

Article

Initial Population Analysis and Mycorrhizal Fungi of the Leafless Epiphytic Orchid, *Campylocentrum pachyrrhizum*, in Florida

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Abstract: *Campylocentrum pachyrrhizum* Rchb.f. Rolfe, also known as the ribbon orchid, is native to southern Florida, the West Indies, and northern South America. In Florida, it is restricted to the Fakahatchee Strand and is currently state-listed as endangered, but virtually nothing is known about the orchid's biology, ecology, and current status. The purpose of this study was to document ribbon orchid populations within the northern portion of the Fakahatchee Strand (Florida Panther NWR) and to identify some of the biotic and abiotic factors critical to its survival. During 2016–2022, a total of 118 ribbon orchids were recorded at five sites on 21 host trees comprising two species (*Annona glabra*, *Fraxinus caroliniana*). The majority (80%) were rooted on pop ash, and over half (54%) were rooted at the base of trees 100–150 cm above the high water mark. The number of orchids decreased from year to year, with the largest drop (2017–2018) coinciding with damage from Hurricane Irma. At the end of the 2022 survey, only 44 orchids remained. Mycorrhizal fungi isolated from two plants were identified as a *Ceratobasidium* species. We urge land managers to take immediate action to safeguard this species throughout southern Florida.

Keywords: *Ceratobasidium*; conservation; tropical cyclones; habitat preservation; Fakahatchee Strand



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1. Introduction

With over 27,000 species, the Orchidaceae constitutes one of the world's most diverse plant families, but they are also among the most vulnerable to extinction. About half (52%) of North America's 250+ species, for example, are currently threatened or endangered [1], and this number is expected to rise this century. The state of Florida is especially rich in orchid diversity, with over 100 species and varieties [2], half of which are confined to the Big Cypress-basin Ecoregion in the southernmost counties. Within a narrow (8 km × 30 km) section of Collier County lies the Fakahatchee Strand, North America's 'hotspot' for orchid diversity, where nearly 50 species are found, including numerous cold-sensitive epiphytes with a Caribbean distribution. Most of these species grow on trees within cypress domes, sloughs, and strand swamps and are insulated from subfreezing temperatures by high water levels [2,3]. Several, such as the leafless ghost orchid, *Dendrophylax lindenii*, produce appealing floral displays and continue to be the target of poachers.

Despite a wealth of the literature on orchids dating back over a century, there remains a sizeable knowledge gap in our understanding of how environmental factors and species traits may influence population numbers over time. Considering that orchids are highly vulnerable to acute environmental changes [4], filling this void has added meaning in a world undergoing rapid change. Unfortunately, securing long-term monitoring data for this purpose requires several years of annual field observations to detect population declines through time. For epiphytic orchids rooted in the tree canopy, gaining access to individual plants is a cumbersome process made worse because small seedling stages are

often difficult to spot, let alone identify, to species level. This is especially true of the leafless orchids recognized solely by their roots when not in flower. Despite such difficulties, Mújica et al. [5] successfully monitored ghost orchid populations within the 10,768-ha Florida Panther National Wildlife Refuge during a six-year period. They concluded that *D. lindenii* would decline by 20% during the next decade in the absence of external adverse factors and that seedling recruitment was not expected to keep pace with the decline. This sobering finding raises the possibility that other orchid species in this important ecoregion may also be in decline.

Campylocentrum pachyrrhizum Rchb.f. Rolfe, also known as the ribbon orchid, is native to southern Florida, the West Indies, and northern South America [1]. In Florida, it is restricted to the Fakahatchee Strand and is currently state-listed as endangered [6,7]. Like *D. lindenii*, it is also a leafless epiphytic orchid that grows on the same host tree species within cypress domes, namely pop ash (*Fraxinus caroliniana* Mill.) and pond apple (*Annona glabra* L.) (Figure 1). Despite years of research on other orchid species within the same environment, virtually nothing is known about the biology, ecology, and population numbers of *C. pachyrrhizum*. Although it is assumed to be more common than *D. lindenii*, population viability analysis is needed to document the number of existing plants and determine if populations are stable, increasing, or declining within the Fakahatchee Strand.



