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Mycorrhizal status of *Guarianthe skinneri* (Orchidaceae) in urban trees in Tapachula, Chiapas, Mexico

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ABSTRACT

Objective: To isolate and classify morphologically and molecularly mycorrhizal fungi associated with adult plants of *Guarianthe skinneri* (Bateman) Dressler & W.E. Higgins (Orchidaceae), distributed in different phorophytes, exotic and native trees, in the City of Tapachula, Chiapas, Mexico.

Design/Methodology/Approach: We sampled roots from adult plants growing in two native phorophytes, *Byrsonima crassifolia* (L.) KUNTH and *Tabebuia rosea* (BERTOL.) BERTERO EX A. DC. and two exotic phorophytes, *Terminalia catappa* L. and *Ficus benjamina* L. located in the city's road. By the isolation of mycorrhizal strains, we had diagnosed them by morpho-physiological attributes, and molecularly (Sanger sequencing of the ITS1-4 region).

Results: Forty-three fungal strains of two anamorphic mycorrhizal genus *Epulorhiza* and *Ceratorhiza* were obtained: 50% of the isolates came from plants growing in the exotic tree *T. catappa* with undigested pelotons and the highest molecular diversity (three contigs of the genus *Tulasnella*). *Ficus benjamina* had one molecular species shared with the native *B. crassifolia*. Roots growing in the native *T. rosea* tree, even though few isolates could be purified.

Study Limitations/Implications: Even if the research was exploratory, it was possible to highlight the diverse mycorrhizal partners that urban phorophytes of *G. skinneri* harbor, showing their potential in the *ex situ* conservation of this species.

Findings/Conclusions: The large number of the anamorph *Epulorhiza* isolates obtained from all phorophytes, reinforces previous observations suggesting that candelaria is preferentially associated with species of the Family Tulasnellaceae.

Keywords: *Ceratobasidium*, *Ceratorhiza*, *Epulorhiza*, *ex situ* conservation phorophyte, *Tulasnella*.

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INTRODUCTION

The epiphytic orchid *Guarianthe skinneri* (Bateman) Dressler & W. E. Higgins is naturally distributed from southeastern Mexico to Panama (Bertolini *et al.*, 2016) and it is listed as threatened in the Mexican Official Legislations (NOM-059-SEMARNAT-2010) (SEMARNAT, 2010), due to illegal trade, land-use change and logging (Coello *et al.*, 2010).

Image by John French at Pixabay

Coutiño-Cortés *et al.* (2018) showed that the genetic diversity of this species may be higher in the urban population of Tapachula, Chiapas, compared to some wild populations of Guatemala. This has been attributed to the diverse provenances of the different established individuals. Backyard and urban cultivation on existing host trees allows this "urban domestication" to occur in transition to colonize new phorophyte species (Cortés-Anzúres *et al.*, 2020). However, the long-term survival, reproduction, and recruitment of these urban populations may be influenced by the tree species that harbor them (phorophyte) and the capability of seed germination on phorophytes (Emeterio-Lara *et al.*, 2021). Part of this adaptive ability depends on their associations with mycorrhizal fungi living in the different phorophyte species (Hossain *et al.*, 2013; Idris and Zaman, 2020; Izuddin *et al.*, 2019). Mycorrhizae that performs symbiosis with orchids belong to the anamorphic artificial polyphyletic group *Rhizoctonia* (López-Chávez *et al.*, 2016), which *Ceratorhiza*, *Moniliopsis*, *Epulorhiza* are recognized as their mainly anamorphic (asexual) genera and whose teleomorphic (sexual) form *Ceratobasidium*, *Thanatephorus*, *Tulasnella* and *Sebacina* are identified through molecular tools by the absence of sexual structures (Nontachaiyapoom *et al.*, 2010; Shimura *et al.*, 2009). The identification of fungi associated in already established orchids is fundamental for the conservation, and ecological restoration of endangered or threatened species (Etanke *et al.*, 2021; Ortega-Larrocea and Rangel-Villafranco, 2015; Suryantini *et al.*, 2015; Zettler *et al.*, 2000). It has been documented that host-mycorrhizal fungus specificity may be determined under natural conditions by forest species (Martins *et al.*, 2020). Therefore, the objective of this research was to know if the native orchid *G. skinneri* develops mycorrhizal associations in their roots in relationship to his phorophytes, exotic or native trees, in the urban context of Tapachula, Chiapas, Mexico. This provides a better understanding about ecological requirements to guarantee mycorrhizal relationships for the conservation and management of this orchid in a strongly anthropic environment.

