to the culture characters of type *T. simmonsii*

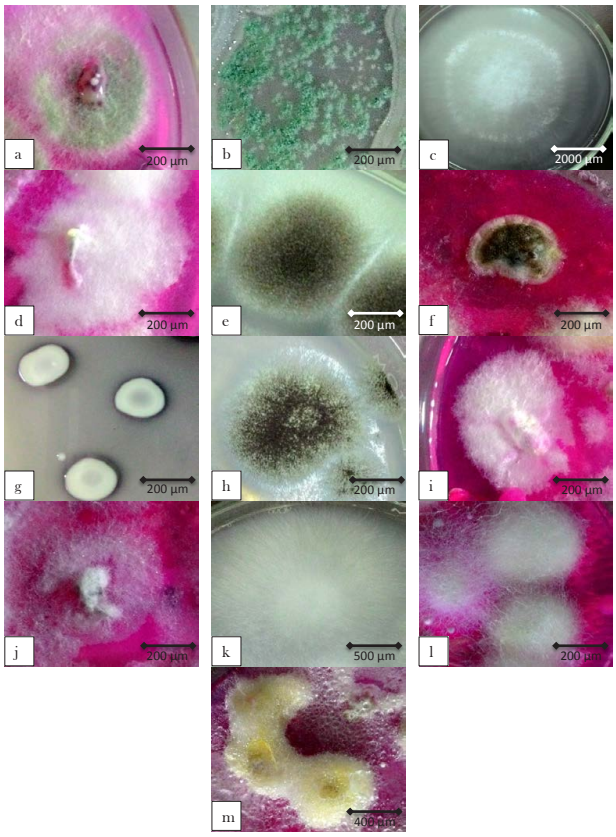

Figure 2. Colony morphology of the RSF isolates viewed under the stereomicroscope (Swift SM100).

investigated by Chaverri et al. (2015). *T. hispanicum* ($F_4P_2RSF_{13}$) is a white filamentous fungus with a crateriform elevation similar to the *T. hispanicum* isolated by Qin and Zhuang (2016). The colony characters of isolated *T. lixii* ($F_4P_1RSF_{15}$) is similar to the culture characters of the type *T. lixii* described by Chaverri et al. (2015) having a cottony mycelia radiating and beginning to turn green in one or two concentric rings. *T. scalesiae* ($F_9P_3RSF_{18}$) is characterized by a wavy circular form a rugose elevation and an undulated margin. The wrinkled grayish brown surface with yellowish white mycelial margin is also distinct. These colony features agrees to the type *T. scalesiae* described by Jaklitsch et al. (2006). The *M. guilliermondii* ($F_1P_3RSF_3$) isolated from *D. quercifolia* is characterized with an entire flat colony and yellowish white colony color that matches the *M. guilliermondii* type material ATCC® 6260™. The isolated *A. brunneoviolaceus* ($F_3P_2RSF_{10}$) possesses a wavy circular form and a rugose and wrinkled surface dark brown color and white margin, which resemble the type *A. brunneoviolaceus* described by Jurjevic et al. (2012). The unidentified Sordariomycetes ($F_3P_2RSF_9$) isolate has a filamentous colony form and a raised elevation. The surface colony of this isolate is dull and with a whitish appearance. $F_4P_2RSF_{14}$ is an unidentified Mucoromycotina with a flat circular and filiform appearance. The surface is dull with a grayish white colour. $F_5P_1RSF_{16}$ is an unidentified Mucoromycotina, filamentous in form with a convex elevation. The isolate's white concentric surface is also evident. Isolate $F_8P_3RSF_{17}$ is an unidentified Mucoromycotina with a filamentous form and convex elevation. The isolate's surface possesses a very distinct buff white appearance. Isolate $F_9P_2RSF_{21}$ is an unidentified Mucoromycotina with a filamentous circular form and convex elevation. The isolate also possess a distinct yellow colour with a buff surface appearance.

Phylogenetic Relationship of RSF

The relationship of the RSF isolates was determined using Molecular Evolutionary Genetics Analysis (MEGA) software version 6.06 (Tamura et al., 2011). The colonization and occurrence rates were computed using the equations by Alias et al. (2010), and biodiversity indices were computed using the Paleontological Statistics (PAST) software by Hammer et al. (2001).

