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Culture-based and Sanger sequencing approaches to uncover the diversity of leaf-fungal endophytes in neotropical gymnosperms

Enfoques basados en el cultivo y la secuenciación Sanger para descubrir la diversidad de endófitos fúngicos de hoja en el neotropical gimnosperma

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ABSTRACT

Endophytic fungi play a crucial role in the survival and adaptation of their host plants. In recent decades, unprecedented diversity of endophytic fungi has been reported in angiosperms, but knowledge about this diversity in tropical gymnosperms is lacking. Among gymnosperms, cycads (Cycadales) are important components of Neotropical forests with high levels of endemism in Central and South America. We used a combination of axenic culture and Sanger sequencing to isolate and identify leaf endophytes of two endemic cycads from humid forests of Panama, *Zamia nana* and *Z. pseudoparasitica*. The latter is the only known epiphytic gymnosperm. In *Z. pseudoparasitica*, 50 morphotypes were isolated from El Copé, 58 from Santa Fe, and 22 from Cerro Marta. We sampled one wild population of *Z. nana* (El Valle de Antón), from which we recovered 74 morphotypes. We also sampled *Z. nana* from the International Garden of Cycads in Panama City. Sequencing of 69 cultures with the ITS rRNA locus identified several ascomycetes: *Colletotrichum*, *Cercophora*, *Hypoxylon*, *Phyllosticta*, *Xylaria*, and a basidiomycete: *Tritirachium*. This is one of the first studies to document the diversity of endophytic fungi in a Neotropical gymnosperm and provides a window into the endophytic fungi that inhabit the leaves of tropical plants.

KEYWORDS

Endophytic fungi, *Colletotrichum, Tritirachium, Xylaria, Zamia*.

RESUMEN

Los hongos endófitos desempeñan un papel crucial en la supervivencia y adaptación de sus plantas huésped. En las últimas décadas, se ha reportado una diversidad sin precedentes de hongos endófitos en angiospermas, pero se carece de conocimiento sobre esta diversidad en las gimnospermas tropicales. Entre las gimnospermas, las cícadas (Cycadales) son componentes importantes de los bosques neotropicales con altos niveles de endemismo en América Central y del Sur. Utilizamos una combinación de cultivo axénico y secuenciación Sanger para aislar e identificar endófitos foliares de dos cícadas endémicas de bosques húmedos de Panamá,

Zamia y *Z. pseudoparasitica*. Esta última es la única gimnosperma epífita conocida. En Z. pseudoparasitica se aislaron 50 morfotipos de El Copé, 58 de Santa Fe y 22 de Cerro Marta. Se muestreó una población silvestre de Z. nana (El Valle de Antón), de la cual se recuperaron 74 morfotipos y también se muestreó Z. nana del Jardín Internacional de Cícadas en la Ciudad de Panamá. La secuenciación de 69 cultivos con el locus ITS rRNA identificó varios ascomicetos: Colletotrichum, Cercophora, Hypoxylon, Phyllosticta, Xylaria y un basidiomiceto: Tritirachium. Este es uno de los primeros estudios en documentar la diversidad de hongos endófitos en una gimnosperma neotropical y proporciona una ventana a los hongos endófitos que habitan en las hojas de las plantas tropicales.

PALABRAS CLAVE

Hongos endófitos, *Colletotrichum, Tritirachium, Xylaria, Zamia*

INTRODUCTION

Endophytic non-pathogenic fungi live asymptomatically within healthy plant tissues (Rodriguez, *et al.*, 2009; U'Ren *et al.*, 2012). Fungal endophytes are a highly diverse and polyphyletic assemblage of mainly ascomycete fungi with diverse and often poorly defined or unknown ecological roles. Endophytic fungi have been recovered from all major land plant lineages and from all terrestrial ecosystems, in natural and anthropogenic communities ranging from the arctic to the tropics (Arnold *et al.*, 2000; Arnold, 2007; Franco *et al.*, 2022). Their role in plant fitness and evolution has been widely recognized (Rodriguez *et al.*, 2009; González–Teuber *et al.* 2021).