MATERIALS AND METHODS

Collection of biological material. Root sampling of *G. skinneri* was carried out during January 2021 from two native phorophytes: *Byrsonima crassifolia* (L.) KUNTH and *Tabebuia rosea* (BERTOL.) BERTERO EX A. DC; as well as from two exotic ones: *Terminalia catappa* L. and *Ficus benjamina* L., both from South and Southeast Asia. The exotic phorophytes were located further in the North part of the city of Tapachula de Córdova y Ordoñez Chiapas, Mexico, while native trees were located slightly further South (Figure 1).

To know the micro-climate of sampling sites, we used data from Worldclim (http://www.worldclim.org/). From each plant, three main roots were collected and preserved under refrigeration for immediate analysis of mycorrhizal fungi in the laboratory of Ecology and Sustainable Cultivation of Soconusco Orchids at El Colegio de la Frontera Sur, Tapachula campus (Figure 2).

Isolation and characterization of mycorrhizal fungal isolates. Collected roots were washed with tap water to eliminate organic matter; velamen was removed with a scalpel under a dissecting microscope. To evince pelotons as mycorrhizal structures in the cortex, transversal cuts about 1 mm thick were made every 2 cm along the root and stained with 0.1% acid fuchsin in polyethylene glycol. Segments were analyzed

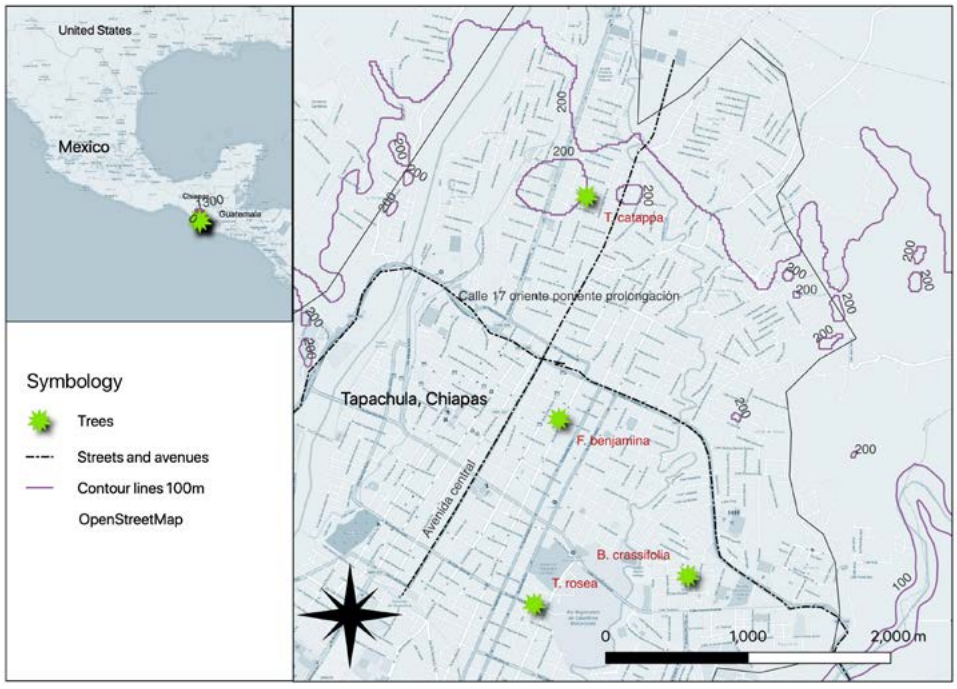

Figure 1. Location of sampled sporophytes were adult *G. skinneri* that were stablished/found in Tapachula, Chiapas, Mexico.