The ML tree depicts the phylogenetic relationship of the RSF isolates from *D. quercifolia* showing four distinct monophyletic groups: Sordariomycetes, Eurotiomycetes, Saccharomycetes and Mucoromycotina (Fig. 3).

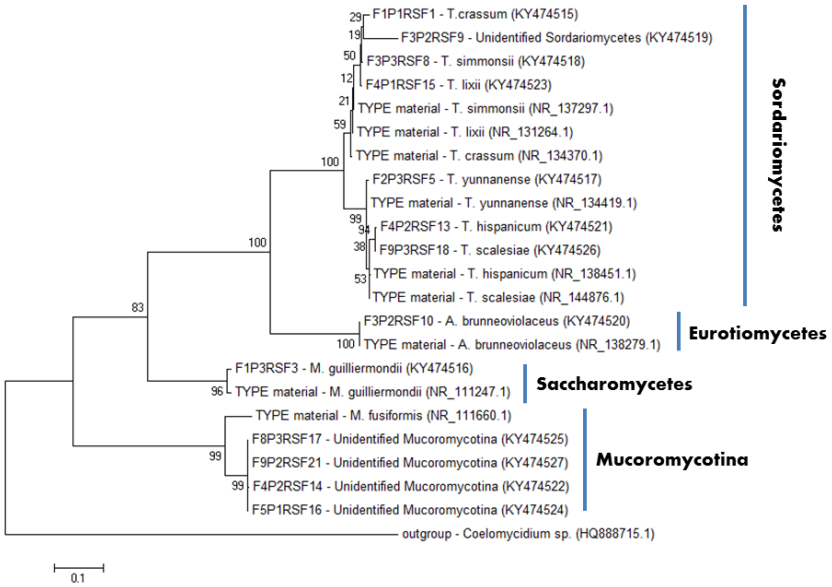

Figure 3. Overall RSF evolutionary history and phylogenetic relationship using maximum likelihood (ML) molecular inference.

While the monophyly in the *Trichoderma* species is evident, several RSF isolates did not cluster with the type materials. Instead of clustering with their respective types, the *T. crassum* isolate appears to cluster closely with the unidentified Sordariomycetes isolate, the *T. simmonsii* isolate seems to cluster closely with the *T. lixii* isolate, and the *T. yunnanense* isolate appears to cluster closely with the *T. hispanicum* isolate. The perceived species diversity in the genus *Trichoderma* confirms the general hyperdiverse nature of the genus as mentioned by Jaklitsch and Voglmayr (2015). However, it was also observed that the placements of the species in this genus are conflicting and unclear. This was termed as "*Trichoderma* aggregate species" by Siddiquee et al. (2007) because of the unclear phylogenetic relationships of many of its members. Despite the ambiguous placements of the species in the genus *Trichoderma*, monophyly is still manifested on a higher taxonomic level. The RSF isolates seem to cluster together to form the Sordariomycetes group. According to Zhang et al. (2006), the monophyly of the Sordariomycetes inferred on multigene sequences were vastly supported on many studies. The presence of perithecial ascoma is the synapomorphy that resolves the clade's monophyly (Spatafora et al., 2006).

Another apparent phylogenetic relationship observed in the ML tree is the sister clade formed by Sordariomycetes and Eurotiomycetes. This is an indication of their close phylogenetic affinity compared to the other groups. This result is supported by a 5-class level clade in the CV Tree reconstructed by Wang et al. (2009) within Pezizomycotina revealing the close relationship of the two groups. This is supported by another hypothesis by Fitzpatrick et al. (2006), Robbertse et al. (2006), and Spatafora et al. (2006) where they proposed that the Sordariomycetes and Eurotiomycetes are monophyletic.

