

Characterization of mycorrhiza associating with *Encyclia tampensis* and its effect on plant health

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Abstract

Starting in the late 1800's orchids were heavily poached, leaving many species to reach critically low numbers. Coupled with habitat loss and urbanization many orchid populations were extirpated in southern Florida. Due to lack of endosperm, orchid seeds are reliant on specific mycorrhizal fungi to obtain nutrients to enable embryo development resulting in very low germination rates in nature. This obligatory relationship with mycorrhizal fungi complicates orchid re-establishment. The research project aims are (1) to evaluate the correlation between phenotypic traits and optimal growing conditions in various micro-climate conditions, and (2) to assess the range of mycorrhizal diversity in urban and botanic garden settings. The native orchid, *Encyclia tampensis*, was sampled from naturally occurring and lab propagated orchids at Fairchild Tropical Botanic Garden (FTBG), Naples Botanical Garden (NBG), and Downtown Doral Park (DDP) on oak, cypress, and palm trees. Phenotypic measurements (chlorophyll content, light intensity, height on the tree, number of leaves, root and shoot lengths) and root sampling (fungal isolations, DNA identification) were conducted. Increased SPAD and NDVI values measuring chlorophyll content as a plant health parameter were observed at FTBG, while DDP and NBG had comparable values indicating the suitability of botanic gardens and urban spaces as reintroduction sites for orchid. Across all 3 sites and host trees, two distinct clades of mycorrhizal fungi within the genus *Tulasnella* were recovered. The results of this study will help maximize current conservation and reintroduction programs within developed urban areas and botanical gardens for best management practices on orchids.

Introduction

Orchidaceae is one of the largest and most diverse families of flowering plants and contains approximately 25,000 species and over 880 genera worldwide (Mullin et al., 2022). Orchids are also well known for having a wide range of uses such as: ornamental, medicinal, and cultural values (De Stefano et al., 2022; Vendrame and Khoddamzadeh, 2016). Habitat loss, environmental degradation, over-collection, and extreme weather events has led many orchid species to reach critically low population numbers (Newman et al., 2013; McCormick and Jacquemyn, 2014). It has been approximated that more than 10% of all orchid species worldwide are threatened (Coile and Garland, 2003; Yang et al. 2017). Florida is home to 106 native orchid species, of which 77 are listed as endangered or threatened (De Stefano et al. 2022). For species that typically grow as epiphytes on a host tree, only seeds that land on trees containing the compatible mycorrhizal fungi will germinate and develop into seedlings (De la Rosa-Manzano et al., 2014; Gangjun et al., 2020). This can further decrease the already small chance of seeds to become established and develop (Knudson, 1992; Rasmussen et al. 2015). Therefore, it is important to understand the impacts of both abiotic and biotic factors on the abundance and distribution of orchid mycorrhiza and how these factors such as host tree species, light intensity, and diversity of mycorrhizal fungi can affect the overall orchid survivorship and recruitment (Jacquemyn et al., 2014; Yang et al. 2017).

The importance of orchid conservation is not only intrinsic but also for their role as potential indicators of changes in ecosystem health. Several biotas depend on epiphytes for their various needs, either for moisture, nectar, pollen, fruits, and seeds. Epiphytes also serve as water storage, provide supplements of secondary metabolites, nesting, and breeding spaces (Phillips et al., 2011). In forestry management, they have been used to indicate increasing levels of atmospheric pollution. Epiphytic communities are sensitive to changes in atmosphere and climate and are expected to be the first communities to show drastic responses to impending climate change (Shashidhar and Kumar, 2009; Landi et al., 2009; Ibrahim et al., 2011; Kaur et al., 2020). Even with this strong sensitivity to environmental changes, orchids have been observed to colonize in urban and non-natural landscapes (Bhatt et al. 2015). This suggests the potential of using urban landscapes and botanic gardens for orchid refuges (Scade et al., 2006). To be able to improve the success of conservation programs in urban and botanical garden settings, comprehensive data collection is imperative (Izuddin et al., 2018 and 2019; Fekete et al., 2020).

Epiphytic orchids rely on the microbiome of the bark on certain host trees for root attachment and nutrient uptake. The mycorrhizal fungi that associate with orchids within the root cortex can be identified using molecular based techniques to give insight on mycorrhizal diversity (Givnish et al., 2015). This novel research can contribute very important knowledge that opens the door to new questions to be asked such as: what are the factors impacting orchid diversity and abundance? To what degree do abiotic and biotic factors, and host tree type, influence mycorrhizal diversity and therefore orchid establishment, health, and growth? This new frontier in orchid biology is shedding light and guiding the way for future scientists to expand knowledge, and it is uncovering the way in which the collective microbiome of a plant plays a key role in global ecosystems and species diversity (Herrera et al. 2019) and the way in which microorganisms have co-evolved with plants to guide the range of expansion and biodiversity in nature. Studying this topic provides evidence for the importance of conserving nature for species with a narrow niche range. This is especially critical in the face of climate change, since many vulnerable rare species are sensitive to environmental conditions.

This research is focused specifically on the abiotic and biotic factors affecting establishment of the commercially exploited orchid, *Encyclia tampensis* Lindl., on varying host trees in urban landscapes. Through the Million Orchid Project conservation program, native orchids have been successfully reintroduced into urban landscapes throughout south Florida. However, the program has yet to determine whether associations with mycorrhizal fungi have been formed in aymbiotically propagated outplants within these urban environments. Mycorrhizal fungi are required for the germination of orchid seeds, so the isolation and identification of mycorrhizal fungi and their location within the environment is critical for future recruitment and creating the best conservation practices (McCormick et al., 2016). Specifically, we seek to understand which mycorrhizal species they might be interacting with and to what extent, as well as how this varies across different habitats. In this study, *E. tampensis* was sampled at Fairchild Tropical Botanic Garden, Naples Botanic Garden, and Downtown Doral Park on oak, cypress, and palm trees. We hypothesize that the site locations due to different climate classification and host tree species will result in significant differences in the plant growth characteristics (shoot length, root

length, number of leaves), plant health parameters (leaf chlorophyll content) and mycorrhizal diversity in *E. tampensis*.