Figure 2. Appearance of the orchid *Guarianthe skinneri* over different host trees in the city of Tapachula, Chiapas, Mexico. a: Phorophyte *Ficus benjamina*; b. Phorophyte *Byrsonima crassifolia*; c. Phorophyte *Tabebuia rosea*; d. Root growth on *F. benjamina* bark (arrow). e. *In situ* recruitment of a near-rooted seedling growing on *Terminalia catappa* bark. f. Cross section of a root growed on *Terminalia catappa* tree, showing undigested mycorrhizal fungal structures (pelotons) stained with acid fuchsin.

under a microscope at 20X and percentage of colonization was calculated according to Rasmussen and Whigham (2002) (Figure 2f). Another 3 mm thin cut was made immediately to the root section where pelotons were observed and sections were placed over MCD LAB® bacteriological agar at 33 g/L in 9 cm Petri dishes (Bertolini *et al.*, 2012). Dishes were incubated at 25±2 °C in the dark. Hyphal tips that emerged within 24 to 36 hours were subcultured twice on the same medium to obtain pure cultures. To observe colony development, they were transferred to BIOXON® potato dextrose agar (PDA) medium at 39 g/L and pH 6.8 under the same incubation conditions. Four-points radial daily growth, colony color and shape at 14 days after culture were recorded. The number of nuclei were observed on hyphae growing in a slide placed on a wet filter inside Petri dishes under axenic conditions incubated under the same conditions for four days. Slides were stained with 200 μL of solution containing 1 μL of 10 000X SYBR®Green I in 10 mM KH_2PO_4 and 18% glycerol for 8 min and washed with 2 mL of sterile water. Observation was done under the Axio Imager2 fluorescence microscope coupled to an AxioVision® image analyzer (CarlZeiss®) at 40 X. Morphometric characters were recorded in light microscopy on hyphae stained with acid fuchsin-stained. The enzymatic polyphenol oxidase test was performed by inoculating each strain in 7.5 g of bacteriological agar and 5 g of malt agar in 425 mL of distilled water with 2 g of tannic acid dissolved in sterile 75 mL distilled water passed through a GELMAN® acrodisc filter with pore size of 0.45 μm. Plates were incubated under the same conditions for five days to record the reaction. A one-way ANOVA was performed to evaluate the differences between the isolates obtained for hyphal width and growth with R Studio® software.

DNA isolation and molecular identification of mycorrhizal fungal isolates. DNA extraction from previously diagnosed strains was carried out with the Promega Wizar Genomic DNA Purification® kit, previously subcultured for one week in potatoe dextrose Broth in sterile 2 mL Eppendorf tubes with apical explants of approximately 3 mm^3. The explant was transferred to microtubes after a rinse with sterile distilled water in a vortex; extraction was performed according to the supplier's instructions. PCR amplification was made with the specific primers ITS1-ITS1F/ITS4. The amplification conditions were PDN 95 °C −2', DN 95 °C −1", AL 55 °C −1", EX 72 °C −1" (22 cycles), EX 72 °C −1" EX final 72 °C −8'. The successfully amplified samples were selected for Sanger sequencing and assembled with Geneious Prime® software (version 2020.0.5), using the Geneious Assembler. Initial regions with weak signals were removed generating a total of six contigs from which consensus sequences were drawn and aligned using MAFFT plugin (Katoh *et al.*, 2002; Katoh and Standley, 2013) to submit to GenBank database using Basic Local Alignment Search Tool (BLAST) (Max E-Value:10, Word size: 11, Gap cost: 5 2) (Altschul *et al.*, 1997). For some of the strains, no sequences were obtained because they could not be assigned to any contig due to low assembly quality and errors (strain 18) or because they were contaminated and not submitted (strains 16 and 34). Phylogenetic reconstruction was done under the Maximum Similarity Procedure (Tamura-Nei substitution model, 1000 node support bootstrap) (Guindon *et al.*, 2010), using MEGA X® (version 10.2.6) and Geneious® program (Version 8.0.4) to

obtain phylogenetic trees. The outgroups selected were *Trementella mesenterica* (AY463475) (Veldre *et al*., 2013) and *Auricularia auricula-judae* (AF291289) (Weiss and Oberwinkler, 2001). Analyses were done for each of the families obtained.