In addition, the close phylogenetic relationship of Saccharomycetes to Eurotiomycetes and Sordariomycetes is also remarkable. The monophyly of these

three groups is due to some of their shared physiochemical activities including the urea amidolyase activity (Strope et al., 2011) and the presence of FHbs fungal globins (Hoogewijs et al., 2012). Consequently, the Mucoromycotina clade is the most distinct as it forms its own separate monophyletic group. Although a monophyletic group is formed by Mucoromycotina on the ML tree, Hibbett et al. (2007) reiterated that this division still has an uncertain phylogenetic placement.

Colonization, Occurrence, Abundance, and Diversity of RSF

The overall colonization and taxon occurrence rate can be obtained in Figure 4. From the 300 root segments collected, 278 were colonized equivalent to a relatively high colonization percentage of 92.67%. Four species are found to occur very frequently in the five sampling sites: two *Trichoderma* species (*T. yunnanense and T. simmonsii*) and two undescribed Mucoromycotina species ($F_5P_1RSF_{16}$, $F_9P_2RSF_{21}$). Two species are common: *T. lixii* and an undescribed Mucoromycotina ($F_3P_2RSF_9$). Seven species are considered rare: three *Trichoderma* species (*T. hispanicum, T. scalesiae,* and *T. crassum*), *M. guilliermondii*, *A. brunneoviolaceus*, and two undescribed Mucoromycotina species ($F_8P_3RSF_{17}$ and $F_9P_3RSF_{18}$).

Figure 5 shows the abundance and the five most dominant (bordered in red) RSF in the five collection sites. *T. yunnanense* is the most dominant in site 1 (n = 17). *T. simmonsii* (n = 18), undescribed Mucoromycotina isolate $F_9P_2RSF_{21}$ (n=21), and another undescribed Mucoromycotina isolate $F_5P_1RSF_{16}$ (n = 34) are the dominant RSF isolates in sites 2, 3, and 4, respectively. The most dominant RSF isolate in site 5 is *M. guilliermondii* (n = 14). The abundance of *T. yunnanense* and *T. simmonsii* presented here is comparable to the surveillance of Jaklitsch et al. (2013), where they regarded *T. yunnanense* as cosmopolitan particularly in subtropical climates. They can be obtained from decaying wood or in tree barks, endophytes from deciduous trees, mushroom compost, and drinking water and can also be found in the soil. On the other hand, Zachow et al. (2009) observed the fungal diversity of *T. simmonsii* on the Canary Island Tenerife. This species was found diverse in the rhizosphere of endemic plants in the different climatic and vegetation zones. In another study conducted by Jacklitsch and Voglmayr (2015), they noted that *T. simmonsii* were collected from various trees and shrubs found in the warmer regions of Southern European countries. Their observations were similar to the preferred high temperature niche of the *T. simmonsii* in this study. Xia et al. (2011) also found the dominance of the genus *Trichoderma*

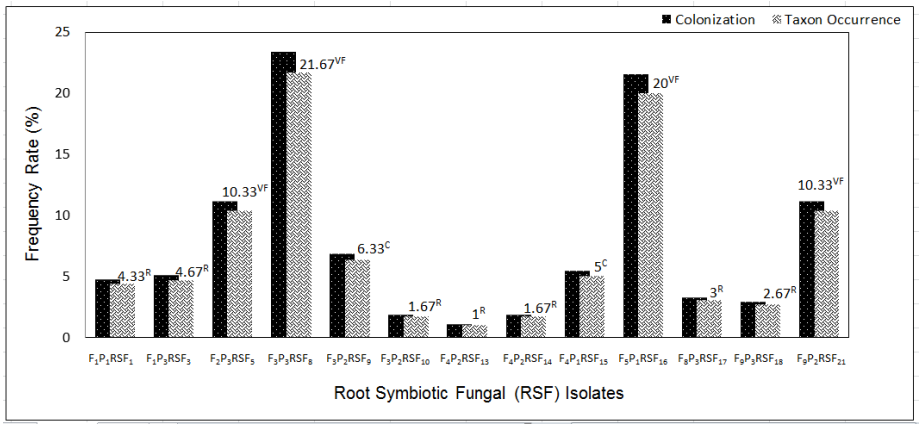

Figure 4. Comparison of the colonization and taxon occurrence rate of the 13 RSF isolates.