Materials and Methods

Study species and sites

Encyclia tampensis is a perennial, epiphytic monocot that is and has native ranges from Florida to the Bahamas. The global status is apparently secure, the state of Florida status is commercially exploited and regionally it is depicted as secure (IRC, 2020). Although this orchid is one of the most common in the region it has been largely extirpated from urban landscapes. The study sites were selected based on world map of the Köppen-Geiger climate classification (Kottek et al., 2006) and included botanic gardens and public parks with varying levels of urbanization; Fairchild Tropical Botanic Garden, Downtown Doral Park, and Naples Botanical Garden. Individual orchids were sampled two years post outplanting on 3 host trees: Oak (*Quercus virginiana* at FTBG and *Quercus germinata* at NBG), Palm (*Roystonea regia*), and Bald Cypress (*Taxodium ascendens*). In total, 25 individual plants were sampled. Phenotypic measurements were taken, and a 1-inch root cutting was collected from each plant. At Fairchild Tropical Botanic Garden, 10 plants were samples, 5 on live oak (naturally occurring) and 5 on palm trees (reintroduced and grown at the lab). Located in Downtown Doral Park within an urban area, 10 outplants were sampled: 5 on live oak and 5 plants on dwarf cypress trees. At Naples Botanical Garden, 5 plants were sampled, all on live oak and naturally occurring within a pine rockland conservation area (Fig. 1).

Ecophysiological Measurements

To evaluate the ecophysiological characteristics, various parameters such as chlorophyll content using SPAD and GreenSeeker™ (NDVI) optical non-destructive handheld devices, light intensity, height and orientation on the trees, number of leaves, and root and shoot lengths were recorded. Using the SPAD device, 3 mature leaves were selected randomly from opposing sides of the plant and the measurements were averaged. Chlorophyll content of a leaf can be estimated by inserting a leaf between the clamps of the SPAD aperture. In order to convert these values into absolute units of chlorophyll concentration, calibration curves must be derived and utilized. The equation shown below can be used to convert SPAD into total chlorophyll per unit in leaf area (Inman et al., 2005).

NIR – reflection in the near-infrared spectrum

RED – reflection in the red range of the spectrum

$$NDVI = \frac{(NIR - Red)}{(NIR + Red)}$$

Readings obtained with the GreenSeeker™ exhibit a robust correlation with the plant's chlorophyll content, enabling an estimation of plant health, nutrient status, and potential mycorrhizal fungi

development. Distinguished as a pioneer among spectral sensors, the GreenSeeker™ sensor uniquely leverages the reflectance qualities of the canopy, facilitating readings over the entire canopy rather than individual leaves. To account for foliage variation, measurements were taken three times for each plant. During readings, the GreenSeeker™ was positioned approximately 45 cm above the sample plant, targeting the green leaves to minimize background noise. This ensured a consistent field of view for the center, measuring approximately 11.85 cm in diameter (Khoddamzadeh and Dunn, 2016; Mullin et al., 2022; Souza Costa et al., 2023; Khoddamzadeh and Souza Costa, 2023). Comprehensive data collection on the host tree and orchid included orchid chlorophyll content using SPAD and GreenSeeker™ handheld devices, light intensity, tree height and orientation, leaf count, and root and shoot lengths. With the SPAD device, three mature leaves were randomly selected from opposing sides of the plant, and measurements were averaged. The SPAD electrode was positioned in the central region of each measured leaf, with readings proportional to the chlorophyll present. To convert these values into absolute units of chlorophyll concentration, calibration curves were derived and applied, as per the equation provided by Ling, Huang, and Jarvis (2017).

For the GreenSeeker™ device to obtain NDVI values, measurements were taken three times for each plant, considering foliage variation, and the mean of the three measurements was used for data analysis. Similar to the GreenSeeker™ readings, the device was positioned about 45 cm above the sample plant, focusing on green leaves to minimize background noise and ensuring a consistent field of view for the center, approximately 11.85 cm in diameter (Samseemoung et al., 2017).

Isolation of root fungi

The isolation and culture of *E. tampensis* mycorrhizal fungi was done using an agar based basal salts media with and without antibiotics. The media constituents included glucose, ammonium tartrate, KH_2PO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, yeast extract, ferric citrate, $\text{MnSO}_4 \cdot 1\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and agar dissolved in 1L deionized water with Novobiocin antibiotic in the initial isolation of individual fungal pelotons. For subculturing, the same media was used without containing the Novobiocin antibiotic.

Samples were gathered from three locations: Fairchild Tropical Botanic Garden, Downtown Doral Park, and Naples Botanic Garden. Following collection, the samples were stored in moist paper towels at 4°C until the isolation of fungi. To eliminate coarse organic material and debris from the root surface, the samples underwent a washing process with deionized (DI) water. Subsequently, the roots were transferred to a laminar flow cabinet, and sterile equipment was utilized to isolate mycorrhizal fungi pelotons.

Tissues underwent rinsing in three sterile water tubes through a serial dilution technique. Each tube containing the root sample was immersed in water for 3 minutes and then vortexed for 1 minute to further remove debris from the root surface. An approximately one-inch root cutting was placed in a petri dish, covered with sterile DI water, and examined under a dissecting microscope. Using a sterile scalpel and forceps, the outer layer of the orchid tissue was scraped. Fungal pelotons, visible under the

microscope as small, white, or cream, oblong to ovoid, granular structures, began to disperse in the sterile water droplet after scraping. The pelotons were also observable as hyphae coils within the root cortex.

The subsequent step involved completing a serial dilution of pelotons. Using a pipette, five water droplets of 400 µl were placed in one petri dish. Individual pelotons were then transferred from one water droplet to another at 1 µl increments using a pipette. Pelotons were collected from the isolation droplet with as little water as possible and ejected into the fresh droplet. This peloton transfer procedure was repeated for each droplet, with a fresh pipette employed between droplet changes. While some pelotons were lost in the water changes, a significant percentage were successfully recovered. Each root cutting was stored in Eppendorf tubes for subsequent use in DNA extraction procedures, following the methodology outlined by Swarts et al. (2010) with minor adjustments.

