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Two novel endophytic *Tolypocladium* species identified from native pines in south Florida

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Abstract: This study investigated the incidence and diversity of *Tolypocladium* within trunks of south Florida slash pines (*Pinus densa*). Thirty-five isolates were recovered from trunk tissue including living phloem, cambium, and sapwood. Two novel species of *Tolypocladium* (*T. subtropicale* and *T. trecense*) are described here based on morphological and molecular analysis of concatenated LSU, ITS, *tef*-1, *tub*, and *RPB1* sequences. Our findings expand our understanding of the distribution, diversity, and ecology of this genus and confirm that it is widely spread as an endophyte across ecosystems and hosts. Strains collected in this survey will be used in future bioassays to determine their potential ecological roles as mycoparasites or entomopathogens.

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INTRODUCTION

Research across biomes has demonstrated the importance of fungal endophytes not only in respect to their impact on host fitness but also at the ecosystem level (Hardoim *et al.* 2015, Cline *et al.* 2018, Zanne *et al.* 2020). Moreover, the endophyte guild has been recognized as an important reservoir of fungal diversity (Blackwell 2011, Gazis *et al.* 2011, Porras-Alfaro & Bayman 2011) and of novel molecules with industrial relevance (Strobel 2006, Alvin *et al.* 2014, Sandhu *et al.* 2017). Through molecular studies (Promputtha *et al.* 2007, Chaverri & Gazis 2011, Tadych *et al.* 2012, Martin *et al.* 2015, Gilmartin *et al.* 2022) and bioassays (Photita *et al.* 2004, Oses *et al.* 2006, Pujade-Renaud *et al.* 2019) connections between the endophytic lifestyle with fungi known as saprotrophic, parasitic, and mutualistic nutritional modes have been established, suggesting that our concepts of the ecological roles of many fungal taxa need revision. Several lineages closely related to entomopathogenic fungi, have been recovered as endophytes from inner leaf, stem, and root tissues (Vega *et al.* 2008, Gazis *et al.* 2014, Vidal & Jaber 2015). However, whether these fungal strains harbor the complete version of functional genes involved in entomopathogenicity and protect their host through anti-herbivory mechanisms such as the production of metabolites or direct parasitism is not known. Additional experiments that test the true ecological role or nutritional mode of these strains are needed, such as the study by Wang *et al.* (2020) which showed that the entomopathogenic caterpillar fungus *Ophiocordyceps sinensis* has an endophytic stage that facilitates infection of the host's larvae through the consumption of endophytically colonized roots.

Fungal strains recovered as endophytes from cultivated plants and commonly used in biological control (*e.g.*, *Beauveria bassiana*, *Isaria fumosorosea, Metarhizium brunneum*, *M. anisopliae*, and *Purpureocillium lilacinum*) often conserve their entomopathogenic capability (Castillo Lopez *et al.* 2014, Carrillo *et al.* 2015, Gange *et al.* 2019, Ramakuwela *et al.* 2020) and field studies have successfully shown pest suppression as a consequence of endophytic colonization (Canassa *et al.* 2020, Russo *et al.* 2021). Conversely, other lineages may have lost the ability to parasitize or antagonize insects and in turn acquired a commensal endophytic habit. So far, direct evidence is lacking in most cases.

Tolypocladium (*Ophiocordycipitaceae*, *Hypocreales*, *Sordariomycetes*) currently contains 49 species registered in MycoBank (www.mycobank.org) (MycoBank database, January 2023). *Tolypocladium* species have diverse lifestyles that range from insect and fungal parasites to endolichens and soil-dwelling inhabitants (Bushley *et al.* 2013, Quandt *et al.* 2015, Blount 2018, Yu *et al.* 2021). Several species (*i.e.*, *T. inflatum*, *T. cylindrosporum*, *T. geodes*) are known as prolific producers of secondary metabolites and used widely in medicine and agriculture (Bushley *et al.* 2013, Li *et al.* 2015, Kebede *et al.* 2017). Due to its importance in human medicine, the most studied species is the cyclosporin and efrapeptin metabolite-producing, *T. inflatum* (Hodge *et al.* 1996, Bushley *et al.* 2013). Another species with medical importance is *T. cylindrosporum*, which besides being a pathogen of insects and ticks and an endophyte, produces cyclosporin (Matsuda & Koyasu 2000, Scholte *et al.* 2004, Herrero *et al.* 2011, Montalva *et al.* 2019). Multiple nutritional modes have also been found in *T. ophioglossoides,* which genome encodes genes involved in both mycoparasitism

and entomopathogenicity, differentially expressed depending on substrate utilization (Quandt *et al.* 2015, 2016).

Using culture-based and metabarcoding approaches, *Tolypocladium* species have been detected as leaf and trunk endophytes of wild and cultivated *Hevea* and closely related species (Gazis *et al.* 2014, Gazis & Chaverri 2015, Skaltsas *et al.* 2019) and from other hosts (Hanada *et al.* 2010, Sánchez Márquez *et al.* 2010). Currently, there are three species (*T. amazonense*, *T. endophyticum, T. tropicale*) known only as endophytes (Gazis *et al.* 2014). Species with an endophytic habit form a well-supported clade (Gazis *et al.* 2014, Yu *et al.* 2021). However, this clade does not exclusively contain strictly endophytic species as it also comprises *T. album*, a generalist species known from soil and dead and living plant material (Gazis *et al.* 2014, Yu *et al.* 2021). Outside the "endophytic clade", *T. pustulatum* has been isolated from wounds of *Pinus contorta* and was originally described from unidentified twigs in an oak forest (holotype BPI 748466, ex-type culture ATCC 74192); however, there is no evidence that these isolates were behaving as true endophytes and not as pathogens or saprotrophs (Bills *et al.* 2002, Arhipova *et al.* 2015). Additionally, *T. cylindrosporum* has been isolated from leaves of grasses (Sánchez Márquez *et al.* 2010) and from woody tissues of cultivated *Theobroma cacao* trees (Hanada *et al.* 2010).