Endophytes can confer great adaptive potential to their host plant species against adverse abiotic conditions (Rodriguez *et al.*, 2009) and provide defensive characteristics against microbial pathogens for the host (González-Teuber *et al.*, 2021), producing secondary metabolites that inhibit a particular pathogen or other endophytic fungi (Rodriguez *et al.*, 2009; Franco *et al.*, 2022). Some endophytes allow host plants to survive in hostile environments where neither the host nor the endophyte can survive alone. This emergent property suggests that interactions with endophytes could influence the response of plants to man-made climate change (Porras-Alfaro and Bayman, 2011). Other fungi, such as members of the family Botryosphaeriaceae (e.g., *Neofusicoccum* spp., *Lasiplodia* spp.), are aggressive phytopathogenic fungi that stay latent in the leaves without causing apparent damage to the host (Belair *et al.*, 2023). In recent years there has been great interest on endophytic fungi, due to the ease of isolation and identification methods. Such methods range from Sanger Sequencing (especially the internal transcribed spacer, ITS), metabarcoding and whole genome sequencing (Belair *et al.*, 2023; Franco *et al.*, 2022; Sierra *et al.* 2024). Such genetic resources are still mostly restricted to phytopathogenic strains with renewed efforts to isolate fungi to uncover the genome properties of non-pathogenic fungal endophytes (U'Ren *et al.*,

2012; Xiong *et al.*, 2013; Franco *et al.*, 2022 Hill *et al.*, 2023; Villarreal *et al.* 2024). Despite the great advances in the biology of fungal leaf endophytes, there is still a large gap in tropical gymnosperms, especially cycadophytes.

The Order Cycadales is a group of plants that have inhabited the planet for 300 million years, with a current diversity of about 377 species (Osborne *et al.*, 2012; Nagalingum *et al.*, 2011; https://www.cycadlist.org/index.php). Cycads harbor a repertoire of anti-herbivorous toxins and other compounds of unknown function (Whittaker and Salzman, 2020; Sierra *et al.*, 2024). Most species have a restricted geographic distribution, and there are high levels of endemism within the group (Calonje *et al.*, 2019). The genus *Zamia* is restricted to the New World and is the most ecologically diverse lineage within the order (Calonje *et al.*, 2019). The most recent phylogeny of *Zamia* proposed five clades with a clear geographic pattern, one of which is restricted to the Central American isthmus (Costa Rica and Panama) with 17 species (Calonje *et al.*, 2019). To date, 16 Zamia species have been described in Panama, twelve of which are endemic to the country (Taylor *et al.*, 2012). Unfortunately, these species, as most cycads, suffer anthropogenic threats such as deforestation and poaching, and cycads are now considered to be the most endangered group of seed plants (Fragnière *et al.*, 2015).

We isolated endophytic fungi from leaves from two Panamanian endemics: *Zamia pseudoparasitica* J. Yates and *Zamia nana* A. Lindstr., Calonje, D.W.Stev. & A.S. Taylor. *Zamia pseudoparasitica* is the only obligately epiphytic gymnosperm known and is endemic to the humid tropical forests of the eastern Atlantic coastal zone of Panama (Bell–Doyon *et al.*, 2020). Plants can be found between seven and twenty meters high up on canopy trees (Bell–Doyon *et al.*, 2020; Monteza–Moreno *et al.*, 2022). This species is listed as "Near Threatened" by the IUCN. *Zamia nana* is found in the mountains of the province of Coclé, within the remnant crater of the extinct volcano El Valle de Antón and also in Cerro Turega (Lindström *et al.*, 2013). *Zamia nana* is classified as "Endangered" (EN) in the IUCN Red List due to its restricted distribution and a dwindling population size.

To fill out the gap in knowledge of endophytic fungi inhabiting Neotropical cycads, we seek to answer the following research questions:

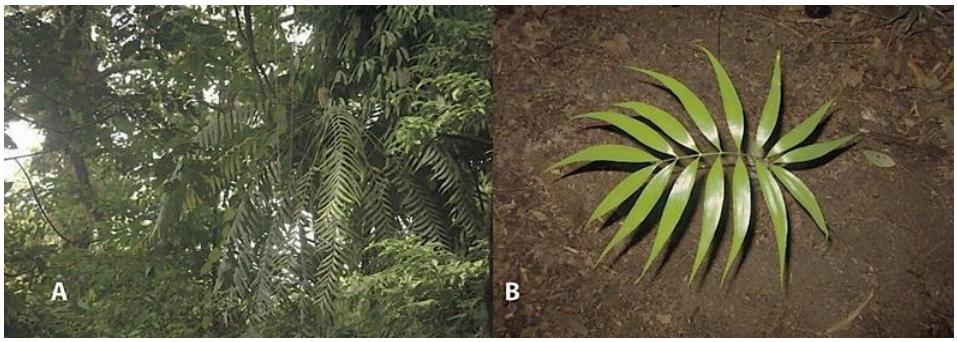
1. Using a culture-based approach, what are the differences between the leaf endophyte morphospecies of *Z. nana* and *Z. pseudoparasitica*?
2. Using a culture-based approach, how do *Z. pseudoparasitica* endophyte morphospecies vary between sites?
3. Using a Sanger-based approach (ITS marker), which fungal genera are found in the leaves of both species?

METHODS

Study area

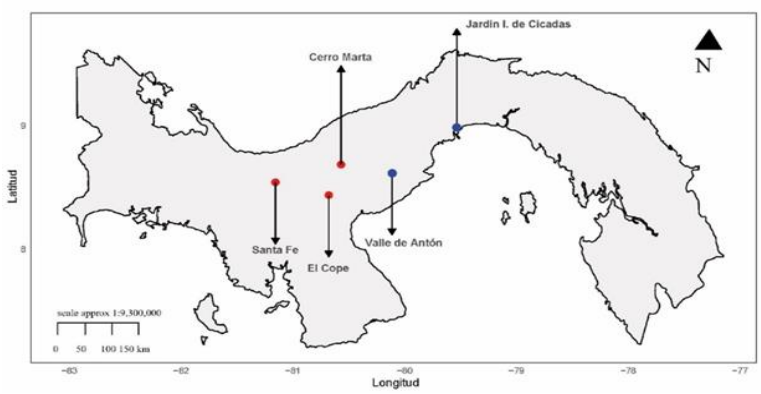
We sampled leaves from three natural populations of *Z. pseudoparasitica* and two populations of of *Z. nana*, one natural and one cultivated (Figure 1).

Figure 1.
Zamia pseudoparasitica in El Copé. B. Zamia nana in El Valle de Antón Photos: P. Castillo and M. Madrid



The collection sites for *Z. pseudoparasitica* were Santa Fe (Veraguas Province), El Copé (Coclé Province) and Cerro Marta (Coclé Province). Samples of *Z. nana* were collected in El Valle de Antón (Coclé Province) and in the International Cycad Garden located within the Campus of the University of Panama (UP, Panama Province) (Figure 2,). Mature and healthy leaflets of *Z. pseudoparasitica* and *Z. nana* were collected, then transported and maintained on ice at an approximate temperature of 10°C until they were processed in the laboratory.

Figure 2
Sampling sites of Zamia pseudoparasitica (red) and Zamia nana (blue) in Panamá



Isolation of endophytic fungi

To isolate endophytic fungi, leaflets were cut into three sections and 50 2×2 mm^2 fragments were obtained from the middle section, which were then disinfected as in Bethancourt (2000). In brief, leaflet fragments were placed in a small strainer, submerged, and shaken constantly while they were passed through a disinfection battery composed of ethyl alcohol (70%) for one-minute, commercial Clorox (10%) (5.25% Na Hypochlorite) for two minutes, ethyl alcohol (70%) for 30 seconds, and finally rinsed in ethyl alcohol (40%).

Of the 50 disinfected fragments per leaflet, 12 random fragments were seeded in large Petri dishes (90mm x 14mm) containing Potato Dextrose Agar (PDA) and incubated at 24°- 26°C for approximately one week to allow fungal growth to emerge. To isolate pure cultures, a fragment of mycelium was taken from each cultivar and transferred to a test tube with inclined PDA which was incubated further at 24 - 26°C.

Morphotyping and cloning of the endophytic fungi.