Figure 1. *Campylocentrum pachyrrhizum* is found in the Fakahatchee strand, which consists of seasonally flooded swamps and sloughs (Left). Easily mistaken for the ghost orchid, the ribbon orchid (Right) has roots that are greenish-grey and appear as though they “ripple”. Left photo courtesy of Larry Richardson.

The purpose of this study was to document ribbon orchid populations within the northern portion of the Fakahatchee Strand (Florida Panther NWR) using the methods outlined by Mújica et al. [5] applied to *D. lindenii*. Special attention was given to studying the growth stages of the ribbon orchid in situ and to document some of the biotic and abiotic factors that may be crucial to its survival. Among the biotic factors studied and described herein were the mycorrhizal associates of *C. pachyrrhizum* acquired from the roots of mature plants.

2. Materials and Methods

2.1. Study Site

The Florida Panther National Wildlife Refuge, located in remote Collier County, FL (26.1925 N 81.4051 W), was chosen because it harbored sizable populations of *C. pachyrrhizum* in areas that were relatively free from human disturbances. These populations were confined to strand swamps and sloughs shaded by bald cypress (*Taxodium distichum* (L.) Rich.) and two primary understory trees (*A. glabra*, *F. caroliniana*), that served as hosts for the orchids. An extensive description of the study site is described

by Mújica et al. [5,8]. Five populations (sites) in the Florida Panther NWR were chosen because they harbored high densities of other co-habiting orchid species. For the purposes of this study and to preserve the anonymity of these locations, they were labeled as Sites A, B, C, D, E. Surveys at other sites within the Florida Panther NWR were ruled out simply because they either lacked *C. pachyrrhizum* or lacked the necessary hydrology needed to support the species. In total, the Florida Panther NWR was revisited six times, all during the month of July, between the years 2016–2018 and 2020–2022. Sites A, B, and C were inventoried every year for five years. Sites D and E were discovered after the 2018 survey and recorded for the final three and two years of the survey, respectively.

2.2. Survey Methods

The survey method used in this study followed the protocol described by Mújica et al. [5]. All potential host trees were searched annually at each site for the presence of *C. pachyrrhizum* in all three growth stages assigned to 4 size/age classes (discussed below). Because *C. pachyrrhizum* is often mistaken for the ghost orchid (*D. lindenii*) when not in flower, care was taken to distinguish the two species using three morphological traits. Briefly, roots of ribbon orchids are laterally compressed, lack pneumatodes, and have bronze-colored root tips leading up to the velamen layer (Figure 1).

When a host tree was identified with a ribbon orchid present, it was marked utilizing flagging, aluminum tags, and GPS technology. The redundancy ensured that each host tree was located every year, even if mortality had occurred. After the initial inventory of the ribbon orchid population, only the host trees with ribbon orchids were revisited in each consecutive year. When a mature ribbon orchid showed signs that a seed capsule had formed and released seeds, the surrounding trees were searched as possible hosts for additional orchids. Because several ribbon orchids grew together in clumps on the same host tree, thumbtacks were placed by each individual orchid to ensure accurate data collection. In cases where an individual orchid perished during the surveys, the thumb tack remained in place to maintain a record of its occurrence.

Each host tree was recorded according to characteristics that were thought to be significant to the orchid's ecology (e.g., bark texture, host tree species). Corrugated bark was characterized as having groves at least 1 cm wide and deep, whereas smooth/semi-corrugated bark had groves < 1 cm deep and wide. Additional information, such as height, orientation, and root growth, was also recorded. Each year, the root lengths of all living and dead roots were measured to gather information on growth. The approximate age of the seedlings that were discovered during the course of the surveys was estimated by locating mature plants with capsules in the vicinity. To ensure that no seed capsules were recorded twice, old inflorescences with dehiscent capsules were detached from these mature plants.