As *Tolypocladium* becomes more speciose, several studies have made efforts to unveil the evolutionary history of substrate association within the genus. Phylogenetic analyses based on standard fungal markers (LSU, ITS, *tef*-1, *tub*, *RPB1*, *RPB2*) and genome-scale approaches support the inter-kingdom host jump hypothesis (Nikoh & Fukatsu 2000); however, have resulted in the reconstruction of contradictory ancestral states. Ancestral state reconstruction using multilocus sequence data supports the hypothesis that insect-associated *Tolypocladium* species derived from mycoparasitic ancestors (Gazis *et al*. 2014, Yu *et al.* 2021), while phylogenomic approaches suggest a single ecological and nutritional transition from insect to fungi (Quandt *et al.* 2016). The endophytic clade described by Gazis *et al.* (2014) still lacks sufficient data to generate robust hypotheses regarding its diversification history; however, it has consistently been recovered as "derived" (Gazis *et al.* 2014, Wang *et al.* 2019, Yu *et al.* 2021, Tehan *et al.* 2022).

This study represents the first attempt to determine if *Tolypocladium* species are part of the trunk endophytic community of pine trees. Sampled trees were distributed in Pine Rocklands habitats in south Florida, a critically endangered tropical dry forest ecosystem (Snyder *et al.* 1990). This ecosystem is extremely important for animals, birds, reptiles, plants, and microorganisms including mycorrhizae and endophytes (Platt *et al.* 2010, Lloyd & Slater 2012, Possley *et al.* 2016, Karlsen-Ayala *et al.* 2022). Based on morphological and molecular data, we introduce two novel *Tolypocladium* species. In addition, we report few strains that cluster with previously described endophytic *Tolypocladium,* confirming that this genus is widely spread as an endophyte across ecosystems and hosts. Strains collected in this survey will be used in future bioassays to determine their potential ecological roles as mycoparasites or entomopathogens.

MATERIALS AND METHODS

Sample collection and endophyte isolation

A total of 30 individual *Pinus densa* (south Florida slash pine; Syn: *Pinus elliottii* var. *densa*) trees were sampled in five different Pine Rockland native forests, (i) University of Florida Tropical Research and Education Center (TREC), Homestead, Florida, USA (n=10), (ii) a Homestead private property (B&P), Homestead, Florida, USA (n = 5), (iii) Pine Ridge Sanctuary (PRS), Homestead, Florida, USA (n = 5) (iv) a Miami private property located in the Pinecrest area (PCA), Miami, Florida, USA (n = 5) and (v) Zoo Miami (ZM), Miami, Florida, USA (n = 5) (Table 1).

A sterile increment borer (Mora Borer, Haglöfs Sweden, Bromma, Stockholm, Sweden) was used to sample trunk cores. Before sampling each tree, the increment borer was sterilized by submerging it in 10 % bleach for one minute and brushed with a straw brush to remove any debris. Afterwards, it was rinsed with deionized water, submerged in 95 % ethanol, and flamed. Trees were randomly selected with a minimum distance of 5 m and were sampled from three different parts of their circumference. The increment borer was inserted at a slight upward angle (~30°) to prevent contamination by water entering the sampling hole (Fig. 1A). The increment borer auger without the handle was attached to an electric drill and used to enter the first 5 cm of the tree (Fig. 1A, B). Once the first layer was bored, the handle was placed in the auger and rotated until the borer penetrated approximately 14 cm of the tree, and then the core was removed with the extractor (Fig. 1C, D). The first core was sampled at an approximate height of 1.5 m and the second and third were taken at 20 cm above and below the initial core. The cores were collected in Ziplock bags and labeled as pine 1 (P1) through pine 30 (P30) and core 1 (C1) through core 3 (C3), (Table 1). Samples were kept in a cooler until brought to the laboratory where they were processed immediately.

Table 1. Sampling localities and summarized *Tolypocladium* isolation results.

	TREC[1]	B&P	PRS	PCA	ZM	Total
GPS coordinates	25° 30' 23.634'' N, 80° 29' 56.8968'' W	26° 31' 8.7492'' N, 80° 29' 57.7068'' W	25° 29' 18.888'' N, 80° 32' 27.492'' W	25° 40' 21.8712'' N, 80° 17' 41.4636'' W	25° 36' 43.1604'' N, 80° 23' 39.624'' W	-
Sample ID	P1–P5; P16–P20	P6–P10	P11–P15	P21–P25	P26–P30	P1–P30
Number of trees	10	5	5	5	5	30
Number of cores	30	15	15	15	15	90
Incidence	5	0	0	5	0	10
Isolates	21	0	0	14	0	35

[1]TREC: Tropical Research and Education Center; B&P: Bruce and Pam Forest; PRS: Pine Ridge Sanctuary; PCA: Pinecrest area; ZM: Zoo Miami.

Fig. 1. Step-by-Step sampling process, from the field to the laboratory. **A.** Increment borer auger without handle attached to an electric drill sampling at approximately 1.5 m. **B.** Borer entered ~5 cm into the tree in an upright 30° angle. **C.** Removal of the electric drill and replacement by the instrument borer handle. **D.** Core taken from the tree. **E.** Photo showing intact core, (**F**) core trimmed ~2 cm from both edges, and (**G**) cut in pieces of ~0.2 cm. **H, I.** Core pieces plated in ½ PDA and incubated at 25 °C in the dark until colonies resembling *Tolypocladium* were observed. **J.** Subculture of tolypocladium-like colonies.