Molecular survey of root fungi

To identify any fungus that might not be culturable axenically, we extracted fungal DNA directly from the plant roots. Extraction of genomic fungal DNA was carried out using the DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany). The PCR amplifications were conducted for a selective screening process to identify mycorrhizal and non-mycorrhizal fungi. Three fungal specific primer pairs were used: ITS1F/4, ITS1OF/4OF, and ITS5/4Tul. primer sets ranged from general to specific, ITS 1F/4 is a universal fungal primer for entire Kingdom Fungi, the second pair is used to screen for known orchid fungi groups, and the third is used to selectively amplify fungi within the genus *Tulasnella*, one of the most common orchid mycorrhizal taxa. Primer mix consists of 6.25 µl Master Mix, 2.5 µl TBT buffer, 1.75 µl DD H₂O, 0.75 µl reverse primer, 0.75 forward primer, and 1.0 µl root or liquid culture DNA added. To prepare the PCR product for sequencing, a PCR clean up procedure and sequencing reaction was done. The PCR cleanup is done to remove enzymes, nucleotides, primers, and buffers. PCR cleanup was done using 2 µl ExoSAP-IT mix and 5 µl of PCR output and run on a thermocycler at 37°C for 15 min and then 80°C for another 15 min. The sequencing reaction is done to generate longer and higher quality reads by sequencing only primer at a time (forward or reverse primer for each of the 3 sets). This is done using the BigDye Terminator v3.1 Cycle Sequencing kit. This consists of 2 µl of DNA added to a PCR tube and a master mix of: 5.5 µl DD H₂O, 2.0 µl BigDye Buffer, 0.5 µl of 1.87 µM forward or reverse primer, 1.0 µl of Big Dye Terminator and 1.0 µl of PCR product post PCR clean up (Downing et al., 2020).

Statistical analysis

All statistical analysis was performed at $P < 0.05$ level using SPSS statistical software (Version 22.0., IBM Corp., Armonk, NY, USA). Data trends will be compared using one-way ANOVA and correlation tests, significant differences between variables will be found using post-hoc least significant differences (LSD) method. This data will be used to assess best management practices and optimal growing conditions for planting orchids from the lab into their native ranges.

Bioinformatics

Sequences obtained via sanger sequencing and distinct operational taxonomic units (OTUs) were identified using NCBI nucleotide BLAST with 97% similarity thresholds. Basic Local Alignment search tool was utilized to find the known fungal sequences with the highest similarities greater than 97%. All sequences were analyzed using Geneious software (Kearse et al., 2012). Fungal sequences were visualized and manually trimmed and aligned using the MAFFT plugin. PHYML plug-in was used to create estimations of phylogenies by generating consensus trees using neighbor-joining, and maximum likelihood with 100 random addition replicates.

Results

Effects of Host Trees on Ecophysiological characteristics and Plant Health in *Encyclia tampensis*

Demographic data, including orchid growth parameters such as height on the tree, shoot length, root length, and number of leaves, were collected to assess plant growth on different host trees. Plant health was evaluated based on chlorophyll content using SPAD and GreenSeekerTM handheld devices, as well as the abundance of OTUs of basidiomycetes and ascomycetes recovered per sample. No significant differences ($p > 0.05$) were observed among the various host trees in terms of orchid growth properties (Table 1). The highest mean heights on the tree were recorded on cypress trees ($176.60 \pm 11.28a$), followed by oak ($143.90 \pm 73.36a$), and palm ($154.38 \pm 10.39a$). Orchids on palms displayed the highest mean shoot length ($12.65 \pm 2.53a$), while cypress trees exhibited the greatest mean root length ($21.00 \pm 6.07a$) and the highest mean number of leaves ($16.40 \pm 9.71a$). Samples collected on oak showed the lowest mean shoot length (17.43 ± 15.10) and the lowest mean number of leaves ($12.33 \pm 7.29a$).

Table 1
Host Tree ANOVA Analysis of *Encyclia tampensis* Growth Properties

Host Trees	Height on the Tree (cm)	Shoot Length (cm)	Root Length (cm)	# of Leaves
Palm	154.38 ± 10.39a	12.65 ± 2.53a	18.7 ± 10.10a	15.20 ± 11.19a
Oak	143.90 ± 73.36a	10.94 ± 5.34a	17.43 ± 15.10a	12.33 ± 7.29a
Cypress	176.60 ± 11.28a	7.60 ± 1.64a	21.00 ± 6.07a	16.40 ± 9.71a

Effect of host tree on orchid growth properties. Independent variable is characterized as the host tree the orchid was sampled on (Palm at FTBG n = 5, Oak n = 5 at FTBG, n = 5 at DDP and n = 5 at NBG, and Cypress n = 5 at DDP). Values are expressed as Mean ± Standard Deviation. One-way ANOVAs were performed on the mean value of the factor evaluated. Means within a column followed by the same letter are not significantly different at $p \leq 0.0500$. Significantly different treatments were highlighted by adding a letter next to the mean value.

Additional parameters, including SPAD values, NDVI values, basidiomycetes, and ascomycetes, are detailed in Table 2. Orchids positioned on palms exhibited the highest average values for both SPAD and

NDVI, registering (56.18 ± 11.70) and (0.62 ± 0.06), respectively. Cypress trees recorded the lowest SPAD values at (33.90 ± 7.73). In contrast, oak trees displayed the lowest mean NDVI value at (0.51 ± 0.10). Significant differences in NDVI values ($F = 5.8676$, $DF = 2$, $P \leq 0.05$) were observed, with orchids on palm trees displaying significantly greater values compared to those on cypress and oak trees (Table 2).

Table 2
Host Tree ANOVA Analysis of *Encyclia tampensis* Plant Health Parameters

Host Trees	SPAD values	NDVI values	Basidiomycetes	Ascomycetes
Palm	$56.18 \pm 11.70a$	$0.62 \pm 0.06a$	$2.00 \pm 1.73a$	$0 \pm 0b$
Oak	$45.80 \pm 16.34ab$	$0.51 \pm 0.10b$	$1.40 \pm 1.30a$	$0.53 \pm 0.83ab$
Cypress	$33.90 \pm 7.73 b$	$0.42 \pm 0.07b$	$0.80 \pm 0.45a$	$1.20 \pm 1.10a$

Effect of host tree on orchid plant health parameters. Independent variable is characterized as the host tree the orchid was sampled on (Palm at FTBG $n = 5$, Oak $n = 5$ at FTBG, $n = 5$ at DDP and $n = 5$ at NBG, and Cypress $n = 5$ at DDP). Values are expressed as Mean $\pm$ Standard Deviation. One-way ANOVAs were performed on the mean value of the factor evaluated. Means within a column followed by the same letter are not significantly different at $p \leq 0.0500$. Significantly different treatments were highlighted by adding a letter next to the mean value.