Based on the mean root lengths recorded, ribbon orchid individuals were characterized using the same 4 classes described by Raventós et al. [9] and Mújica et al. [5] so that future comparisons could be made and homogeneity of data maintained. Newly recruited seedlings were recorded separately because the stage classes were based on root length, and die-off could interfere with this number. Classes were organized as follows: Class 1, = 0–6.33 cm; Class 2, = 6.33–12.8 cm; Class 3, = 12.8–19.6 cm; Class 4 = 19.6+.

When an individual orchid was discovered, its directional orientation (N, S, E, W) was recorded to determine if, and to what extent, ribbon orchids may colonize one side of the tree versus another. The individual orchids were classified into the four cardinal headings so that characterization might be obtained from the small population. These data were analyzed for each individual orchid using a contingency table to compare survivorship to the direction the orchid faced. A similar process was repeated for the height of each individual orchid. When each ribbon orchid was recorded, the height of the individual on the tree from the ground was noted. These heights were broken up into classes of 50 cm so that the analysis could work easily with the contingency tables constructed for the orientation. The survivorship rates for the overall population were analyzed against these classes to derive the Chi-Square values and significance.

2.3. Isolation and Identification of Mycorrhizal Fungi

Prior to the onset of field surveys (July 2014), roots of two mature *C. pachyrrhizum* individuals, each from a different site (A and B), were detached for the isolation of mycorrhizal fungi in the laboratory. Care was taken to detach one root from each plant 3 cm from the growing tip using a sterile scalpel without causing harm to the rest of the plant. Each root segment was then placed into a pre-sterilized glass vial and taken to the laboratory the same day. Roots were placed in a refrigerator (4 °C) for 24–48 h leading up to fungal isolation. The procedure for fungal isolation followed the methods described by Zettler and Corey [10] for epiphytic orchids. Roots were removed from refrigeration and scraped gently to remove surface debris under running tap water. They were then measured and photographed to document where mycorrhizal fungi were obtained along the root length. Roots were surface sterilized for 1 min in a mixture of 90 mL sterile water, 5 mL Clorox® (Oakland, CA, USA) (8.25% NaOCl), and 5 mL of ethanol (95%), followed by two 1 min rinses in sterile deionized water. Each root was cut into 1 cm length segments, and each segment was placed into a separate sterile petri dish containing 5 mL of sterile deionized water. Using sterile forceps and scalpel, each root segment was macerated in the water droplet liberating clumps of cortical cells containing fungal pelotons. Warm molten Fungal Isolation Medium (FIM) containing streptomycin sulfate antibiotic [11] was slowly poured into the plate, followed by gentle swirling in a circular motion to facilitate separation of pelotons from one another. After the agar cooled and solidified at ambient temperature, the plates were inspected for signs of active fungal growth after 24–48 h using a dissection microscope with a light source from below the plate. Pelotons were counted visually for each plant to quantify their density for each 1 cm root segment. Fungal hyphae that were observed growing from pelotons were subcultured onto potato dextrose agar (PDA, Difco, Becton Dickinson and Co., Sparks, MD, USA) and incubated at ambient temperature for 1–3 weeks.

Orchid mycorrhizal fungi in pure culture were provisionally identified using light microscopy and colony characteristics on PDA. Monilioid cell morphology, hyphal widths, growth rates, and branching patterns that matched published descriptions for basidiomycetes in the *Rhizoctonia* complex [12–14].