The cores consisted of (i) outer bark, (ii) living phloem, (iii) vascular cambium, (iv) sapwood containing functional xylem, and (v) heartwood composed of old xylem (Fig. 1E). The core size was approximately 14 cm (5.5 inches); 2 cm (0.8 inches) from each end of the core, containing the outer bark and inner heartwood, were discarded (Fig. 1F). Under a biosafety cabinet, the remaining core was dissected into 16–20 pieces of 0.2 cm (0.08 inches), using a flame sterilized scalpel (Fig. 1G) and quickly transferred to 140 mm Petri dishes containing ½ Difco™ potato dextrose agar (PDA) amended with 1 % neomycin-penicillin-streptomycin (Sigma-Aldrich, St Louis, Missouri, USA) (Fig. 1H). Petri dishes were incubated for up to 2 mo at room temperature in darkness (Fig. 1I). Emerging colonies were sub-cultured onto fresh ½ PDA plates to obtain pure isolates (Fig. 1J).

Isolate identification

A total of 35 fungal strains showing tolypocladium-like colony characteristics, such as slow-growth and white cottony appearance (Gams 1971), were recovered. Cultures were purified either by single-spore or hyphal tip technique and grown in ½ PDA until purity was confirmed. Genomic DNA was extracted from mycelial mats using a GeneJET genomic DNA purification kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA), according to the manufacturer's instructions with few modifications (Parra *et al.* 2020). The internal transcribed spacer (ITS) region was amplified using the primers ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990) and used to confirm the generic placement of the isolates. Four other loci were amplified: nuclear large subunit (LSU), largest subunit of RNA polymerase II (*RPB1*), partial *ß*-tubulin gene (*tub*), and elongation factor 1a

(*tef*-1), following protocols described in Gazis *et al.* (2014) and Quandt *et al.* (2014). PCR products were cleaned and sequenced at MCLAB laboratories (MCLAB, South San Francisco, California, USA - www.mclab.com). Sequence chromatograms were assessed, and bi-directional reads were trimmed, assembled, and checked for quality using default settings in Geneious v. 9.0.5 (Geneious Computer Software, Newark, New Jersey, USA). Newly generated sequences were deposited in GenBank (Supplementary Table S1).

A combined dataset was constructed by concatenating the newly generated ITS, LSU, *tef*-1, *RPB1,* and *tub* sequences and vouchered sequences retrieved from GenBank (NCBI) (Supplementary Table S1). Taxa included in the analysis represent lineages used in the following systematic studies of the genus: Gazis *et al.* (2014), Sung *et al.* (2007), Quandt *et al.* (2014), Yu *et al.* (2021), Li *et al.* (2018), Wijayawardene *et al.* (2021), Yamamoto *et al.* (2022), and Wang *et al.* (2022). A total of 26 *Tolypocladium* species were included, representing 53 % of the currently described species. Each locus was aligned separately using MAFFT v. 7 under default settings (Katoh & Standley 2013). Ambiguously aligned regions were excluded from the alignments using Gblocks v. 0.91b (Talavera & Castresana 2007) under reduced stringency settings. A combined 106-taxa dataset was constructed with five-loci combined LSU (805 bp), ITS (464 bp), *tef*-1 (825 bp), *RPB1* (576 bp), and *tub* (597 bp), for a total base pair length of 3 267 nucleotides. Dataset completeness was as follows: 99/106 for ITS, 94/106 for LSU, 84/106 for *tef*-1, 80/106 for *tub*, and 83/106 for *RPB1* (Supplementary Fig. S1, S2). A separate dataset, based only on the ITS marker, included data from 19 species (39 % of described species). Maximum Likelihood (ML) phylogenies based on the ITS and on the concatenated dataset were constructed using RaxML v. 8.2.10 (Stamatakis 2014) under the GTRGAMMA evolutionary model. Branch support values were estimated using 1 000 bootstrap (BS) replicates. *Ophiocordyceps agriotidis*, *Ophiocordyceps acicularis*, and *Ophiocordyceps stylophora* were used as outgroup. Alignments were deposited in the Open Science Framework(OSF); the alignments used to construct the phylogenetic trees are available through this URL: https:/osf.io/t8uz5/?view_only=6eb775b84a53410aabe9525ddcebecaa.

Morphological studies

Four representative strains from each of the new species being described were selected for morphological description. In addition, two isolates of *T. amazonense* (MS308 [type] and LA100) were examined for comparison purposes. Single-spored isolates were grown on PDA, MEA (DifcoTM malt extract agar, Franklin Lakes, New Jersey, USA), and SNA (synthetic nutrient-poor agar) (Nirenberg 1976) and incubated at 25 °C with a photoperiod of 12 h/12 h fluorescent light/dark for 3 wk. Colony characteristics such as shape, size, and color were documented at 14 and 21 d after media inoculation. Colony size measurements were taken in two linear measurements (separated by a 45° angle) of mycelial growth and recorded from each plate at 4, 7 and 10 d after inoculation by measuring the distance between the edge of the inoculum plug and the edge of the colony. Conidia and phialides (length and width) measurements were obtained using a Motic Panthera microscope and Excelis HDS software (Micro Optics of Florida, St. Petersburg, Florida, USA).

Continuous measurements were based on 100 conidia and 30 phialides. Terms used to describe the morphology of the reproductive structures follow Seifert *et al.* (2011). Images were captured with an Accu-scope digital camera accoupled with Excelis HDS HD camera & monitor system (Micro Optics of Florida, St. Petersburg, Florida, USA). A dried culture of the type specimen corresponding to each novel species was deposited in the U.S. National Fungus Collections (BPI) and additional representatives and ex-type cultures were deposited in the Agricultural Research Service Culture Collection. Copies of all isolates are stored at -80 °C as part of Gazis' laboratory strain collection at the University of Florida, Tropical Research and Education Center.

RESULTS

Isolation of *Tolypocladium*

The presence of *Tolypocladium* within trunk tissue of pine trees was confirmed in two of the five locations sampled, Tropical Research and Education Center (TREC) and Pinecrest Area (PCA). From the 30 trees sampled and ~ 2 000 pieces plated, a total of 35 *Tolypocladium* isolates were recovered. At TREC, *Tolypocladium* was isolated from four of the 10 trees sampled (P4: n = 7; P5: n = 1; P16: n = 2; P19: n = 11) and at PCA, *Tolypocladium* was isolated from all five trees sampled (P21: n = 3; P22: n = 4; P23: n = 4; P24: n = 1; P25: n = 2) (Table 1).