Effects of Site Location on Ecophysiological characteristics and Plant Health in *Encyclia tampensis*

The phenotypic data was further analyzed based on the site, focusing on orchids sampled from oak trees. At FTBG, the site primarily featured naturally occurring orchids on old-growth and large oak trees. Naples Botanic Garden (NBG) had samples from naturally occurring orchids on dwarf sand live oaks, while the orchid samples from DDP were outplants grown in a laboratory setting and later transplanted onto smaller and less mature oak trees than those at FTBG, approximately two years prior to sampling. Orchids sampled at DDP displayed the highest mean height on the tree after being transplanted from laboratory growth (217.40 ± 55.33) but had the lowest mean shoot length (7.50 ± 2.57) and the highest mean number of leaves (16.60 ± 10.31). Orchids from NBG (120.30 ± 8.14) and FTBG (94.00 ± 71.72), which were naturally occurring on oak trees, had significantly lower mean heights on the trees compared to those in DDP ($F = 7.6607$, $DF = 2$, $P \leq 0.05$). FTBG samples ($17.40 \pm 2.75a$) had a significantly greater mean shoot length than those in DDP and NBG ($F = 21.9243$, $DF = 2$, $P \leq 0.05$) (Table 3). Samples from DDP had the highest average number of leaves (16.60 ± 10.31), while NBG had the lowest average number of leaves ($7.2 \pm 4.15b$), although this mean difference was not significant ($F = 2.63$, $DF = 2$, $P \leq 0.05$) (Table 3).

Table 3
Site Location ANOVA Analysis of *Encyclia tampensis* Growth Properties

Sites	Height on the Tree (cm)	Shoot Length (cm)	# of Leaves
FTBG	120.30 ± 8.14b	17.40 ± 2.75a	13.20 ± 2.39ab
DDP	217.40 ± 55.33a	7.50 ± 2.57b	16.60 ± 10.31a
NBG	94.00 ± 71.72b	7.92 ± 2.70b	7.2 ± 4.15b

Comparison of growth properties of orchids growing on oak trees within different site locations. Independent variable is characterized as the site location the orchid growing on oak was sampled from (n = 5 at FTBG, n = 5 at DDP and n = 5 at NBG). Values are expressed as Mean ± Standard Deviation. One-way ANOVAs were performed on the mean value of the factor evaluated. Means within a column followed by the same letter are not significantly different at $p \leq 0.0500$. Significantly different treatments were highlighted by adding a letter next to the mean value.

Significant variations were observed across plant health parameters at different site locations, with the exception of the number of ascomycetes (putative root pathogens) detected. Counts of basidiomycetes and ascomycetes were determined based on the number of OTUs recovered per sample. In some root samples, mycorrhiza was not recovered for various reasons, even though mycorrhiza may have been present. All three sites had a notable impact on chlorophyll content in the leaves, with FTBG exhibiting the highest SPAD values ($65.02 \pm 5.45a$) and DDP displaying the lowest ($30.90 \pm 6.38c$) (Table 4). The differences among all three sites were statistically significant ($F = 26.6956$, $DF = 2$, $P \leq 0.05$). Furthermore, FTBG's NDVI values were significantly higher than those at DDP and NBG ($F = 23.9122$, $DF = 2$, $P \leq 0.05$). The count of basidiomycetes was significantly higher at NBG compared to FTBG and DDP ($F = 6.6429$, $DF = 2$, $P \leq 0.05$) (Table 4).

Table 4
Site Location ANOVA Analysis of *Encyclia tampensis* Plant Health Parameters

Sites	SPAD values	NDVI values	Basidiomycetes	Ascomycetes
FTBG	65.02 ± 5.45a	0.63 ± 0.07a	0.40 ± 0.55b	0.40 ± 0.55a
DDP	30.90 ± 6.38c	0.43 ± 0.02b	1.20 ± 0.45b	0.8 ± 1.10a
NBG	41.48 ± 10.05b	0.45 ± 0.04b	2.60 ± 1.52a	0.40 ± 0.89a

Comparison of plant health parameters of orchids growing on oak trees within different site locations. Independent variable is characterized as the site location the orchid growing on oak was sampled from (n = 5 at FTBG, n = 5 at DDP and n = 5 at NBG). Values are expressed as Mean ± Standard Deviation. One-way ANOVAs were performed on the mean value of the factor evaluated. Means within a column followed by the same letter are not significantly different at $p \leq 0.0500$. Significantly different treatments were highlighted by adding a letter next to the mean value.

Correlation Table

SPAD values did not exhibit a correlation with the host tree from which the orchid was sampled. However, NDVI values demonstrated a positive correlation with palm trees and a negative correlation with cypress trees, with these correlations being the only ones significant to the host trees. As both SPAD levels and NDVI values increased, there was a significant decrease in measured light levels, accompanied by an increase in average leaf length. Ascomycetes count exhibited a negative correlation with NDVI values (Table 5). Higher levels of height on the tree were associated with significantly shorter average leaf and root lengths. There was a positive correlation between average leaf length and root length. Conversely, a negative correlation was observed between average root length and basidiomycetes count (Table 5).

Table 5 Bivariate correlation of all recorded orchid growth parameters.

	NDVI ^a	Light	Height on tree	Leaf Length	Root Length	No. of leaves	Basidio -mycetes	Asco -mycetes
SPAD	.660**	-.647*	-0.301	.687**	0.389	-0.058	0.032	-0.153
NDVI		-.730*	-0.281	.695**	0.428	0.262	-0.178	-.416*
Light Intensity			-0.304	-.777**	-0.997	-.687*	0.375	-0.016
Height on tree				-.440*	-.641**	0.21	-0.172	0.2
Leaf Length					.594*	-0.12	-0.232	-0.202
Root Length						0.114	-.531*	-0.145
No. of leaves							-0.235	0.131
Basidio -mycetes								-0.022

^a Normalized Difference Vegetation Index. ^b Representing Pearson's correlation coefficient (r) significant at $p \leq 0.05$ (*), $p \leq 0.01$ (**), or $p \leq 0.001$ (***).

Mycorrhizal Diversity

From 25 root samples, a total of 47 sequences were obtained, representing 42 distinct endophytic fungi, including 27 basidiomycetes OTUs (Table 6) and 15 ascomycetes OTUs (Table 7) with > 97% sequence similarity thresholds. Some sampled individuals hosted more than one fungus simultaneously. Tables 6 and 7 illustrate the distribution of different OTUs across various site locations and host tree species.

Table 6

List of fungal operational taxonomic units (OTUs) identified used to create Basidiomycetes phylogenetic trees. Fungi were grouped into OTUs defined by 97% internal transcribed spacer (ITS) sequence similarity.