2.4. Molecular Confirmation

We used ribosomal DNA internal transcribed spacer (ITS) amplification and Sanger Sequencing to confirm the identity of the fungal isolates using procedures outlined in Zettler et al. [15], Yokoya et al. [16], and Thixton et al. [17]. Fungal inoculum (1 cm³) from each isolate in pure culture was added to potato dextrose broth (PDB; Sigma Aldrich, St. Louis, MO, USA) on a shaker at ambient temperature for 3–4 weeks. Globular fungal colonies in the broth were then removed and used as the material for extracting and amplifying DNA using Extract-N-Amp™ Plant PCR Kit (Sigma Aldrich). The primers for DNA amplification and PCR conditions were chosen from White et al. [18] and Taylor and McCormick [19]. The DNA's quality was checked (gel electrophoresis on 1.5% agarose gel at 150–175 milliamps, 15–20 min.) and then quantified using a Nanodrop® (Wilmington, DE, USA) spectrophotometer (Thermo Scientific). Ethidium bromide was used to stain gels that were then photographed using a BIO-RAD ChemiDoc™ (Hercules, CA, USA) MP Imaging System. DNA extracted from the fungal samples was then amplified (20 µL) using primers (ITS 1 and 4) based on [18] purchased from Integrated DNA Technologies (Skokie, IL, USA). JumpStart Taq-Ready Mix (Sigma) was used for PCR and final primer concentrations (0.4 µmol/µL). A LabNET™ (Edison, NJ, USA) thermocycler was used for PCR. The conditions for this process were 94 °C for 5 min. The DNA that was successfully amplified was cleaned and PCR products were sent to the University of Illinois Urbana-Champaign's DNA Sequencing Facility. All samples prepared for submission adhered to guidelines posted at <https://unicorn.biotech.illinois.edu/> (accessed on 15 November 2022). An NCBI BLAST search was performed on GenBank to identify potential fungal sequencing matches. A consensus of sequences was obtained from reactions using forward

and reversed primers aligned with top matches from the BLAST search. One fungal isolate was sent to the University of Missouri Division of Biological Sciences (Columbia, MO, USA) for shallow genome sequencing (performed at the Joint Genome Institute, JGI) for further confirmation and to gain a better understanding of its phylogenetic placement amongst other orchid mycorrhizal fungi acquired throughout North America.

3. Results

3.1. Orchid Observations

During the six-year survey on the Florida Panther NWR (2016–2022), a total of 118 ribbon orchids were recorded at five sites on 21 host trees comprising two species (*A. glabra*, *F. caroliniana*). Of the smallest seedlings observed (1–2 cm in length), all possessed a dorsal crest that then yielded one or two true leaves (Figure 2). These leaves were <1 cm in length (typically 6–7 mm) and persisted as the seedling developed longer root systems (Figure 2). In some instances, a third leaf was present. When root systems elongated in length, these leaves senesced, and the plant remained leafless throughout the remainder of its life. At maturity, *C. pachyrrhizum* root systems were observed as a tangled, intertwined mixture of newer and younger roots forming a distinct dome on the host tree bark. It was unclear whether the youngest roots and inflorescences originated from above or below the root mass.



Figure 2. *Campylocentrum pachyrrhizum* seedlings are often found with 2–3 leaves that will fall off as the orchid grows; some leaves have been observed to remain after years. Although it is unknown how large the orchid must be before the leaves fall off, all the large individuals do not have leaves present.

3.2. Host Tree Numbers and Interactions

Overall, *C. pachyrrhizum* numbers between 2016 and 2022 steadily declined during the survey period (Figure 3a,b). The total number of living individuals decreased from 103 to 44, representing a 43% drop in the total number for all five populations within the Florida Panther NWR (Figure 3a). The average rate of decline from year to year was 8.43 individuals. The largest drop occurred during the 2017–2018 period when 28 orchids were lost, coinciding with damage and ca. 257 cm of rainfall from Hurricane Irma on 10 September 2017 [20]. Fewer than 50 ribbon orchids were present at each of the five sites (Figure 3b). Two of the populations (Sites B and C) had the highest number at the time of the first survey (2016). Site C had the highest number, and it was also one of the wettest of the five populations. It also had the highest mortality (38 individuals) observed at the conclusion of the surveys (2022). Although Sites D and E showed an increase in the number of ribbon orchids, the net gain was minimal, with only 10 new individuals recorded.