Six weeks after the first isolation carried out, we began the grouping and morphotyping process. Group morphotypes were obtained by grouping isolates with similar morphological characteristics while individual morphotypes were classified when the morphological characteristics were not shared with any other fungus. The morphological characteristics considered for the groupings included: color, texture, colony margin, presence of aerial or immersed mycelium in the agar, pigmentation of the culture medium caused by the fungus, presence or absence of reproductive structures, and the coloration and growth rate of each colony (fast, moderate, or slow). After scoring, fungi were incubated again for two weeks in the new tubes after which the groupings were reviewed and both group and individual morphotypes were confirmed.

Density and relative diversity estimate of endophytic fungi.

The density of endophytic fungi in this study was obtained by dividing the total number of isolates by the total number of incubated leaf fragments, then multiplying by 100:
DENSITY = (Total number of isolated endophytic fungi) / (Total number of sown fragments) ×100

The diversity of endophytic fungi was estimated by dividing the total number of morphotypes (both group and individual) by the total number of endophytic fungi that were isolated, then multiplying by 100.

DIVERSITY = (Total number of morphotypes) / (Total number of isolated endophytic fungi) ×100

Molecular methods

The Covid-19 pandemic greatly impacted our research since we could not access our cultures for over a year, during which time they were subjected to uncontrolled temperatures and humidity levels and most of our isolates were lost. Before sequencing, we revived our stored cultivars on PDA and prepared three replicates of each isolate to avoid confusion regarding the morphology of each one and also to recognize possible contamination.

We used direct PCR on most samples, where a slice of mycelium was placed directly into the PCR amplification reaction without any prior DNA extraction procedure. DNA from fungal isolates that did not amplify directly were extracted using PrepMan Ultra reagent (Thermo Fisher). The internal transcribed spacer (ITS) rRNA region was amplified (between 400-500 bp) with primers ITS4 and ITS5 (White, 1990). Each reaction included a volume of 20 µL composed of: sterile water, Platinum II 2X PCR Mastermix (Life Technologies), primers (ITS 4 and ITS 5, 10 mM (White et al. 1990), and a fragment of mycelium or a DNA aliquot of the corresponding endophyte. The amplification conditions were 95°C for 3 minutes, followed by 25 cycles of 95°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute, with a final incubation at 72°C for 10 minutes. We sequenced the samples using BigDye chemistry on an ABI 3500XL sequencer at the Naos Molecular Laboratory using the same PCR primers (Smithsonian Tropical Research Institute, Panama). We used Geneious Prime 8.0.5 (https://www.geneious.com) to clean and align the DNA sequences and used BLAST (Basic Local Alignment Search Tool, Astchul *et al.*, 1990), as implemented in Geneious Prime, to identify taxonomy.

RESULTS

We isolated 733 fungal cultures, 433 from *Z. pseudoparasitica* and 300 from *Z. nana*, which we classified into over 200 morphotypes. Fungal endophytes were isolated from over half of the leaflet fragments that we cultured and both grouped and unique morphotypes were found at each site. Within *Z. pseudoparasitica*, the diversity and density of morphotypes varied across sites, with Cerro Marta having the highest diversity despite having fewer leaflets sampled than the other sites (Table S1). In contrast, El Copé had the lowest morphotype diversity despite having more leaflets sampled. More morphotypes were isolated from *Z. nana* cultivars at both sampling locations (Table 1).

Fungal morphotypes

Table 1:
Density and diversity of cultured fungal endophytes of Zamia pseudoparasitica and Zamia nana identified by morphology.

Species	Site	No. leaflet fragments	No. of isolates	No. of morphotypes	Endophyte Density (%)	Endophyte Diversity (%)
Z. pseudoparasitica	El Copé	480	272	50	56.7	18.4
	Santa Fe	180	130	58	72.2	44.6
	Cerro Marta	108	31	22	28.7	71.0
Z. nana	El Valle de Antón	240	144	74	60.0	51.4
	Cycad Garden	240	156	63	65.0	40.4