Identification and phylogenetic placement

The topology of the phylogenetic tree, based on the combined dataset (ITS, LSU, *RPB1*, *tub*, and *tef*-1), was congruent with previous studies (Gazis *et al.* 2014, Quandt *et al.* 2014, Li *et al.* 2018, Wijayawardene *et al.* 2021, Yu *et al.* 2021, Wang *et al.* 2022) and revealed two novel *Tolypocladium* species (described here as *T. subtropicale* and *T. trecense*). *Tolypocladium subtropicale* (BS = 99) comprised 14 isolates collected from TREC and PCA sites (Fig. 2). A group containing three isolates (JMS361, JMS364, and JMS365) (BS = 98), all recovered from the same tree (PCA: P21), was resolved as sister to *T. subtropicale* and based on molecular and morphological data may represent an undescribed species (here labeled as *Tolypocladium* sp. 1). *Tolypocladium trecense* (BS = 100) comprised 13 isolates collected from TREC and PCA sites. A group composed of three isolates (JMS376, JMS373, and JMS372) (BS = 100), all recovered from the same tree (PCA: P23), formed a sister group to *T. amazonense* (BS = 95) and based on molecular and morphological data may represent a second undescribed species (here labeled as *Tolypocladium* sp. 2). Two other *Tolypocladium* strains recovered in this study (JMS377 and JMS381) grouped with the previously described species *T. tropicale* (BS = 97) (Fig. 2). The phylogeny, based on the ITS sequences, was congruent with the multilocus phylogenetic analysis (Supplementary Fig. S3).

Diagnostic morphological characters were scarce, but the four distinctive and previously undescribed clades can be separated from closely related species by differences in one or more of the following characters: color of the underside of colony grown in PDA, conidiophore branching type, and phialide and conidia size and shape (Table 2).

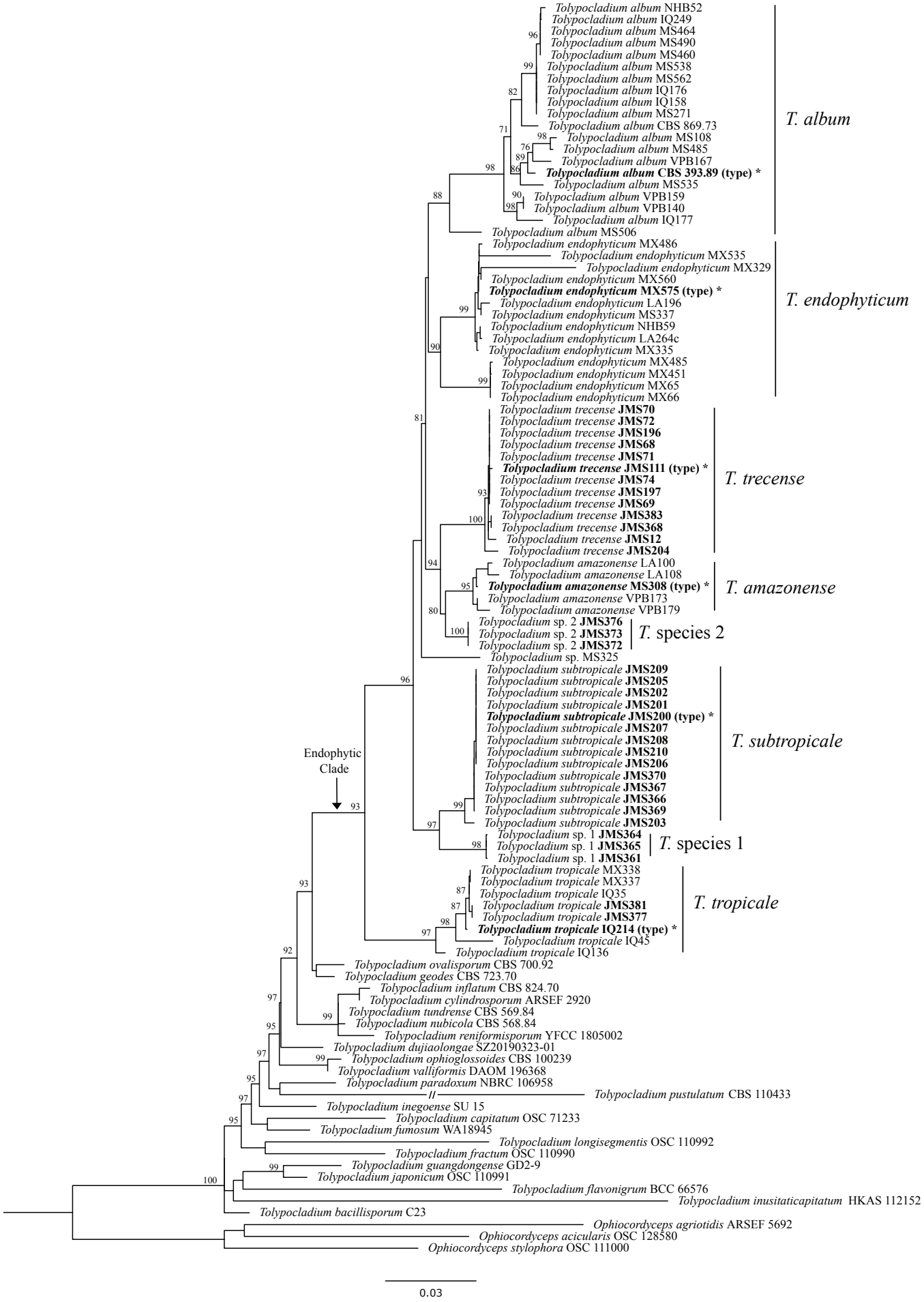

Fig. 2. Phylogenetic tree based on a Maximum Likelihood analysis using five concatenated loci (LSU, ITS, *tef-1*, *tub*, and *RPB1*) showing relationship amongst *Tolypocladium* isolates collected in this study and previously described species. Bootstrap support (BS) values > 75 % are indicated.