ID	Location	Species	Host Tree	Primer	Outplant or natural	Related Taxonomic Group	Accession Number
R8	FTBG	ET	Oak	C1	N	<i>Tulasnella</i>	MH0644468.1
F10	FTBG	ET	Oak	C2	N	<i>Ipomoea</i> Chloroplast	NC_042939.1
R5	FTBG	ET	Palm	C2	O	<i>Tulasnella</i>	MH0644468.1
R4	FTBG	ET	Palm	C1	O	<i>Tulasnella</i>	MH0644472.1
R3	FTBG	ET	Palm	B2	O	<i>Tulasnella</i>	MH0644467.1
R2	FTBG	ET	Palm	C2	O	<i>Tulasnella</i>	MH0644468.1
R2	FTBG	ET	Palm	B2	O	<i>Tulasnella</i>	MH0644468.1
R1	FTBG	ET	Palm	C1	O	<i>Tulasnella</i>	MH0644468.1
D9	DDP	ET	Cypress	C2	O	<i>Tulasnella</i>	MH0644466.1
D8	DDP	ET	Cypress	C2	O	<i>Tulasnella</i>	MH0644467.1
D7	DDP	ET	Cypress	C2	O	<i>Tulasnella</i>	MH0644468.1
D6	DDP	ET	Cypress	C2	O	<i>Tulasnella</i>	MH0644468.1
L4 5 - 2	DDP	ET	Oak	C2	O	<i>Tulasnella</i>	MH0644468.1
L3 5 - 1	DDP	ET	Oak	C1	O	<i>Tulasnella</i>	MH0644468.1
D5	DDP	ET	Oak	C1	O	<i>Tulasnella</i>	KM211339.1
D4	DDP	ET	Oak	C2	O	<i>Tulasnella</i>	KM211335.1
D3	DDP	ET	Oak	C1	O	<i>Tulasnella</i>	KM211339.1
D2	DDP	ET	Oak	C1	O	<i>Tulasnella</i>	KM211339.1
D1	DDP	ET	Oak	C1	O	<i>Tulasnella</i>	JF691303.1
N5B	NBG	ET	Oak	C2	N	<i>Tulasnella</i>	MH0644466.1
N5B	NBG	ET	Oak	B2	N	<i>Tulasnella</i>	MH0644468.1
N5	NBG	ET	Oak	C2	N	<i>Tulasnella</i>	MH0644466.1
N5	NBG	ET	Oak	B2	N	<i>Tulasnella</i>	MH0644466.1

ID	Location	Species	Host Tree	Primer	Outplant or natural	Related Taxonomic Group	Accession Number
N2	NBG	ET	Oak	C1	N	<i>Tulasnella</i>	MH0644468.1
N2	NBG	ET	Oak	B2	N	<i>Tulasnella</i>	MH0644468.1
N1	NBG	ET	Oak	C1	N	<i>Tulasnella</i>	MH0644468.1
L6 4 - 2	NBG	ET	Oak	C2	N	<i>Tulasnella</i>	MH0644466.1

Table 7

List of fungal operational taxonomic units (OTUs) identified used to create Ascomycetes phylogenetic trees. Fungi were grouped into OTUs defined by 97% internal transcribed spacer (ITS) sequence similarity.

Source	Location	Species	Host Tree	Primer	Outplant or natural	Related Taxonomic Group	Genbank Accession Number
Root	FTBG	ET	Oak	A1	O	<i>Fusarium</i>	MN540868.1
F10	FTBG	ET	Oak	B2	N	<i>Anthrinium</i>	KF746108.1
F10	FTBG	ET	Oak	B1	N	<i>Arthrinium</i>	KF746108.1
L4 5 - 2	DDP	ET	Oak	A1	O	<i>Fusarium</i>	MT447548.1
L3 5 - 1	DDP	ET	Oak	A2	O	<i>Fusarium</i>	MT447548.1
D9	DDP	ET	Cypress	A2	O	<i>Sordaria</i>	MN341415.1
D9	DDP	ET	Cypress	B1	O	<i>Neurospora</i>	MH675494.1
D8	DDP	ET	Cypress	B2	O	<i>Alternaria</i>	MK649900.1
D8	DDP	ET	Cypress	A1	O	<i>Alternatia</i>	MT635195.1
D7	DDP	ET	Cypress	A1	O	<i>Alternaria</i>	MW478644.1
D7	DDP	ET	Cypress	B1	O	<i>Alternaria</i>	MW764299.1
D1	DDP	ET	Oak	B1	O	<i>Alternaria</i>	MT557073.1
D1	DDP	ET	Oak	A1	O	<i>Alternatia</i>	MN615420.1
L6 4 - 2	NBG	ET	Oak	A2	N	<i>Talaromyces</i>	MH856399.1
L5 4 - 2	NBG	ET	oak	A1	N	<i>Talaromyces</i>	MK849928.1

When considering differences in sample sizes, there were no discernible variations in the diversity of fungal associations between outplanted and naturally occurring individuals, featuring 10 and 17 OTUs, respectively. However, distinct but ecologically similar fungal assemblages were identified between host tree types and sites. For basidiomycetes, a total of 32 sequences were recovered from individuals across all sites and host tree types, encompassing 26 distinct OTUs, all within the genus *Tulasnella* (Fig. 2).

Tulasnella fungi were categorized into two closely related clades, Clades A and B. Both outplanted and naturally occurring plants were found to form associations with both *Tulasnella* clades A and B. Clade A, the more diverse group with 18 OTUs, was present in root samples collected across all sites and host tree types. Clade B fungi were exclusively identified in samples from southeastern Florida at FTBG and DDP, primarily from plants on hardwood trees, with one exception from a palm tree at FTBG.

The identified ascomycete fungi comprised a total of 15 OTUs representing six pathogenic genera. The most prevalent group was the genus *Alternaria*, featuring six OTUs, followed by *Fusarium* with three OTUs, and *Anthrinium* and *Talaromyces* with two OTUs each.