in the roots of banana. Several *Trichoderma* species were isolated ecto- and endophytically. Their results suggest the colonizing ability of *Trichoderma* in the roots of banana plants. This present research consequently shows that the genus *Trichoderma* is also a common root colonizer of *D. quercifolia*. In one of the collection sites (site 5), the dominance of *M. guilliermondii* is evident (CF = 14, Relative CF = 26.92%; Taxon Occurrence % = 23.33; Occurrence Class = Very frequent). This shows that it is a ubiquitous species as mentioned by Kurtzman et al. (2010) having isolates found in the soil, in water bodies, on plant materials, and on many invertebrates. The high colonization rates of RSF isolates in the roots of *D. quercifolia* are considered plant–fungus symbioses that have been known to occur even during plants' early terrestrial migration (Redecker et al., 2000). There were assumptions that Mucoromycotina–host symbiosis emerged during plant's premature land colonization (Bidartondo et al., 2011). A mycorrhizal mutualism between Mucoromycotina and liverworts was even demonstrated by Field et al. (2015) to portray early plant–fungus symbiosis. Similarly, in this study, the dominance of unknown species of Mucoromycotina and a relatively high colonization rate in *D. quercifolia* support their possible importance in fungi–plant host symbiosis.

The overall biodiversity of the five sampling sites is summarized in Table 3. The Simpson indices (D and 1-D) are measures of diversity. In the table above, it is apparent that site 2 (Simpson's D = 0.1855; Simpson's 1-D = 0.8145) and site 5 (Simpson's D = 0.1686; Simpson's 1-D = 0.8314) are the most diverse communities while site 4 (Simpson's D = 0.3783; Simpson's 1-D = 0.6217) has the least diversity. The Shannon index (H) also measures evenness and richness (0 = no diversity, 5 = infinite diversity). Table 3 also shows that site 2 and site 5 have the highest Shannon's (H) values of 1.91 and 1.911, respectively, thus manifesting the highest diversity among the different sampling sites. The two sites with the highest diversity (site 2 and site 5) also have the highest temperature (site 2 = 31.7°C; site 5 = 27.10°C), highest light intensity (site 2 = 10,871.30 lux; site 5 = 9,123 lux), and the lowest substrate moisture content (site 2 = 12.20%; site 5 = 10%). The Equitability index (J) quantifies how equal the community is numerically with values constrained between 0 and 1. The most even communities are found in site 3 (J = 0.9174) and site 5 (J = 0.9192), which implies the lack of a dominant species on these sites. On the other

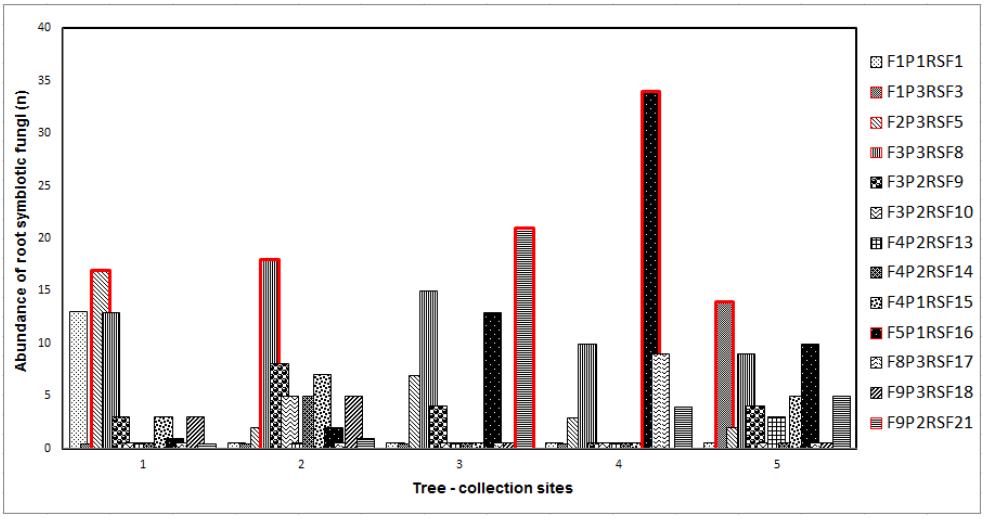

Figure 5. Abundance of RSF in the different tree collection sites.