Table 2. Distinguishing morphological characteristics of previously described endophytic *Tolypocladium*, the two novel species (*T. subtropicale* and *T. trecense*), and the two unidentified independent lineages (*Tolypocladium* sp. 1 & 2).

Species	Colony in PDA (F/B)[1]	Conidia		Conidiophore Type[2]	Phialide Shape[3] and Size (µm)
		Shape	Size (µm)		
T. album[†]					
MS 506 = CBS 136902	W/Y-	Globose to Subglobose	2.3 ± 0.5	T	L/C; 8.2 ± 1.7 × 3.5 ± 1.3
T. amazonense[#]					
MS 308	W/Y	Globose	1.4 ± 0.2	T	L; 4.6 ± 1.2 × 1.5 ± 0.3
T. endophyticum[#]					
MX 575	W/P	Globose	1.3 ± 0.2	T	L; 4.1 ± 0.9 × 1.6 ± 0.2
T. subtropicale					
JMS 200*	W/Y-	Globose to Ellipsoidal	2.0 ± 0.4	UB	C; 3.5–19 × 1–2.5
JMS 370	W/W	Ellipsoidal	2.5 ± 0.3 × 1.8 ± 0.2	UB	C; 6.2–15.5 × 1–2.5
Tolypocadium tropicale[#]					
IQ 214	W/W	Globose	1.5 ± 0.1	T	L; 4.6 ± 1.2 × 1.5 ± 0.3
T. trecense					
JMS 111*	W/Y-	Globose	2.0 ± 0.6	UB	C; 6.5–13 × 0.9–2.4
JMS 196	W/Y-	Globose	2.2 ± 0.1	UB	C; 8.5–21.6 × 0.6–2.8
Tolypocadium sp. 1					
JMS 361	W/Y+	Ellipsoidal	2.8 ± 0.3 × 1.9 ± 0.3	UB	C; 9.2–36 × 2–2.6
JMS 364	W/Y+	Ellipsoidal	2.6 ± 0.3 × 1.6 ± 0.3	UB	C; no data
JMS 365	W/Y+	Ellipsoidal	2.6 ± 0.4 × 1.6 ± 0.2	UB	C; 13.9–42 × 1.5–2.7
Tolypocadium sp. 2					
JMS 372	W/P	Ellipsoidal	2.1 ± 0.3 × 1.4 ± 0.3	T	L; 6.3 ± 1.5 × 1.9 ± 0.3
JMS 373	W/P	Ellipsoidal	2.2 ± 0.2 × 1.7 ± 0.2	T	L; 6.3 ± 1.7 × 2.5 ± 0.2
JMS 376	W/P	Ellipsoidal	2.3 ± 0.2 × 1.7 ± 0.2	T	L; 6.1 ± 1.5 × 2.3 ± 0.6

[1]W = white; Y = yellow; Y+ = dark yellow; Y- = pale yellow; P = pink. [2]UB = unbranched conidiophore; T = trichodermatoid. [3]C = Cylindrical; L = Lageniform. *Ex-type isolate. [#]Data from Gazis *et al.* (2014); [†]Data from Gams (1980).

TAXONOMY

Tolypocladium subtropicale JM Soares & Gazis, ***sp. nov.*** MycoBank MB 845563. Fig. 3.

Etymology: Epithet refers to the climatic region where the strains were collected.

Diagnosis: *Tolypocladium subtropicale* forms a distinct species strongly supported by molecular data and can be distinguished from other closely related endophytic *Tolypocladium* by a combination of morphological characters, including conidiophore type (unbranched) and phialide (cylindrical) and conidia (ellipsoidal) shape (Table 2).

Colonies on MEA circular, forming abundant white pruinose mycelium, forming concentric rings with pale yellow plate reverse. Colonies on PDA forming abundant white floccose mycelium and radially sulcate colonies with bright yellow plate reverse. Colonies on SNA, circular, scarce, colorless to white. *Hyphae* hyaline, branched, smooth-walled (Fig. 3A–F). *Conidiomata* absent. *Conidiophores* unbranched, hyaline. *Conidiogenous cells* phialidic, hyaline, smooth-walled, solitary, intercalary, cylindrical, broad range in size (3.5–19 × 1–2.5 µm). *Conidia* abundant in MEA and PDA, aseptate, mainly globose but also ellipsoidal, hyaline, small (2.0 ± 0.4 µm), single or aggregating in slimy heads at the apex of phialides (Fig. 3G–K). *Chlamydospores* present (Fig. 3L), hyaline, intercalary. *Sexual morph* unknown.

Typus: **USA**, Florida, Tropical Research and Education Center Pine Rocklands, Homestead, Miami-Dade County, isolated from the functional sapwood of *P. densa* (south Florida slash pine; Syn: *Pinus elliottii* var. *densa*), 5 May 2021, *J.M. Soares*, strain JMS200 (**holotype** BPI 911232, culture ex-type NRRL 64456). GenBank accessions: ITS: ON490898; LSU: ON495714; *tef*-1: ON512593; *tub*: ON512656; *RPB1*: ON512625.