Additionally, one OTU each was attributed to *Neurospora* and *Sordaria* (Fig. 3). Orchids growing on oak trees exhibited the most diverse range of ascomycete fungi, including *Fusarium*, *Anthrinium*, *Alternaria*, and *Talaromyces*. Conversely, samples of *Sordaria*, *Alternaria*, and *Neurospora* were exclusively found in orchids on cypress trees. No ascomycetes were detected in orchids growing on palm trees. The phylogenetic analysis in Fig. 3 indicates three distinct clades: Clade A comprises fungi from outplanted orchids sampled from DDP on cypress trees; Clade B includes fungi from both outplanted and naturally occurring individuals at FTBG and DDP on oak trees; and Clade C consists of fungi from outplanted individuals growing on oak trees and cypress at DDP and NBG.

Ascomycete fungi identified consisted of a total of 15 OTUs consisting of six pathogenic genera. The most abundant group was the genus *Alternaria* with six OTUs, followed by *Fusarium* with three OTUs, *Anthrinium*, and *Talaromyces* with two OTU, and one OTU of *Neurospora* and *Sordaria* (Fig. 3). Orchids on oak trees harbored the most diverse ascomycete fungi: *Fusarium*, *Anthrinium*, *Alternaria* and *Talaromyces*. Samples of *Sordaria*, *Alternaria* and *Neurospora* were found only in orchids on cypress trees. No ascomycetes were detected in orchids growing on palm trees. In Fig. 3 the phylogeny suggests three distinct clades; Clade A fungi are from out planted orchids sampled from DDP on cypress trees, Clade B fungi are from outplanted and naturally occurring individuals at FTBG and DDP on oak trees, Clade C consists of fungi from out planted individuals growing on oak trees and cypress at DDP and NBG.

Discussion

The primary objectives of this study were twofold: 1) to investigate the potential influence of site location, microhabitat, and host tree type on the growth properties, plant health parameters, and mycorrhizal associations of *E. tampensis*, and 2) to assess the implications of these findings for enhancing orchid reintroductions into their native ranges. Abiotic factors exhibited variation across

different sites and host tree types (Shin et al., 2018). The results indicate that palm trees serve as particularly effective host trees in urban areas, as evidenced by samples exhibiting the greatest average leaf length, as well as high SPAD and NDVI values for *E. tampensis*. Plants growing on palm trees were also observed to establish diverse associations with beneficial OMF groups while harboring fewer plant pathogens, such as ascomycetes fungi. Regarding biotic factors, the overall findings suggest that diverse assemblages of closely related OMF within the genus *Tulasnella* are prominently utilized by *E. tampensis*. These associations are widespread across the South Florida region, persisting across various habitats and host tree types. This insight contributes to our understanding of the ecological dynamics and preferences of *E. tampensis*, offering valuable considerations for optimizing orchid reintroduction efforts in their native environments.

Implementing a rapid and cost-efficient handheld plant health monitoring sensor to assess chlorophyll content proves invaluable for quantifying various stresses, both biotic and abiotic on various palm several palms (Khoddamzadeh et al., 2023), orchids (Porrás-Alfaro, 2007; Mullin et al., 2022), cacao (Khoddamzadeh and Souza Costa, 2023), red shrimp plant (Freidenreich et al., 2019), and cocoplum (Souza Costa et al., 2023). In this context, palm trees emerged as the most promising host tree for re-establishment, displaying high SPAD and NDVI values, along with the presence of suitable OMF and a low count of ascomycetes. However, for plant health parameters, chlorophyll content was significantly higher on palm trees compared to oak and cypress trees. This suggests that microclimate conditions on palm trunks, expected to be hotter and drier than the canopies of hardwood trees, may influence chlorophyll content (Pérez-Patricio et al., 2018). Previous studies have indicated that microclimate conditions can impact chlorophyll fluorescence measures in tropical epiphytes under drier and hotter conditions than natural settings (McCormick et al., 2009; Crain and Tremblay, 2017; Cevallos et al., 2017). Our findings highlight negative impacts on plant health due to abiotic microclimate conditions, although not necessarily on the ability to form mycorrhizal associations or overall survivorship.

Orchids on oak trees at FTBG exhibited the highest averages for SPAD, NDVI, and shoot length compared to DDP and NBG, which may be attributed to the size of the host trees. A study on Green Fly Orchid host tree selection suggested that Southern Magnolia, with a larger diameter and presumably older than average available trees, was the most common host and critical to the orchid population's viability (Bergstrom and Carter, 2008; Jaspreet et al., 2021). Conversely, outplants at DDP on young cypress and oak trees displayed a greater average number of leaves and lower SPAD values. These results indicate that microclimatic conditions correlate with host tree size and exert more influence on plant growth characteristics than the host tree type.

A study on nursery-grown orchids utilized NDVI values for precision pesticide spraying against harmful pathogens, as healthy and infected orchids exhibited significantly different values (Samseemoung et al., 2017). High light intensity was negatively correlated with leaf length and the number of leaves, aligning with a previous study suggesting optimal light intensity for *Phalaenopsis* orchid between 200 and 300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, with photo inhibition and reduced carbon dioxide fixation occurring above 400 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (Lin et al., 2019). Average NDVI values also correlated with greater leaf length and negatively

correlated with ascomycetes association. Additionally, root average was negatively correlated with basidiomycetes count, implying that roots may proliferate in greater numbers when fungi are absent, promoting increased water and nutrient acquisition.

In this study *E. tampensis* in southern Florida associates predominantly with two closely related clades of *Tulasnella* fungi. The *Tulasnella* OTUs identified from outplants in this study and are consistent with those identified in the naturally occurring populations (Stewart et al. 2003, Zettler et al. 2007, Fuji et al., 2020; Silva et al., 2020, Downing et al. 2020), indicating that urban/ non-natural areas are able to support the appropriate OMF across different native host trees; palm, oak and cypress species. Knowing the presence and identity of OMF on varying host trees and at different sites within orchid roots can facilitate the process of selecting suitable habitats and compatible orchid species for translocation or reintroduction purposes. Florida orchids exhibit differing levels of mycorrhizal specificity (Stewart et al. 2003, Zettler et al. 2007, Downing et al. 2020; Quentin et al., 2020). Here *E. tampensis* only associated with one genus and two distinct clades of fungi. Similar studies show other co-occurring orchid species commonly associated with more than one genus of OMF (Downing et al. 2020). While these specific fungi are seemingly widespread across the region, their prevalence underscores a high degree of mycorrhizal specificity for this species. Understanding this specificity is crucial as it can impact a species' suitability for *ex-situ* conservation efforts.