Taxonomic identification of fungal isolates

We recovered 77 of our isolates, which included both group and unique morphotypes. However, eight of them could not be amplified. In total, 34 isolates were sequenced from *Z. pseudoparasitica* and 35 from *Z. nana*. Each population of *Z. pseudoparasitica* had at least one unique genus of endophyte isolated. Despite this, higher overall richness of endophytes was identified in *Z. nana* than *Z. pseudoparasitica* (14 vs 8 genera, Figure 3); however, five of these genera were only isolated from *Z. nana* plants growing in the International Cycad Garden and three other genera were unique to the population of El Valle de Antón. Most isolates belonged to the Phylum Ascomycota, including the genera *Colletotrichum, Cercophora, Hypoxylon, Xylaria, Phyllosticta, Endomelanconiopsis, Chaetomium, Zasmidium, Acremonium, Parengyodontum, Diaporthe, Biscogniauxia, Hypomontagnella, Pseudocercospora* (Figure. 3; Figure 4). A single member of the Basidiomycota, *Tritirachium,* was isolated from the Jardín de Cicadofitas. Isolates of *Colletotrichum* and *Xylaria* were common in both species and were present in all five *Zamia* populations evaluated (Figure 3; Table 1). One endophyte from the *Z. nana* Valle de Antón population

could only be identified up to the class: Sordariomycetes (Z58), perhaps representing a new ge.nus (Table 1).

Figure 3.
Genera and proportions (%) of cultured fungal endophytes isolated from Zamia species. The identification was made using a 621 bp fragment of the ITS rRNA region.