Additional cultures examined: **USA**, Florida, Tropical Research and Education Center Pine Rocklands, Homestead, Miami-Dade County, isolated from the functional sapwood of *P. densa* (south Florida slash pine; Syn: *Pinus elliottii* var. *densa*), 21 Jun. 2021, *JM Soares*, strain

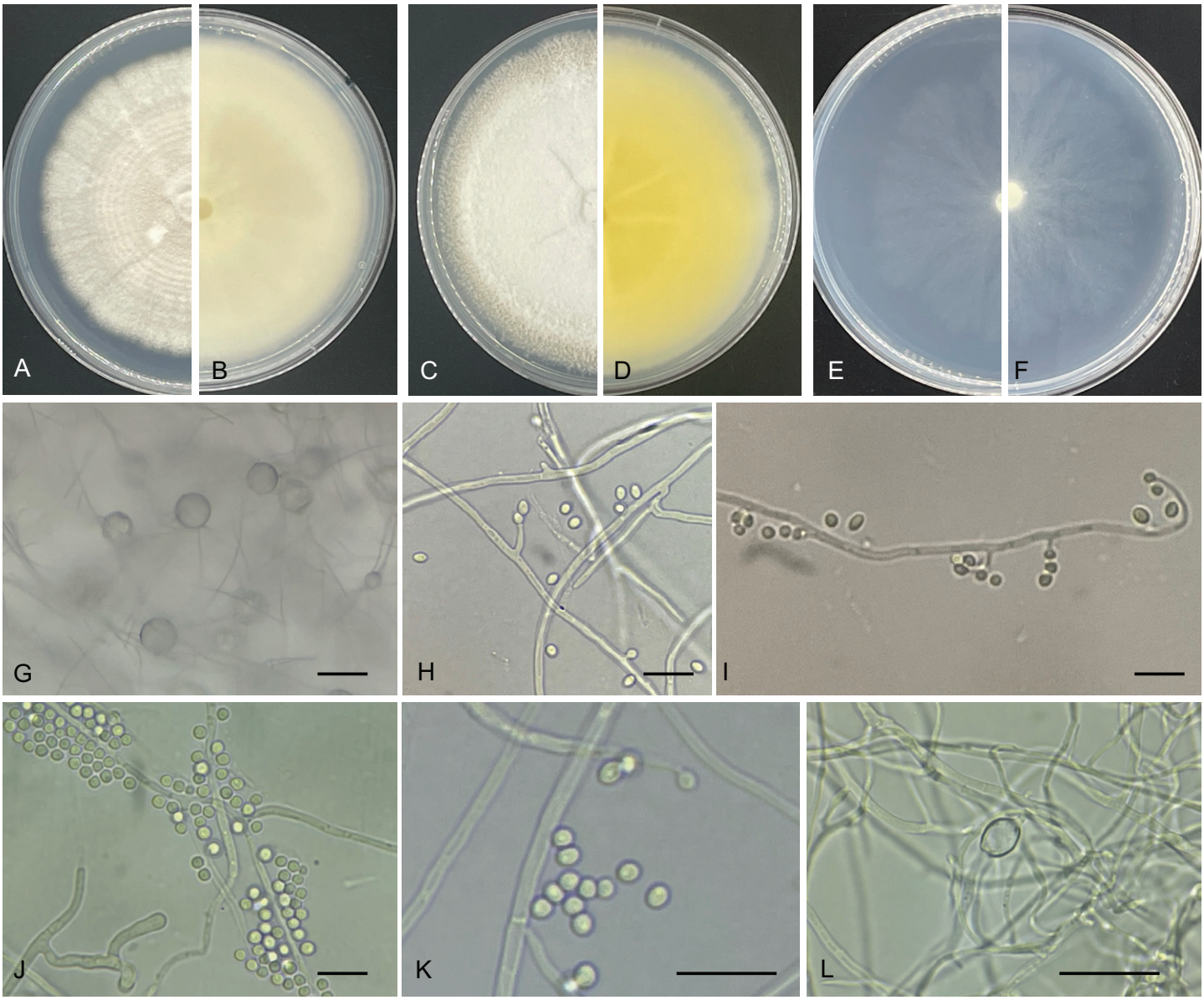

Fig. 3. Micrographs showing the morphological characters of *Tolypocladium subtropicale*. **A, B.** Colony growth in MEA media, (**A**) upper surface, and (**B**) the bottom surface of the plate. **C, D.** Colony growth in PDA media, (**C**) upper surface, and (**D**) the bottom surface of the plate. **E, F.** Colony growth in SNA media, (**E**) upper surface, and (**F**) the bottom surface of the plate. **G.** Slimy heads being produced intercalated to the mycelia where conidia are produced. Photos were taken directly from PDA plates. **H–L.** Photos showing microscopic structures: (**H, I**) conidia and phialides, (**J, K**) conidia and (**L**) chlamydospore. Scale bars: G = 50 µm; H–L = 10 µm.

JMS210, culture accession NRRL 64457; Miami-Dade County, Miami, PCA Pine Rocklands, isolated from the functional sapwood of *P. densa* (south Florida slash pine; Syn: *Pinus elliottii* var. *densa*), 5 May 2021, *J.M. Soares* strain JMS370 culture accession NRRL 64458.

Tolypocladium trecense JM Soares & Gazis, ***sp. nov.*** MycoBank MB 845562. Fig. 4.

Etymology: Epithet refers to the site where the ex-type strain was collected, the University of Florida, Tropical Research and Education Center (TREC).

Diagnosis: *Tolypocladium trecense* forms a distinct species strongly supported by molecular data and can be distinguished from other closely related endophytic *Tolypocladium* by a combination of morphological characters such as conidiophore type (unbranched), phialide shape (cylindrical), and lager conidia size (Table 2).

Colonies on MEA circular, forming abundant white floccose mycelium and light-yellow plate reverse. Colonies on PDA forming abundant white flat mycelium and radially sulcate colonies and yellow plate reserve. Colonies on SNA, circular, white, floccose (Fig. 4A–F). *Hyphae* hyaline, branched, smooth-walled. *Conidiomata* absent. *Conidiogenous cells* phialidic, unbranched, hyaline, smooth-walled, solitary, intercalary, cylindrical, often with bent necks, broad range in size (6.5–13 × 0.9–2.4 µm). *Conidia* abundant in MEA, PDA, and in SNA, aseptate, mainly globose, hyaline, small (2.0 ± 0.6 µm), single or aggregating in slimy heads at the apex of phialides (Fig. 4G–J). *Chlamydospores* present (Fig. 4K, L), hyaline, intercalary. *Sexual morph* unknown.