RESULTS

Mycorrhizal colonization, isolation and characterization of strains. All roots were slightly colonized by mycorrhizae. The highest colonization percentage was found in roots growing over the *T. catappa* tree, with 21%, followed by *F. benjamina*, *B. crassifolia* and *T. rosea*, which showed similar rates (13%, 13% and 12%, respectively). The environmental conditions at each site were very similar for all parameters, only the annual rainfall was slightly higher (150-200 mm) at sites where exotic photophytes *T. catappa* and *F. benjamina* species were located.

A total number of 43 fungal strains were purified. From native phorophytes, four isolated from roots on *B. crassifolia* and seven on *T. rosea*. From exotic ones, 21 strains were obtained from roots grown on *T. catappa* and 11 on *F. benjamina*. The morphometric and appearance characteristics of the colonies are shown in Table 1.

All isolates belonging to anamorphic mycorrhizal fungi *Rhizoctonia* (right angles with bifurcation at 90°) (Figure 3b and 3g) and all measured features correspond to the diagnostic characters. The hyphal width was from 2.4 to 5.9 μm, where statistically, isolates 46-48 were significantly different presenting wider hyphae than the rest. The formation of monilioid cells was present in most fungal strains where in isolates smaller than 4 μm were spherical cells with clear septa typical of *Epulorhiza anamorphs*. Instead, *Ceratorhiza monilioids* were slightly discernible digitiforms without evident septal separation (Figure 3c and 3f, respectively). The number of nuclei of all isolates was two per cell (Figure 3d and 3h).

The strains developed two growth rates; slowly ones growing from 1.2 to 6.0 mm per day and fast growing developed 17.0 to 18.2 mm per day, the latter corresponding to three isolates obtained from the anamorph *Ceratorhiza*. Also, two types of growth appearance were evident: smooth creamy with submerged hyphae, sometimes cottony, which corresponded to the strains with lower growth rates, and cottony with rings, which occurred in the strains with higher growth rates (Figure 3a and 3e). Colony color evaluated after 14 days of development; was white to creamy in the low growth strains and white in the faster growth strains. The polyphenol oxidase test was negative for all strains except for strain 19 corresponding to one fungus isolated from the *T. catappa* phorophyte, which had a weak reaction compared to the three isolates of strains 46-48 obtained from the *T. rosea* phorophyte.

The molecular identity of the 40 sequences corroborated the morpho-physiological diagnosis corresponding to the two teleomorphic families Tulasnellaceae and Ceratobasidiaceae (Figure 4). Within the family Tulasnellaceae, four consensus sequences or contigs were grouped: 17 sequences belonged to contig 1 which was phylogenetically separated from all others in a group apart and represented those isolates with wider hyphae, mostly coming from the native tree *T. rosea* and the exotic one *T. catappa*. In another clade formed by two contigs, 2 and 4 which had slight differences in growth rates, 13 sequences

Table 1. Morphometric features of mycorrhizal fungal strains isolated from the orchid *Guarianthe skinneri* in Tapachula, Chiapas, Mexico growing on the phorophytes *Terminalia catappa* (strains no. 1-25); *Ficus benjamina* (26-37); *Byrsonima crassifolia* (38-41) and *Tabebuia rosea* (42-49). PFO=polyphenol oxidase reaction; CM=monilioid cells, CSB=basal septum constriction. Mean ± standard error, different letters mean significant differences at $p<0.05$.