Table 3. Overall Biodiversity Estimates

	Site 1	Site 2	Site 3	Site 4	Site 5	Overall
No. of species	7	9	5	5	8	13
No. of isolates	53	53	60	60	52	278
Simpson's (D)	0.2332	0.1855	0.25	0.3783	0.1686	0.1411
Simpson's (1-D)	0.7668	0.8145	0.75	0.6217	0.8314	0.8589
Shannon's (H)	1.617	1.91	1.477	1.235	1.911	2.201
Equitability (J)	0.8308	0.8693	0.9174	0.7676	0.9192	0.8582
Bray–Curtis (BC)	0.169	0.298	0.104	0.091	0.261	0.298

hand, the community with the least evenness in species distribution is site 4 (J = 0.7676), which suggests the dominance of the undescribed Mucoromycotina isolate $F_5P_1RSF_{16}$. Lastly, the Bray–Curtis dissimilarity index revealed the similar species composition of the five sites (0 = same site composition, 1 = do not share any species). Sites 3 (BC = 0.104) and 4 (BC = 0.091) have the least value, which means that species within these two sites are highly similar. On the other hand, greater species variation are exhibited by site 2 and site 5, in which their Bray–Curtis values are 0.298 and 0.261, respectively.

Abiotic stresses (high temperature, high light intensity, and low moisture) are common in an epiphytic habitat (Zotz & Hietz, 2001). Several mechanisms allow epiphytes to adapt and favor these stressful environments including CAM utilization, (Winter & Smith, 1996), modified stomatal response, reduced leaf transpiration, and osmotic adjustment (Hietz & Briones, 1998) and the presence of RSF. Dickie (2007) stressed that since epiphytes prefer these "stressful" microhabitats, their abundance on these sites triggers the coexistence of diverse fungal symbionts. He added that each unique microhabitat occupied by fungal symbionts elicit the co-occurrence of more symbionts. Therefore, site conditions play a huge impact in RSF biodiversity on the different *D. quercifolia* collection sites. However, the present understanding of the quantitative changes in niche differentiation-dependent symbiotic functioning is still very limited (Konvalinkova et al., 2015). Previous studies agree on the uncertainty of the effects of numerous ecological parameters on the working symbiosis on fungi–host relationship (Johnson & Graham, 2013; Johnson et al., 2015). On the other hand, Dickie (2007) reiterated that the presence of a dominant species is common in root symbiotic fungal communities and it is important to identify them because they often have the greatest functional role in the ecosystem. It is however more important to understand the overall RSF biodiversity and species composition in an area because each species, regardless if it's abundant or rare, may affect various ecosystem functions and processes including nutrient acquisition and substrate stability, which may possibly boost plant-host performance. Understanding both diversity and dominance of RSF in different microhabitats can reveal their critical roles and impact in their plant host and in the ecosystem. This can be later used for plant biotechnology and ecological conservation measures.

CONCLUSION

Based on molecular evidence, potential novel species of RSF were isolated. It was also established in the study that there is

generally a high colonization rate of symbiotic fungi in the roots of *D. quercifolia* where the species in the genus *Trichoderma* occur very frequently. The distribution of the different RSF isolates on the five tree-collection sites possibly denotes their microclimatic preference and their diversity on niches experiencing abiotic stresses may imply their significance and principal impact on ecosystem functioning that may eventually affect their hosts' nutrient acquisition, productivity, and disease resistance. Many studies have focused on the root symbiotic fungal communities in plant roots in various micro- and large-scale ecosystems. On the contrary, studies on the direct relationship of RSF community composition and their functional roles are still sparse. It is therefore highly recommended that a study that will focus on the functional impact the RSF communities have on their host organisms may also be undertaken.