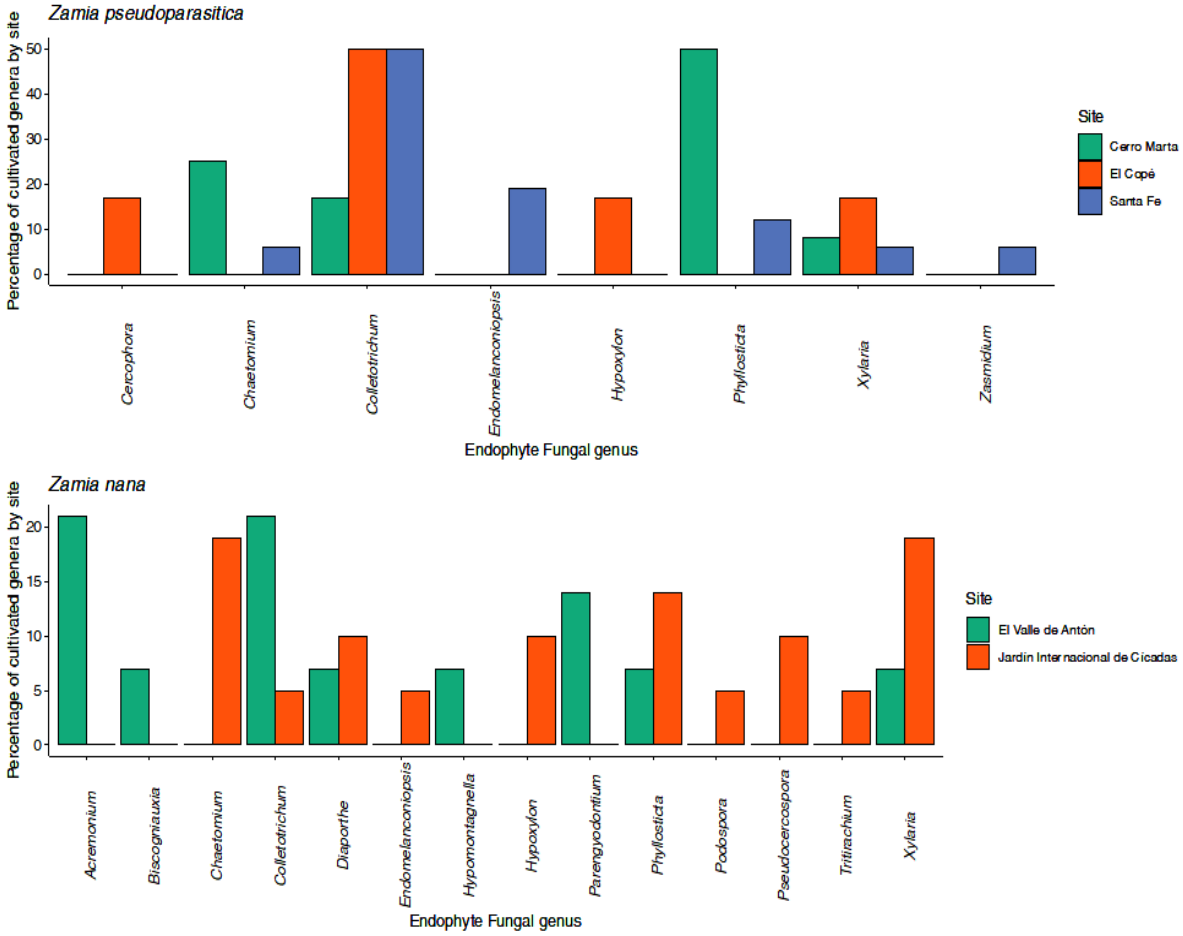
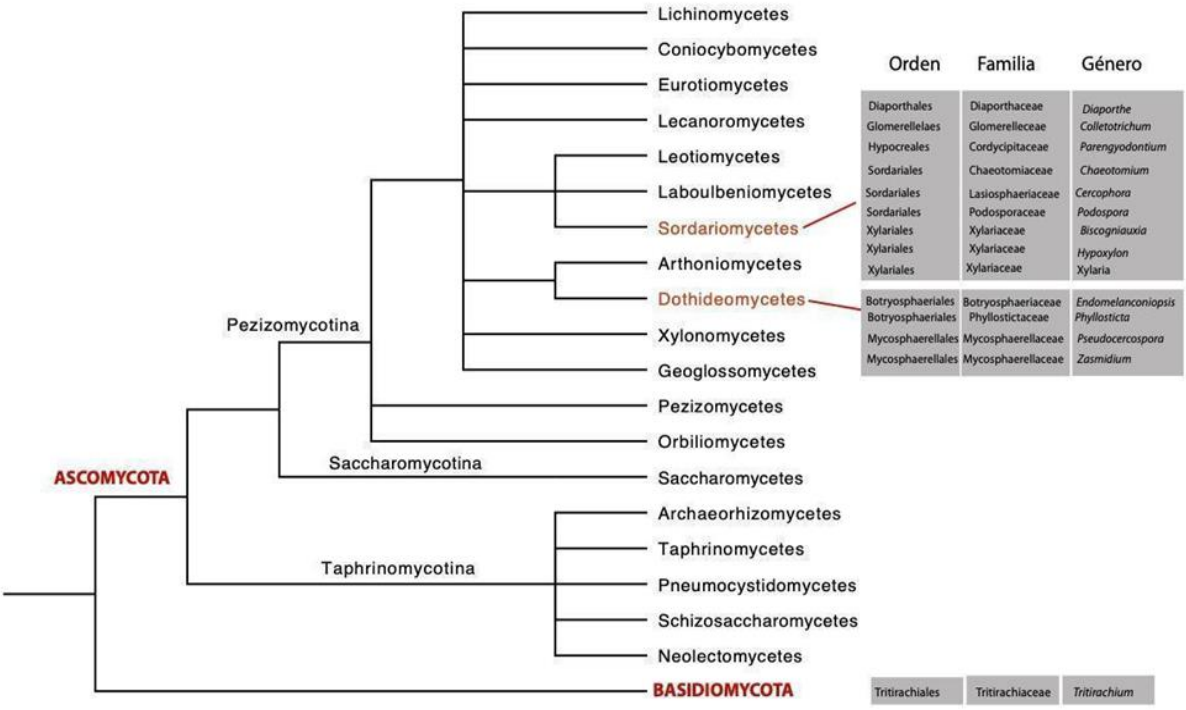


Figure 4.
Phylogeny of Ascomycota and its sister phylum Basidiomycota, with all classes shown. The tree was generated by https://phylot.biobyte.de/ based on the Current knowledge of fungal phylogenetic relationships. The three classes recovered in this study, with their respective genera, are highlighted in red. Most fungi belong to the classes Sordariomycetes y Dothideomycetes.



DISCUSSION

Our results present a high diversity of fungal endophytes in *Zamia*, similar to those reported for flowering plants (Arnold and Lutzoni 2007). Each sampling location had unique genera, but the dominant taxa were common to all sites, suggesting that a core endophytic microbiome exists in these two *Zamia* species, despite their contrasting lifestyles in the canopy and forest understory.