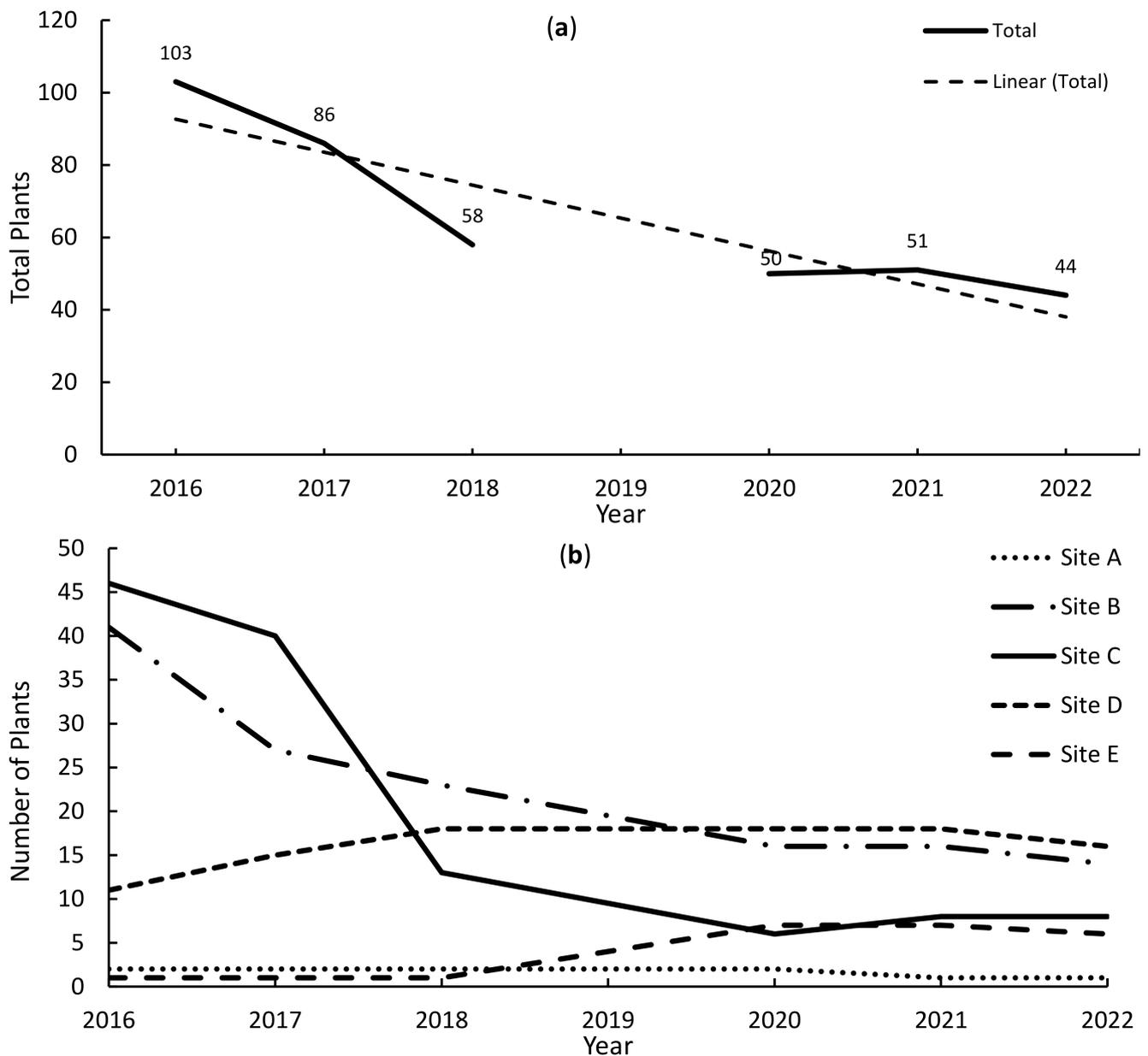


Figure 3. Total observed live populations of *Campylocentrum pachyrrhizum* on the FPNWR across all sites (a). Total observed live individuals for each of the sites that were surveyed (b).