Root samples also yielded Ascomycetes, including *Neurospora*, *Anthrinium*, *Fusarium*, *Alternaria*, *Sordaria*, and *Talaromyces*. These taxa are recognized as potential pathogens, causing issues such as leaf spotting, root rot, and wilt (Samseemoung et al., 2017; Jiang et al., 2019). Future studies are warranted to comprehend the impact of these root pathogens on the health of epiphytic orchids and to determine effective remedies, such as pruning and chemical treatments. Bivariate correlation analysis revealed a negative correlation between NDVI values and the count of ascomycetes. Ascomycetes fungi, identified as putative root pathogens in this context, were significantly more prevalent in hardwood trees compared to palm trees. These findings align with the overarching hypothesis that a species' ability to establish, and spread is influenced by various types of biotic interactions in the recipient range (Compant et al., 2010). A recent study indicated that native orchids were more likely to host pathogenic fungal groups (i.e., co-evolved) than co-occurring non-native congeners, suggesting that the enemy release hypothesis also plays a role in orchid naturalization and reintroductions (Dickie et al., 2017; Pecoraro et al., 2018; Downing et al., 2020). The enemy release theory posits that invasive species experience fewer impacts from enemies than native species, as they are released from parasites that typically regulate their growth in their native environment (Middleton, 2019).

Conclusions

The host tree type, rather than site differences, exerted the most significant impact on growth factors, plant health, and the presence of root pathogens on *E. tampensis*. Previous reintroductions have demonstrated palm trees to be the optimal host for *E. tampensis* establishment. However, the success of outplants on oak and cypress trees in urban areas compares favorably with those in natural habitats.

Analysis of biotic factors indicates that *E. tampensis* specializes in closely related *Tulasnella* fungi, which appear widely distributed on trees in southern Florida. Collectively, these findings underscore the suitability of palm and hardwood trees in urban spaces for supporting future orchid reintroduction efforts. Conservation management practices in urban areas are crucial for contributing to biodiversity preservation in the face of habitat losses. Identifying the mycorrhizal associations of each orchid species is vital for the success of orchid conservation programs. Recommendations for future studies include conducting symbiotic germination tests and cryopreservation of axenically cultured OMF. This would provide insights into whether the OMF enabling germination is present in urban areas. Little is known about the functionality of ascomycetes fungi on orchid seedling growth or their antagonistic interactions with OMF taxa. Further studies would be beneficial in understanding how these pathogens affect orchid growth, OMF acquisition, and determining the most suitable strategies for mitigating any resulting health effects. Testing populations from additional natural habitats, such as Everglades National Park and Big Cypress National Preserve, would offer insight into the role of orchids as potential ecosystem health indicator species and how to better protect vulnerable species, enabling endangered and threatened species to return to a functional, secure, and increasingly abundant population. The findings of this study indicate that the abiotic and biotic factors necessary for the establishment of *E. tampensis* are present in urban environments. Notably, the presence of compatible OMF across multiple host trees and site locations highlights numerous potential suitable orchid habitats. Conservation efforts should prioritize keystone OMF that enable orchid germination and subsequent growth. Research is imperative to enhance our understanding of the biotic and abiotic conditions influencing orchid growth and how these factors shape their geographical distribution and niche requirements. With proper planning, urban environments can indeed serve as viable habitats for epiphytic orchid species, playing a crucial role in mitigating the risk of biodiversity loss.

Declarations

Author Contribution

Author Contributions: Conceptualization, A.K. and J.D.; methodology, E.G., A.K., J.D; software, J.D. and E.G.; validation, A.K. and J.D.; formal analysis, E.G., A.K. and J.D.; investigation, E.G and A.K.; resources, A.K. and J.D.; data curation, E.G.; writing-original draft preparation, E.G.; writing-review and editing, A.K., and J.D; visualization, E.G. A.K., J.D. and supervision, A.K. and J.D.; project administration, A.K.; funding acquisition, A.K. All authors have read and agreed to the published version of the manuscript.

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Figures



Figure 1

Photos taken at sampling sites. (1) *Encyclia tampensis* on *Quercus Germinata* at Naples Botanic Garden, (2) Pine Rockland habitat at Naples Botanic Garden where naturally occurring orchids were sampled from, (3) *Encyclia tampensis* outplant attached to *Quercus virginiana* by means of zip tie, and (4) *Quercus virginiana* host tree at Downtown Doral Park.