ACKNOWLEDGEMENTS

The authors wish to thank the Commission on Higher Education (CHED) for the CHED Dissertation Grant and the Philippine Rice Research Institute (PhilRice) for the Dissertation Fellowship which helped in the financial expenses in this dissertation study. The authors also wish to thank the following academic institutions: Don Mariano Marcos Memorial State University – North La Union Campus, Saint Louis University and University of the Philippines, Baguio for all related dissertation endeavors. Finally, the authors would also like to express their sincere gratitude and appreciation to all staff and research assistant/technicians from PhilRice, SLU-NSRU, and UPB Research Laboratory for all their steadfast support and assistance in the conduct of all the necessary laboratory activities.

REFERENCES

Alias, S. A., N., Zainuddin, N., & Jones, E. B. G. (2010). Biodiversity of marine fungi in Malaysian mangroves. *Botanica Marina*, *53,* 545–554.

Bayat, F., & Mirlohi, A. (2009). Effects of symbiotic fungi o some drought tolerance mechanisms of tall fescue in a hydroponics culture. *Russian Journal of Plant Physiology*, *56*(4), 510–516.

Benzing, D. H. (1990). Vascular epiphytes. *General biology and related biota* (p. 271). Cambridge: Cambridge University Press,.

Benzing, D. H., Friedman, , Peterson, G., & Renfrow, A. (1983). Shootlessness, velamentous roots, and the pre-eminence of Orchidaceae in the epiphytic biotope. *American Journal of Botany*, *70*, 121–133.

Bidartondo, M. I., Read, , Trappe, , Merckx, V., Ligrone, R., & Duckett, (2011). The dawn of symbiosis between plants and fungi. *Biology Letters, 7,* 574–577.

Chaverri, P., Branco-Rocha, F., Jaklitsch, W., Gazis, R., Degenkob, T., & Samuels, G. (2015). Systematics of the *Trichoderma harzianum* species complex and the re-identification of commercial biocontrol strains. *Mycologia*, *107*(3), 558–590.

Chaverri, P., Castlebury, L., Overton, B., & Samuels, G. (2003). *Hypocrea*/*Trichoderma*: Species with conidiophore elongations and green conidia. *Mycologia, 95*(6), 1100–1140.

Cheplick, G. P. (2004). Recovery from drought stress in *Lolium perenne* (Poaceae): Are fungal symbionts detrimental? *American Journal of Botany, 91*(12), 1960–1968.

Dickie, I. (2007). Host preference, niches and fungal diversity. *New Phytologist, 174*(2), 230–233.

Dubuisson, J., Hennequin, S., Rakotondrainibe, F., & Schneider, H. (2003). Ecological diversity and adaptive tendencies in the tropical fern *Tricomanes* L. (Humenophyllaceae) with special reference to climbing and epiphytic habits. *Botanical Journal of the Linnaean Society, 142,* 41–63.

Dubuisson, J., Schneider, H., & Hennequin, S. (2009). Epiphytism in ferns: Diversity and history. *Comptes Rendus Biologies, 332,* 120–128.

Field, K. J., Rimington, , Bidartondo, , Allinson, , Beerling, , Cameron, , Duckett, , Leake, , & Pressel, S. (2015). First evidence of mutualism between ancient plant lineages (Haplomistriopsida liverworts) and Mucoromycotina fungi and its response to simulated Palaeozoic changes in atmospheric CO_2. *New Phytologist, 205*(2), 743–756.

Fitzpatrick, D. A., Logue, , Stajich, , & Butler, G. (2006). A fungal phylogeny based on 42 complete genomes derived from supertree and combined gene analysis. *BMC Evolutionary Biology, 6,* 99–100.

Gilbert, G. S., Reynolds, , & Bethancourt, A. (2007). The patchiness of epifoliar fungi in tropical forests: Host range, host abundance, and environment. *Ecology, 88*(3), 575–581.

Hammer, O., Harper, , & Ryan, (2001). PAST: Paleontological statistics software package for education and data analysis. *Palaeontologica Electronica, 4*(1), 9.

Hawksworth, D. L. (2001). The magnitude of fungal diversity: the 1.5 million species estimate revisited. *Mycological Research, 105*(12), 1422–1432.