Strain	Colour	Appearance	PFO	Growth	Hypal width	CM	CSB	Mycelium rings
1C1	creamy white	creamy smooth	–	5.8±0.8ac	3.39±0.11a	–	–	+
2C1	creamy white	creamy	–	5.4±0.8ac	2.95±0.09b	+	–	–
3C1	white	cottony	–	6.0±0.5c	3.81±0.12c	–	–	+
4C1	creamy white	creamy smooth	–	4.0±0.4b	4.22±0.10d	+	+	+
5C1	creamy white	creamy smooth	–	4.9±0.4a	3.84±0.14c	+	+	–
6C1	white	creamy smooth	–	4.6±0.2a	4.36±0.16d	+	–	+
8C1	creamy white	cottony	–	4.9±0.3a	3.39±0.10a	+	+	+
9C1	creamy white	cottony	–	4.0±0.2b	4.30±0.13d	+	+	+
10C1	creamy white	creamy smooth	–	4.9±0.3a	4.97±0.17e	+	+	+
12C1	creamy white	creamy smooth	–	6.2±0.6c	3.83±0.14c	+	+	+
14C4	creamy white	creamy smooth	–	4.4±0.6ab	3.07±0.10b	+	+	–
16NA	creamy white	cottony	–	3.5±0.1d	4.51±0.18d	+	+	–
17C2	creamy white	cottony	–	1.5±0.4e	3. 01±0.11b	+	+	–
18NA	creamy white	cottony	–	1.8±0.3e	2.80±0.08f	+	+	–
19C2	creamy white	cottony	+	2.3±0.1f	3.05±0.07b	+	+	–
20C4	creamy white	cottony	–	1.6±0.2e	2.76±0.2bf	+	+	–
21C4	creamy	creamy smooth	–	2.0±0.2ef	3.36±0.08a	+	+	+
22C1	creamy white	cottony	–	5.9±0.3c	3.40±0.12a	+	+	–
23C1	white	creamy smooth	–	4.8±0.4[a]	2.89±0.18bf	+	+	+
24C1	white	Algodonoso	–	4.6±0.3ab	2.98±0.09b	+	+	+
25C2	creamy white	creamy smooth	–	1.5±0.2g	2.56±0.08g	+	+	+
26C2	creamy white	creamy smooth	–	2.2±0.2ef	2.57±0.11g	+	+	+
27C2	creamy white	creamy smooth	–	1.7±0.4eg	2.72±0.08gf	+	+	–
28C2	creamy white	creamy smooth	–	2.6±0.5e	2.33±0.08h	+	+	–
29C2	creamy white	creamy smooth	–	1.2±0.1h	2.58±0.07g	+	+	+
30C2	creamy white	creamy smooth	–	2.5±0.3ef	2.81±0.18bfg	–	+	–
31C2	creamy white	creamy smooth	–	2.4±0.1f	2.76±0.03f	–	+	+
32C2	creamy white	creamy smooth	–	1.9±0.2e	2.71±0.10fg	–	+	–
33C2	creamy white	creamy smooth	–	2.5±0.4ef	2.60±0.06g	+	+	+
34NA	creamy white	creamy smooth	–	s.d.	2.48±0.07g	+	+	–
36C2	creamy white	creamy smooth	–	2.1±0.1e	2.97±0.18b	–	+	–
37C2	creamy white	creamy smooth	–	1.8±0.0g	2.34±0.09h	–	+	–
38C3	creamy white	creamy smooth	–	3.1±0.0e	2.64±0.07g	–	+	–
39C3	white	creamy smooth	–	2.4±0.2ef	2.44±0.10hg	–	+	+
40C3	white	cottony	–	3.2±0.2e	2.76±0.03bf	+	+	–
41C3	white	creamy smooth	–	4.0±0.3b	3.79±0.15c	–	+	–

Table 1. Continues...