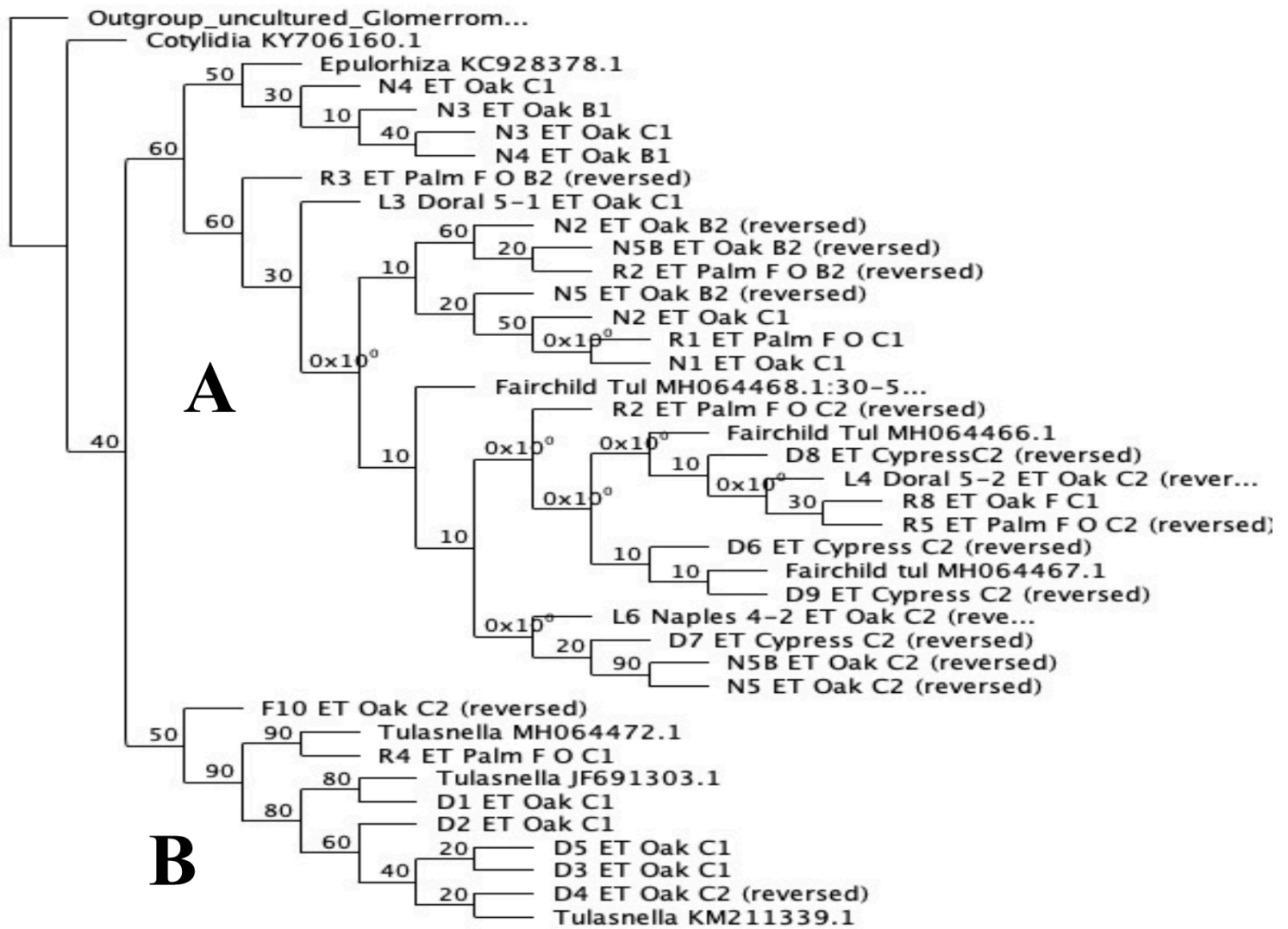


Figure 2

Phylogenetic tree of Basidiomycetes sequences obtained from *Encyclia tampensis* roots. Maximum likelihood trees of ITS sequences of Basidiomycetes mycorrhizal fungi isolated from *Encyclia tampensis* on 3 different host trees: oak, palm and cypress and within 3 different site locations: FTBG, DDP, and NBG. Depicted are the relationships among clades A and B. Numbers show the bootstrapping percentage that supports the branches.

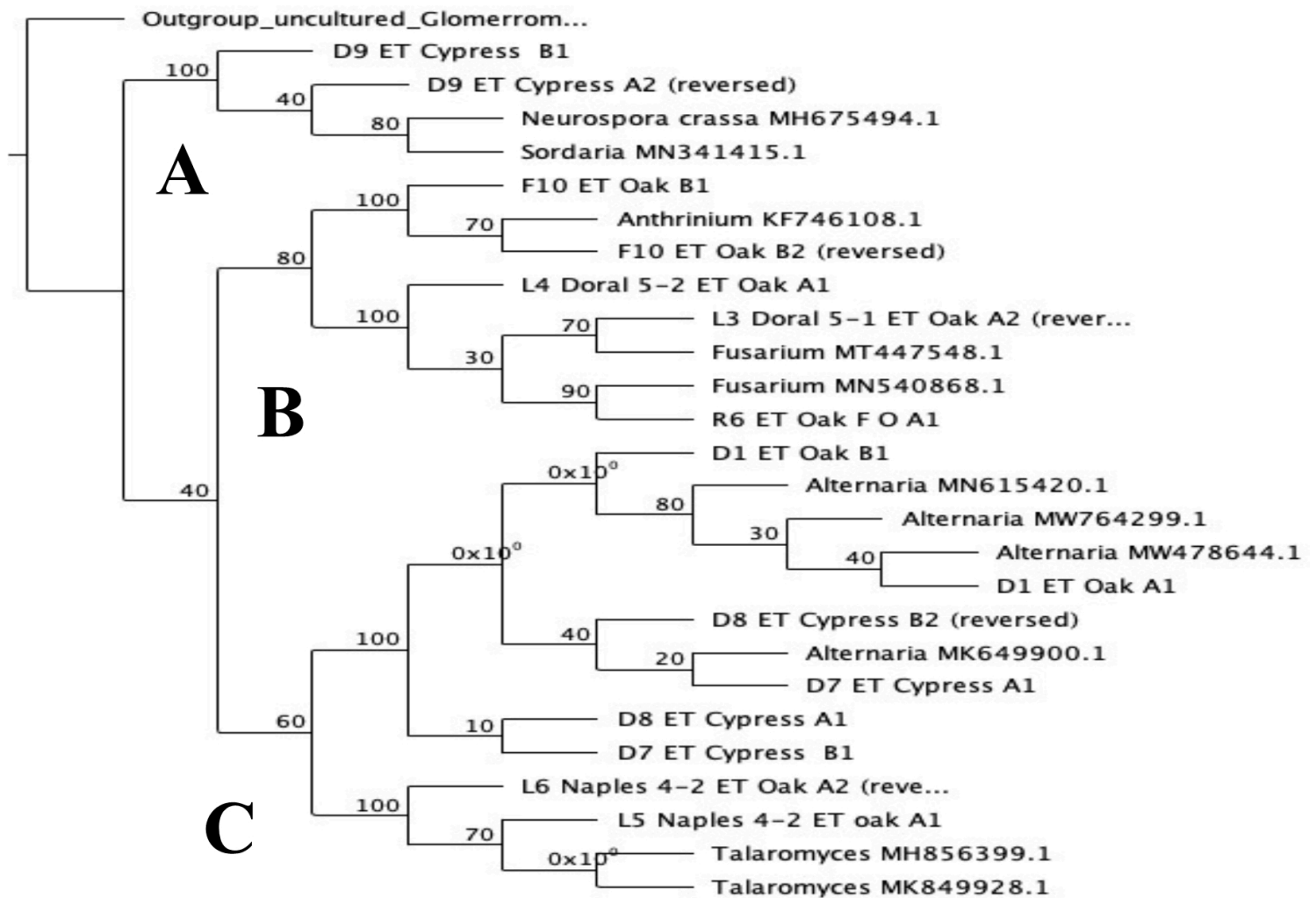


Figure 3

Phylogenetic tree of Ascomycetes sequences obtained from *Encyclia tampensis* roots. Maximum likelihood trees of ITS sequences of Basidiomycetes mycorrhizal fungi isolated from *Encyclia tampensis* on 3 different host trees: oak, palm and cypress and within 3 different site locations: FTBG, DDP, and NBG. Depicted are the relationships among clades A, B, and C. Numbers show the bootstrapping percentage that supports the branches.