When ribbon orchid numbers were pooled for all five populations per year based on the four age classes (Figure 4), the majority of the individuals were either in Class 1 (small, 0–6.33 cm) or Class 4 (mature, >19.6+ cm), and the fewest were in Class 3 (12.8–19.6 cm) (Figure 2). All ribbon orchids that were observed to have dehiscent fruits were in Class 4, meaning the number of individuals that set seed during any given year was fewer than 20.

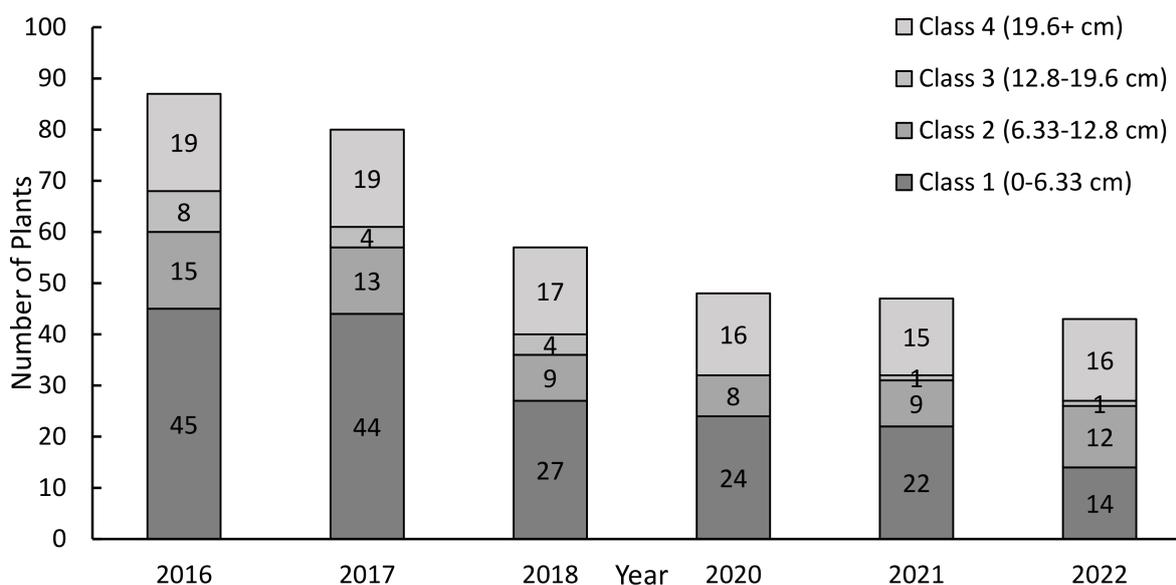


Figure 4. *Campylocentrum pachyrrhizum* class distributions of the total population at the FPNWR. The class breakdown indicates that there is a large population of Class 1 and Class 4 individuals. There were not many individuals observed in the intermediate (class 2–3) stages.

Of the 21 host trees that supported 80% of all *C. pachyrrhizum* throughout the Florida Panther NWR, 17 were pop ash, whereas only four were pond apple. Although pop ash served as host to the majority of *C. pachyrrhizum* surveyed, orchids rooted on pond apple had twice the survivorship (Table 1). Individual orchids were also not evenly distributed across the 21 host trees. For example, one host tree, pop ash, harbored 46 ribbon orchids at the onset of the surveys (2016), encompassing 45% of the total number present at the Florida Panther NWR.

Table 1. Observed *Campylocentrum pachyrrhizum* individuals on host tree species. The *C. pachyrrhizum* found on *A. glabra* (pond apple) had a higher survivorship rate, but the *F. caroliniana* (pop ash) was found to host a majority percentage of the population.

	Pop Ash	Pond Apple
Hosts Observed	17	4
Ind Obs Alive	36	8
Ind Obs Dead	70	4
Ind Obs totals	106	12
% survival of orchids	34	66.7
% of orchid pop	89.8	10.2

Bark texture (corrugated vs. smooth/semi-corrugated) was recorded for each of the individual host trees and corresponded to the 21 host trees recorded in Table 1. Most orchids (91 of 118) were found growing on semi-corrugated bark, but higher orchid survivorship (73.3%) was correlated with corrugated bark (Table 2).