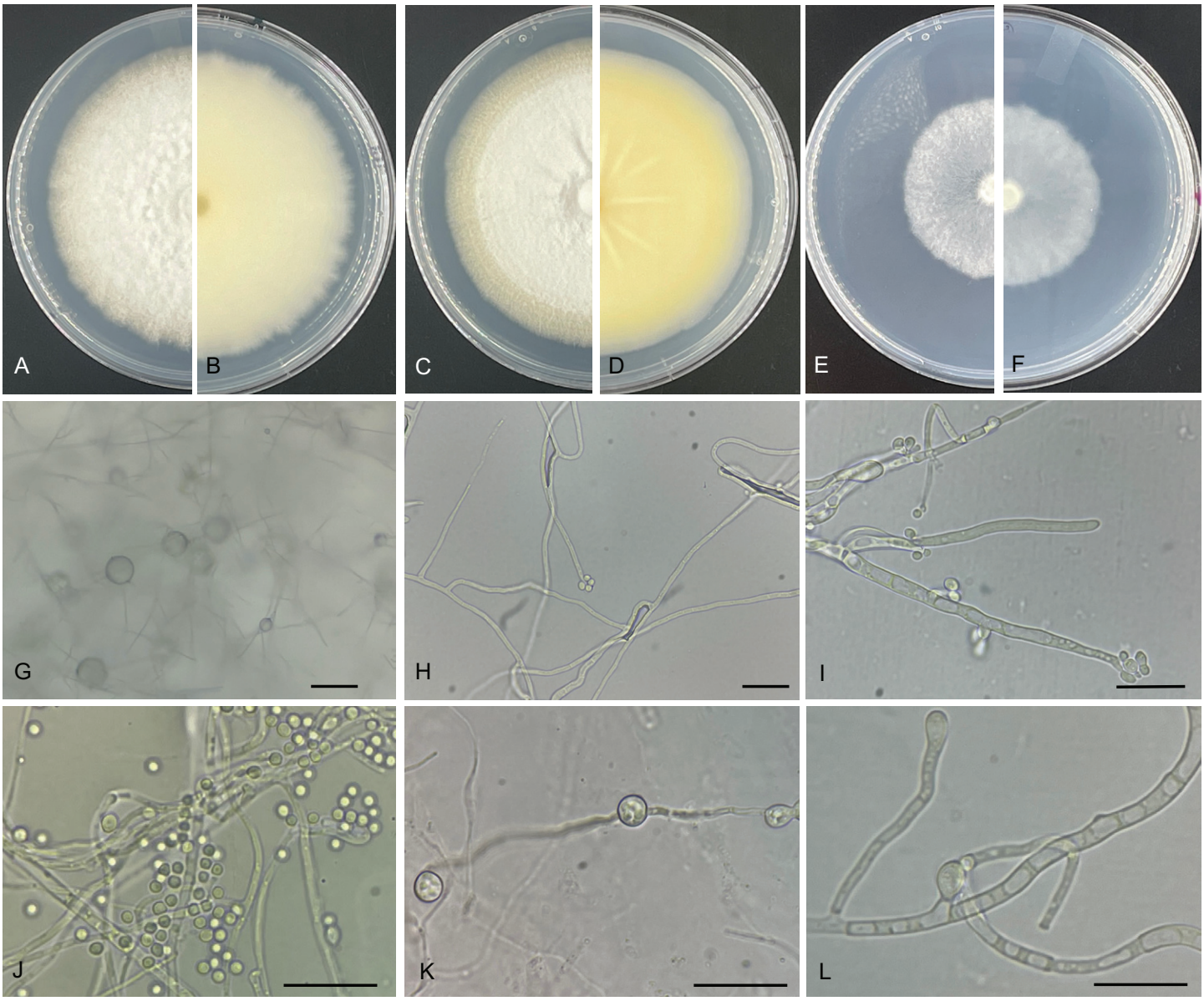

Fig. 4. Micrographs showing the morphological characters of *Tolypocladium trecenses*. **A, B.** Colony growth in MEA media, (**A**) upper surface, and (**B**) the bottom surface of the plate. **C, D.** Colony growth in PDA media, (**C**) upper surface, and (**D**) the bottom surface of the plate. **E, F.** Colony growth in SNA media, (**E**) upper surface, and (**F**) the bottom surface of the plate. **G.** Slimy heads intercalated to the mycelia where conidia are produced. Photos were taken directly from PDA plates. **H–L.** Photos showing microscopic structures: (**H, I**) conidia and phialides, (**J**) conidia and (**K, L**) chlamydospores. Scale bars: G = 50 µm; H–L = 10 µm.

Typus: **USA**, Florida, Tropical Research and Education Center Pine Rocklands, Homestead, Miami-Dade County, isolated from the functional sapwood of *P. densa*, 24 Apr. 2021, *J.M. Soares*, strain JMS111 (**holotype** BPI 91123, culture ex-type NRRL 64453). GenBank accessions: ITS: ON490895; LSU: ON495712; *tef*-1 ON512590; *tub*: ON512653; *RPB1*: ON512645.

Additional cultures examined: **USA**, Florida, Miami-Dade County, Homestead, Tropical Research and Education Center Pine Rocklands, isolated from the functional sapwood of *P. densa* (south Florida slash pine; Syn: *Pinus elliottii* var. *densa*), 24 Apr. 2021, *J.M. Soares*, strain JMS196 culture accession NRRL 64454 and strain JMS204 culture accession NRRL 64455.