Strain	Colour	Appearance	PFO	Growth	Hypal width	CM	CSB	Mycelium rings
42C1	white	creamy smooth	–	4.8±0.6ab	2.86±0.10bf	+	+	+
43C1	white	creamy smooth	–	4.8±0.4a	3.26±0.23ba	–	+	+
44C1	creamy white	creamy smooth	–	3.7±0.4bd	3.16±0.14b	+	+	+
46C5*	white	cottony	++	17.0±0.8i	5.88±0.24i	–	+	–
47C5*	white	cottony	++	17.7±0.4i	5.23±0.17je	–	+	–
48C5*	white	cottony ringed	++	18.2±1.2i	5.62±0.30ij	–	+	–
49C1	creamy white	cottony	–	5.6±0.3ac	3.71±0.16c	+	+	+

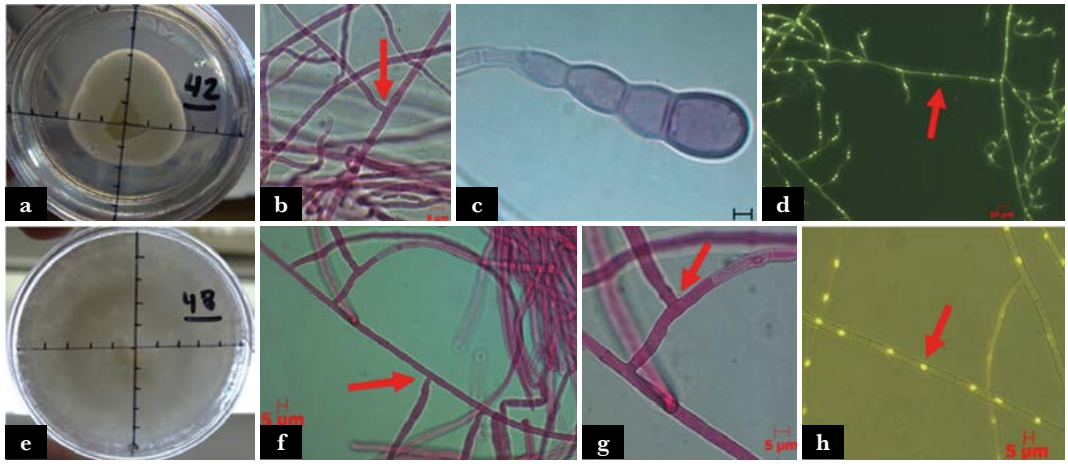

Figure 3. Anamorphs of the mycorrhizal fungi *Epulorhiza* (a-d) and *Ceratorhiza* (e-h) isolated a. Radial growth of strain 42. b. Mycelial growth of strain 9 stained with acid fuchsin. c. Monilioid cells stained with acid fuchsin from strain 20. d. Binucleate cells of hyphae strain 37 under fluorescence. e. Sterile mycelial of strain 48. f. Monilioid hyphae of strain 48 stained with acid fuchsin. g. Hyphal right-angle bifurcation and basal septum of strain 48. h. Binucleate cells of strain 47 seen by fluorescence.

were grouped in contig 2, isolated from the introduced trees *T. catappa* and *F. benjamina* and three to contig 4 isolated from the exotic tree *T. catappa*. A third clade was formed with contig 3 with sequences isolated from native *B. crassifolia*. In the Family Ceratobasidiaceae, only one clade, contig 5, was formed by three isolates obtained from the native phorophyte *T. rosea* (Figure 4).

G. skinneri roots from exotic phorophyte *T. catappa* showed the highest number of isolates (48.8% of total) with characteristics of the anamorph *Rhizoctonia*, or the highest percentage of mycorrhizal colonization and the highest annual precipitation in their location. Roots from *F. benjamina*, located near *T. catappa*, showed 25.5% of the anamorph *Rhizoctonia* strains purified. Concerning native phorophytes, *B. crassifolia* and *T. rosea*, fewer isolates were achieved from them (9.3 % and 16 % respectively), as they were found in a slightly less humid and warmer location with less lush canopy due to pruning. While all phorophytes developed under the same climate, microenvironmental conditions are known to influence the degree of mycorrhizal colonization and species richness on air exposed epiphytic roots