The majority of the ribbon orchids were observed on an N-facing orientation, yet the trends suggest mortality was higher compared to more sun-exposed surfaces (W, S, E orientation; Figure 5). When orchids were grouped into height classes (distance above the high water mark on the host tree), those located between 150–300 cm had the highest survivorship but consisted of a smaller proportion of the total in the FPNWR. Over half (54%) of all ribbon orchids were rooted between 100–150 cm above the high water mark (Figure 6).

Table 2. Survivorship of *Campylocentrum pachyrrhizum* on each substrate texture. Bark texture was recorded at each individual on the host trees. Ribbon orchids that grew on corrugated bark had the highest survivorship, but the majority of the orchids were rooted on smooth/semi-corrugated bark.

	Corrugated Bark	Semi-Corrugated Bark	Smooth Bark
Obs Alive	11	31	2
Obs Dead	4	60	10
Obs totals	15	91	12
% survival	73.3	34.0	16.7
% of pop	12.7	77.1	10.2

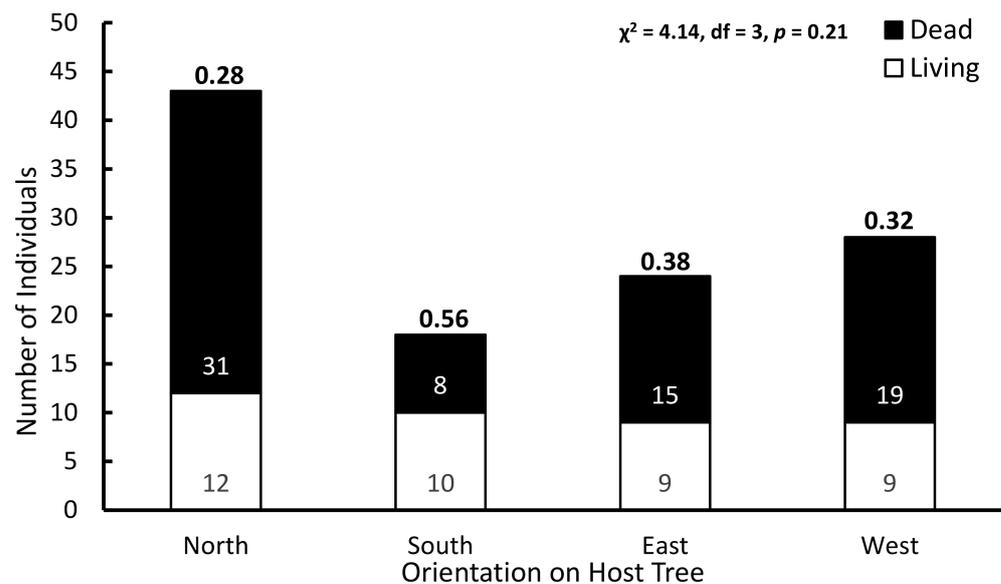


Figure 5. Total *Campylocentrum pachyrrhizum* observed from 2016–2022, organized by orientation on the host tree. Number of living and dead orchids appear inside of the bars. Survivorship of orchids at each orientation is listed at the top of each bar. The north orientation had the most individuals but also the lowest survivorship.