DISCUSSION

In this study, we investigated the incidence of *Tolypocladium* within the functional sapwood of pine trees distributed in the Pine Rocklands ecosystem. Our findings expand our understanding of the distribution, diversity, and ecology of this genus. Multilocus phylogenetic analysis revealed two novel species, here introduced as *T. subtropicale* and *T. trecense*. Both species and six other isolates resolved as two independent undescribed lineages (*Tolypocladium* sp. 1 & 2) clustered within the "endophytic clade", first introduced by Gazis *et al.* (2014). The discovery of *Tolypocladium* as part of the endophytic community of pines expands the host association and geographic distribution of the genus and reinforces the belief that functional sapwood endophytes are a frontier for fungal diversity discovery.

Tolypocladium subtropicale was resolved as sister to *Tolypocladium* sp. 1 and even though these two lineages have overlapping morphological characters, molecular data supports their separation. *Tolypocladium* sp. 2 was resolved as sister to *T. amazonense* and can be distinguished from the later by its ellipsoidal conidia and larger phialides. Together, these two lineages, were resolved as sister to the newly described *Tolypocladium trecense* which can be distinguished by its conidiophore type (unbranched *vs*. trichodermatoid) and the shape of its phialides (cylindrical *vs*. lageniform). Because all isolates from *Tolypocladium* sp. 1 and from *Tolypocladium* sp. 2 were recovered from single trees, and therefore could be clonal, we decided to leave these clades as unnamed independent putative species. Future sampling and recovery of additional conspecific strains may warrant their description as a novel species.

In general, *Tolypocladium* was recovered in low incidence and abundance. Isolates were only recovered from trees at the TREC (5/10 trees) and PCA (5/5 trees) sites. The low incidence and abundance of *Tolypocladium* is likely a reflection of the culture dependent isolation technique used in this study which favors fast growing endophytes (*i.e*., *Pestalotiopsis*, *Phomopsis, Trichoderma*). Multiple studies have shown that culture and molecular (*i.e.*, high throughput sequencing-HTS) based approaches are needed to achieve a realistic characterization of the endophytic community. For instance, HTS analysis of tall grass *Brachypodium rupestre* (shoots, rhizomes, and roots) identified abundant and rare species of endophytes which were 5.8 times more enriched in number of taxa when compared to the traditional culture-based method. However, metabarcoding approaches were unable to detect the most abundant endophyte, *Omnidemptus graminis,* recovered through culturing (Durán *et al.* 2021). In addition, the number of OTUs recovered through HTS approaches may vary depending on the primer employed in the assays. Gilmartin *et al.* (2022) used HTS to examine the functional sapwood of beech trees and detected more OTUs when using LSU than when using ITS and culture-based approaches; however, unique OTUs were detected in each approach emphasizing the importance in the employment of more than one method to characterize endophyte communities. The need for multiple sampling approaches is especially important when working with long-lived hosts (*i.e*., hardwoods) in tropical and subtropical areas (Skaltsas *et al.* 2019, Fonseca *et al.* 2022).

Tolypocladium has been reported in high incidence from wild and cultivated *Hevea* (rubber trees), through culture-dependent (Gazis & Chaverri 2015) and a combination of culture and culture-independent approaches (Skaltsas *et al.* 2019). In fact, in Skaltsas *et al.* (2019), *Tolypocladium* was classified as a core endophyte, being present in more than 50 % of the individual trees of each species sampled (*H. guianensis*, *H. nitida*, *H. pauciflora*, and *Micrandra spruceana*). Furthermore, *Tolypocladium* was the most abundant genus recovered from adult trees through culture-dependent methods (123 isolates out of 2 061 recovered from 125 trees) and the fourth most abundant genus recovered through culture-independent approaches (14 % of sequence reads, 9 % incidence frequency from a total of 1 086 242 reads from 91 trees).

Tolypocladium has been isolated as endophyte in other hosts such as *Theobroma cacao*, *Scapania verrucosa*, and *Nothofagus dombeyi* (Hanada *et al.* 2010, Zeng *et al.* 2011, Molina *et al.* 2020). Although the number of endophyte studies focusing on this genus is low, a search in the Global Fungi Database (short sequence reads database, https://globalfungi.com) revealed that surveys based on high throughput sequencing have recovered this genus as an endophyte of roots and above-ground tissues. Currently, a total of 53 species hypothesis (SH) are identified as *Tolypocladium*, associated with multiple functional guilds (GlobalFungi, October 2022). *Tolypocladium* SHs data originated from studies targeting fungi from forest soil systems in different geographic regions across several continents such as North and South America, Asia, and Australia (Větrovský *et al.* 2020). This information suggests that species of the *Tolypocladium* endophytic clade are widely distributed.

Tolypocladium is a diverse genus with species ranging from tree endophytes, mycoparasites, and entomopathogens. Formulating a robust hypothesis to explain the evolutionary history of host association within the genus is hindered by the incompleteness of available molecular data. Among the 49 described *Tolypocladium* species, only 35 have sequence data available, including eight species with one marker sequenced (either ITS or LSU) and 27 species with more than one ribosomal marker or protein coding gene (*RPB1*, *tub* and *tef*-1) or a combination. The remaining fourteen species have only morphological data available. While the number of available *Tolypocladium* genomes has increased in the last few years, the number of genomes available is only fourteen (Tehan *et al.* 2022).

To fill some of the aforementioned knowledge gaps in regard to the ecology of *Tolypocladium* strains with an endophytic habit, strains collected in this study will be used in future bioassays to address the following pending questions: (i) are the *Tolypocladium* isolates recovered strict endophytes (commensal nutritional mode) or are they capable of parasitizing other species of fungi, nematodes, and/or insects and (ii) are the *Tolypocladium* isolates recovered capable of producing industrially promising metabolites.

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Supplementary Material: http://fuse-journal.org/

Fig. S1. Alignment used to construct the concatenated phylogenetic tree.

Fig. S2. Alignment used to construct the ITS phylogenetic tree.

Fig. S3. Phylogram of *Tolypocladium* species collected in this study and previously described generated from Maximum likelihood analysis of ITS sequence alignment. Bootstrap support (BS) values > 75 % are indicated.

Table S1. Strains used to construct the multilocus phylogeny. Information on isolation source and GenBank accession numbers.