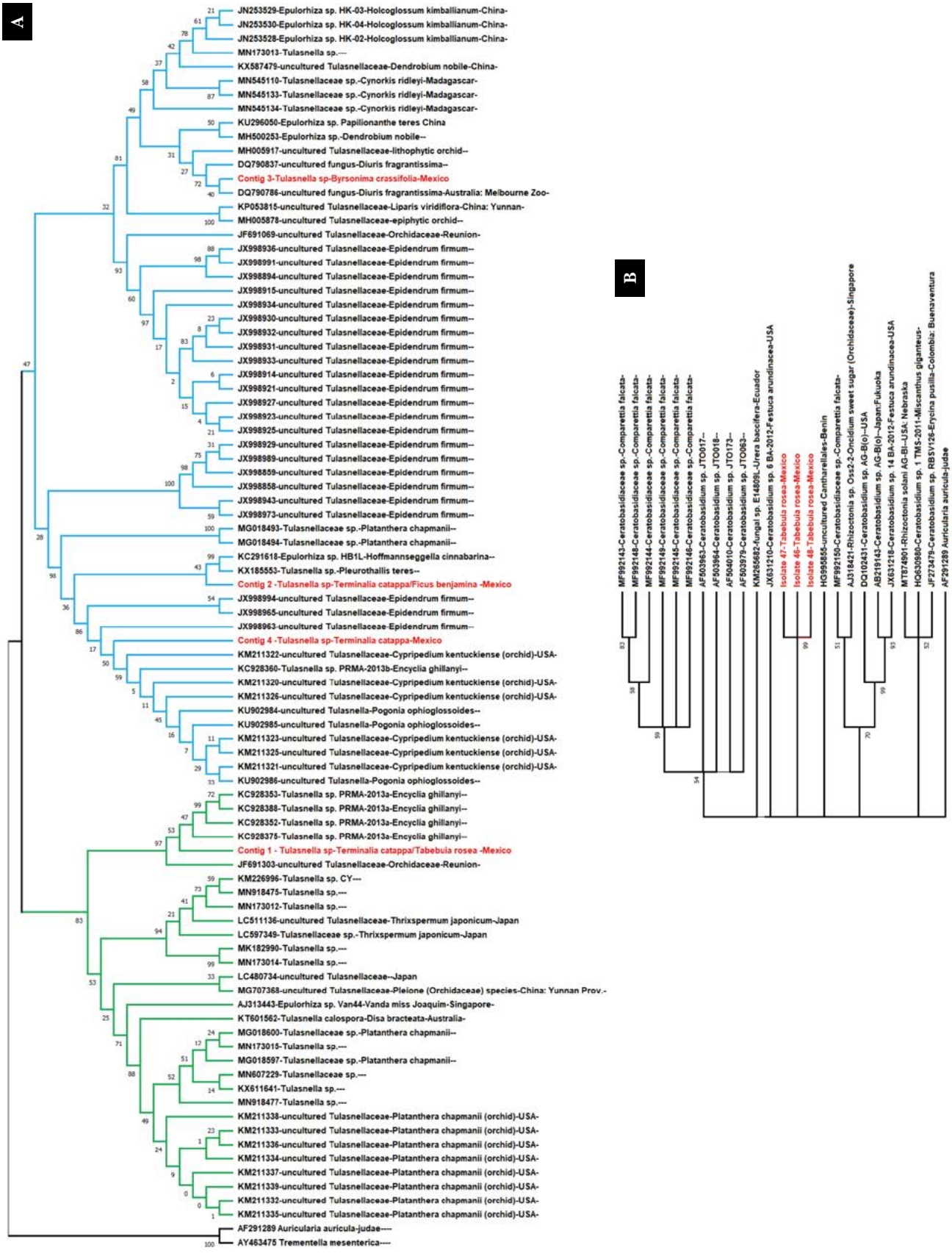

Figure 4. A) Phylogenetic tree of mycorrhizal fungal isolates (contigs 1-4) associated with the Family Tulasnellaceae in the epiphytic orchid *G. skinneri*. The tree was constructed under Maximum Similarity (Tamura-Nei substitution model, 1000 node support bootstrap). B) Phylogenetic tree of contig 5 associated with the Family Ceratobasidiaceae.