Hibbett, D. S., Binder, M., Bischoff, , Blackwell, M., Cannon, P., Eriksson, O.,…Zhang, N. (2007). A higher level of phylogenetic classification of the Fungi. *Mycology Research, 111,* 509–547.

Hietz, P., & Briones, O. (1998). Correlation between water relations and within-canopy distribution of epiphytic ferns in a Mexican cloud forest. *Oecologia, 114*, 305–316.

Hoogewijs, D., Dewildge, S., Vierstraete, A., Moens, L., & Vinogradov, (2012). A phylogenetic analysis of the globins in fungi. *PLOS One, 7*(2), e0031856.

Hubbard, M., Germida, J., & Vujanovic, V. (2012). Fungal symbionts improve wheat seed germination under heat and drought stress. *Botany, 90*(2), 137–149.

Hubbard, M., Germida, J., & Vujanovic, V. (2014). Fungal symbionts enhance wheat heat and drought tolerance in terms of grain yield and second-generation seed viability. *Journal of Applied Microbiology, 116*(1), 109–122.

Jaklitsch, W. M., Samuels, G., Dodd, S., Lu, B., & Druzhinina, I. (2006). *Hypocrea rufa/ Trichoderma viride*: A reassessment, and description of five closely related species with and without warted conidia. *Studies in Mycology, 55*, 135–177.

Jaklitsch, W. M., Samuels, G. J., Ismaiel, A., & Voglmayr, H. (2013). Disentangling the *Trichoderma viridescens* complex. *Persoonia, 31,* 112–146.

Jaklitsch, W. M., & Voglmayr, H. (2015). Biodiversity of *Trichoderma* (Hypocreaceae) in Southern Europe and Macaronesia. *Studies in Mycology, 80*, 1–87.

Johnson, N. C., & Graham, (2013). The continuum concept remains a useful framework for studying mycorrhizal functioning. *Plant Soil, 363,* 411–419.

Johnson, N. C., Wilson, , Wilson, , Miller, , & Bowker, (2015). Mycorrhizal phenotypes and the law of the minimum. *New Phytologist, 205,* 1473–1484.

Jurjevic, Z., Peterson, S., Stea, G., Solfrizzo, , Varga, , Hubka, , & Perrone, G. (2012). Two novel species of *Aspergillus* section Nigri from indoor air. *IMA Fungus, 3*(2), 159–173.

Konvalinkova, T., Puschel, D., Janouskova, , Gryndler, , & Jansa, J. (2015). Duration and intensity of shade differentially affects mycorrhizal growth- and phosphorus uptake responses of *Medicago truncatula*. *Frontiers in Plant Science, 6*(65), 1–11.

Kress, W. J. (1986). A symposium: The biology of tropical epiphytes. *Selbyana, 9,* 1–22.

Kurtzman, C. P., Kurtzman, M., & Suzuki, M. (2010). In C. P. Kurtzman, J. W. Fell, & T. Boekhout. (Eds.), *The yeasts: A taxonomic study* (5th ed., Vol. 2, pp. 621–624). London, UK: Elsevier Science.

Kwasna, H., Bateman, , & Ward, E. (2008). Determining species diversity of microfungal communities in forest tree roots by pure-culture isolation and DNA sequencing. *Applied Soil Ecology, 40,* 44–56.

Lehmann, A., Leathwick, , & Overton, J. M. (2002). Assessing New Zealand fern diversity from spatial predictions of species assemblages. *Biodiversity and Conservation, 11,* 2217–2238.

Liu, D., Coloe, S., Baird, , & Pedersen, J. (2014). Rapid mini-preparation of fungal DNA for PCR. *Journal of Clinical Microbiology, 52*(3).

Ng, C. K-Y., & Hew, (2000). Orchid pseudobulbs—

"False" bulbs with a genuine importance in orchid growth and survival. *Scientia Horticulturae, 83,* 165–172.

Qin, W., & Zhuang, W. (2016). Seven wood-inhabiting new species of genus *Trichoderma* (Fungi, Ascomycota) in Viride clade. *Scientific Reports, 6*, 27074.

Redecker, D., Kodner, R., & Graham, (2000). Glomalean fungi from the Ordovician. *Science, 289,* 1920–1921.