Several genera were common across our samples from *Z. nana* (understory) and *Z. pseudoparasitica* (epiphytic habitat). *Colletotrichum* spp. were the most common isolates across our five sampling sites and can be found in the soil and in plants as pathogenic agents

and endophytic fungi throughout the world (Farr *et al.*, 2006; Baroncelli *et al.* 2017). This genus was also shown to be a dominant member of the leaf fungal microbiome in a recent metabarcoding study of *Z. nana* and *Z. pseudoparasitica* endophytes (Sierra et al. 2024). *Xylaria* spp. were also common in this study, but have been shown to have more diverse lifestyles, ranging from saprophytes to common endophytes in both vascular and non-vascular tropical plants (Bayman *et al.*, al. 1998, Macías-Rubalcava *et al.*, 2017; Villarreal et al. 2024). *Phyllosticta*, another genus that has been identified as both a pathogen and endophyte (Valle Bejarano & Carrillo 2023; Vicent 2021), was found at four of our five sampling sites.

Morphotype and BLAST analyses of a subset of these morphotypes showed a higher diversity of endophytic fungi in *Z. nana* than *Z. pseudoparasitica*. This may be a reflection of their habitat as endophytes colonize plants from their surrounding environment (Arnold 2007). For example, *Z. nana* may be receiving inoculants from both the soil and leaf litter as well as the air column while *Z. pseudoparasitica* may have a more limited source of endophytic inoculants up in the forest canopy. We also found several genera unique to the *Z. nana* plants from the International Garden of Cycads. This location is not representative of the natural habitat of *Z. nana* found in the tropical montane forests of Panama and therefore may have a different inoculant pool, as it is in the lowlands of Panama, with a higher annual temperature, lower annual precipitation, and in an urban environment.

Although *Zamia* species are known to have symbiotic associations with fungi and cyanobacteria (*Nostoc* spp.) in roots, we have a limited understanding of the nature of interactions between leaf endophytes and their host plants (Díaz Pérez *et al*., 2018; Sierra *et al*., 2024). Most of the genera of endophytic fungi identified in *Zamia* species belong to the Ascomycota. Similarly, Arnold and Lutzoni (2007) examined plant tissue of different plants in eight locations from the Canadian arctic to the lowlands of Canada confirming Ascomycota as the predominant phylum. In our work, however, we were able to detect an endophyte belonging to the Basidiomycota. *Tritirachium* has been also found in the soil and decomposing plant material and recently in coastal sediments in the Arabian Sea (Piontelli *et al.*, 2013; Manohar *et al.*, 2014) and here newly reported from *Zamia nana* in the International Cycad Garden of the University of Panama (UP).

Colletotrichum, *Diaporthe* and *Endomelanconiopsis* have shown to be of great importance as antagonists by being reported as producers of bioactive compounds (Costa-Ferreira *et al.*, 2015). For example, *Colletotrichum* demonstrated selective antibacterial activity against the Gram-negative bacterium *Escherichia coli* (Migula) Castellani and Chalmers and was able to inhibit the proliferation of the infection yellow fever virus. (Costa-Ferreira *et al.*, 2015). On the other hand, *Endomelanconiopsis* and *Diaporthe* showed high trypanocidal activity

against amastigotes forms of *Trypanosoma cruzi* Chagas (Costa-Ferreira *et al.*, 2015). Some foliar endophytic fungi can act as pathogens (Arnold 2007) but also as opportunists, such is the case of species of three of the genera identified in this study: *Hypoxylon*, *Neofusicoccum* and *Biscogniauxia*, which were found in oak trees weakened by biotic or abiotic factors. (Moreno-Rico *et al.*, 2010). Our results, based on axenic cultures, surely underestimate the fungal diversity in cycad leaves. Further work using metabarcoding approaches will unveil more of the hidden diversity of fungal endophytes found in tropical cycads.

CONCLUSIONS

Both morphotype and sequence analyses of cultured fungi show that *Z. nana* and *Z. pseudoparasitica* harbor diverse communities of fungal endophytes in their leaves, dominated by the genera *Colletotrichum*, *Xylaria* and *Phyllosticta*. Additional work exploring these fungi's genomic diversity and functional potential is now needed to better understand the dynamics between this unique group of plants and their microbial partners.

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Data availability

Raw sequencing files, images of all sequenced isolates, and associated metadata are available at SI FigShare: https://doi.org/10.25573/data.22782806.v1.

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