very sensitive to dryness (Izunddin *et al.*, 2019). In this urban microclimate at Tapachula City, tree position favored exotic phorophytes by their location, especially in *T. catappa*, exposed to a microenvironment with lower average temperature and receiving a little more precipitation in a year. This had been previously recorded for this species by Ovando *et al.* (2005) who found mycorrhizal colonization favored by the growing conditions and a lower incidence of mycorrhiza under water stress in cultivated plants (Rivas *et al.*, 1998).

Morphological features of strains are quite useful to separate them into the two most common anamorphic mycorrhizal genera: *Epulorhiza* and *Ceratorhiza*. The three isolates belonging to the latter genus were distinguished by forming concentric rings and cottony mycelium, having the highest growth rate, the greatest hyphal width and a positive reaction to polyphenol oxidase. Among the isolates of the anamorph *Epulorhiza*, that were mostly, several of the characters allowed segregating the strains into those with hyphal width less than 3 μm and those around 4 μm and which generally coincided with a lower and higher 3 mm growth rate per day, respectively. The morphology of the monilioid cells also coincided with that previously described for both anamorphs (Freitas *et al.*, 2020; Pereira *et al.*, 2005). Ovando *et al.* (2005) found a high incidence of fungi of the *Epulorhiza* and *Moniliopsis* genera of the Family Tulasnellaceae isolated from different substrates and times of the year on plants cultivated in the Botanical Garden of Tuzantán in Soconusco, Chiapas. Research made by Freitas *et al.* (2020) on two species of the genus *Cattleya* in Brazil showed that this orchid genus in its natural habitat has clear preferences to associate with *Tulasnella* calospora species, the same fungus that was recognized as effective in the symbiotic propagation of *C. purpurata* (Bazzicalupo *et al.*, 2021). The specificity of epiphytic orchids with fungal species of the Families Tulasnellaceae and Ceratobasidiaceae has been previously explored by Otero *et al.* (2002) and Dearnaley (2007). The phylogenetic relatedness of the sequences in GenBank, joint some of these strains (contigs 2, 3 and 4) in a 96-99% identity with terrestrial and epiphytic orchids from the Americas: Costa Rica (*Epidendrum firmum*), Brazil (*Pleurothallis teres*) and the United States (*Diuris fragantisima* and *Cypripedium kentuki*). Contig 1 has 98% similarity to an isolate of *Encyclia ghillani* from Brazil (Kartzinel *et al.*, 2013). In contrast, contig 5 of the Family Ceratobasidiaceae is similar to saprophytic or endophytic fungi colonizing grasses (98% of identity with *Ceratobasidium* sp. isolated from *Festuca arundinacea* from the United States) and closely to other fungi of epiphytic orchids, mainly of the species *Comparettia falcata*. Less than 10% of all characterized strains belonged to the genus *Ceratobasidium* found in only one native phorophyte (*T. rosea*), which reinforces the evidence that this orchid has a greater affinity to associate in the adult stage mostly with species of the genus *Tulasnella*.

CONCLUSIONS

This study shows that *G. skinneri* plants, already established in urban sites, are able to form mycorrhizal relationships with a certain diversity, both on a native or exotic phorophytes. Extending the sampling to more urban phorophytes will allow us to confirm this specificity for a large species of mycorrhizal fungi of the Tulasnellaceae family in adult plants, as well as to test the specificity through symbiotic propagation to implement propagation technologies. Together with previous studies conducted on *G. skinneri* (Aguilar

Diaz *et al.*, 2018; Coello *et al.*, 2010; Coutiño-Cortés *et al.*, 2017;), we propose to employ these species in urban environments as a sustainable strategy in order to conserve the remaining wild populations in nature.

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