Robbertse, B., Reeves, J., Schoch, C., & Spatafora, (2006). A phylogenomic analysis of the Ascomycota. *Fungal Genetic Biology, 43*(10), 715–725.

Romanelli, A. M., Sutton, , Thompson, , Rinald, , & Wickes, (2010). Sequence-based identification of filamentous basidiomycetous fungi from clinical specimens: a cautionary note. *Journal of Clinical Microbiology, 48,* 741–752.

Rudgers, J. A., & Swafford, (2009). Benefits of a fungal symbiont in *Elymus virginicus* decline under drought stress. *Basic and Applied Ecology, 10*(1), 43–51.

Sanchez-Marquez, S., Bills, , & Zabalgogeazcoa, I. (2008). Diversity and structure of fungal endophytic assemblages from two sympatric coastal grasses. *Fungal Diversity, 33,* 87–100.

Selosse, M. A., & Le Tacon, F. (1998). The land flora: A phototroph-fungus partnership? *Trends in Ecology and Evolution, 13*(1), 15–20.

Siddiquee, S., Guan, F., & Aziz, E. (2007). Phylogenetic relationships of *Trichoderma harzianum* based on the sequence analysis of the internal transcribed spacer region-1 of the rDNA. *Journal of Applied Sciences Research, 3*(9), 896–903.

Silvera, K., & Lasso, E. (2016). Ecophysiology and crassulacean acid metabolism of tropical epiphytes. In G. Goldstein & L. S. Santiago (Eds.), *Tropical tree physiology. Adaptations and responses in a changing environment* (pp. 25–43). Switzerland: Springer International Publishing.Spatafora, J. W., Sung, , Johnson, D., Hesse, C., O'Rourke, B., Serdani, M.,… Schoch, (2006). A five-gene phylogeny of Pezizomycotina. *Mycologia, 98*(6), 1018–1028.

Strope, P. K., Nickerson, , Harris, , & Moriyama, (2011). Molecular evolution of urea amidolyase and urea carboxylase in fungi. *BMC Evolutionary Biology, 11*, 80.

Stuntz, S., Simon, U., & Zotz, G. (2002). Rainforest air-conditioning: The moderating influence of epiphytes on the microclimate in tropical tree crowns. *International Journal of Biometeorology, 46,* 53–59.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution, 28,* 2731–2739.

Wang, H., Xu, Z., Gao, L., & Hao, B. (2009). A fungal phylogeny based on 82 complete genomes using the composition vector method. *BMC Evolutionary Biology, 9*, 195.

Winter, K., & Smith, (1996). Crassulacean acid metabolism: Current status and perspectives. In K. Winter & J. A. C. Smith (Eds.), *Crassulacean acid metabolism: Biochemistry, ecophysiology and evolution* (pp. 389–426). Heidelberg: Springer.Xia, X., Lie, , Quian, X., Zheng, Z., Huang, Y., & Shen, Y. (2011). Species diversity, distribution and genetic structure of endophytic and epiphytic *Trichoderma* associated with banana roots. *Microbial Ecology, 61*(3), 619–625.

Yu, Z., Qiao, M., Zhang, Y., & Yang, J. (2007). Two new species of *Trichoderma* from Yunnan, China. *Antonie van Leeuwenhoek, 92*(1), 101–108.

Zachow, C., Berg, C., Muller, H., Meincke, R., Komon-Zelazowska, M., Druzhinina,, , Kubicek, , & Berg, G. (2009). Fungal diversity in the rhizosphere of endemic plant species of Tenerife (Canary Islands): Relationship to vegetation zones and environmental factors. *The ISME Journal, 3,* 79–92.

Zhang, N., Castlebury, , Miller, , Huhndorf, , Schoch, , Seifert, ,…Sung, (2006). An overview of the systematics of the Sordariomycetes based on a four-gene phylogeny. *Mycologia, 98,* 1076–1087.

Zotz, G., & Hietz, P. (2001). The physiological ecology of vascular epiphytes: current knowledge, open questions. *Journal of Experimental Botany, 52*(364), 2067–2078.