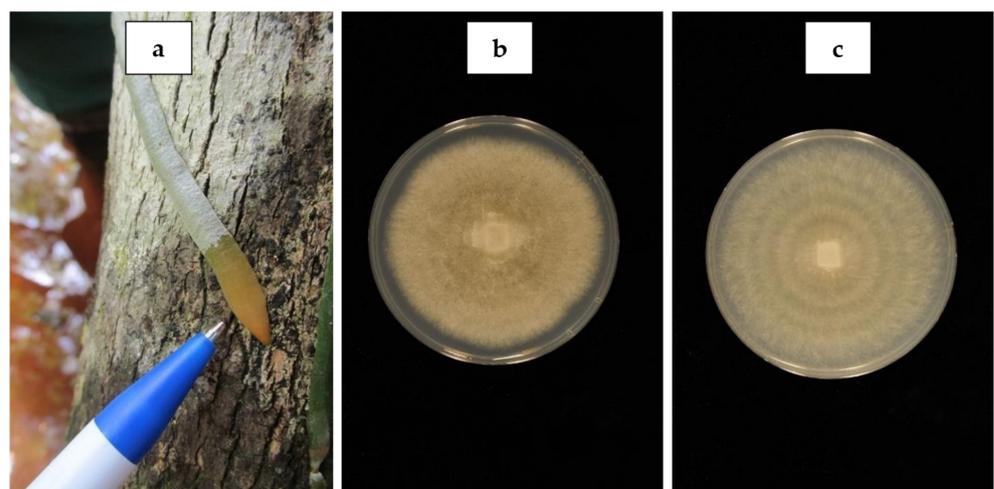


Figure 6. *Campylocentrum pachyrrhizum* pelotons within the bronze-colored growing root tip were isolated from two separate individuals (a). Isolates from these samples were *Ceratobasidium* 390 (b) and *Ceratobasidium* 392 (c). Photos b and c are courtesy of Mike Kane.

3.3. Mycorrhizal Fungi

Pelotons were evident in the roots of *C. pachyrrhiza* collected from mature plants from both sites (A and B). The highest number of pelotons (between 4–8) were recorded in the second cm region at the boundary where the bronze-colored root tip transitioned into the gray velamen layer (Figure 6a). On PDA, these pelotons all yielded pure cultures provisionally identified as *Rhizoctonia*-like fungi in the genus *Ceratobasidium* D. P. Rogers. These cultures exhibited fluffy yellowish tan-colored aerial mycelia with pronounced concentric rings (Figure 6b,c). Hyphal growth at ambient temperature (22 °C) was rapid (0.2–0.5 mm/h). Runner hyphae were consistently broad (4–7 µm) and generally straight. Branch hyphae were constricted at the base of their attachment to runner hyphae. Monilioid cells were barrel-shaped or elliptical and often numerous in aged cultures (>10 days). One fungus, isolated from *C. pachyrrhiza* at Site A (*Ceratobasidium* 390), had cultural characteristics nearly identical to another fungus from Site B (*Ceratobasidium* 392) but had more diffuse concentric zonation on PDA compared to the other isolate (Figure 6b,c). ITS amplification and Sanger sequencing confirmed that both fungal isolates were *Ceratobasidium* via NCBI BLAST search.

4. Discussion

4.1. Hosting a Funeral: Host Trees, Ribbon Orchid Numbers, and Mortality

Unlike the highly coveted and well-known ghost orchid (*D. lindenii*), little is known about the biology and ecology of the ribbon orchid (*C. pachyrrhizum*) that inhabits the same swampy habitats in southern Florida. Both are leafless epiphytes nestled within the same subfamily (Epidendroideae) and tribe (Vandaeae), yet the ribbon orchid appears to have been largely overlooked despite being a state-listed species [7]. Using the same survey methods employed by the present study, Mújica et al. [5] documented 652 *D. lindenii* individuals in the Florida Panther NWR as of 2020, roughly one-third of all ghost orchids thought to remain in the wild, excluding those in Cuba. The ghost orchid is also currently under review by the U.S. Fish and Wildlife Service for Federal listing as an endangered species [21], but to our knowledge, *C. pachyrrhizum* has yet to be considered for further protection. Although it is unclear how many ribbon orchids remain in habitats throughout southern Florida, the highest number of *C. pachyrrhizum* documented within the Florida Panther NWR was 118 individuals, far fewer than *D. lindenii*. As this study also revealed, ribbon orchid populations on the FPNWR are in steady decline, now down to 44 individuals, and this downward trend may have been accelerated by damage incurred from Hurricane Irma in September 2017. Direct evidence for orchid mortality linked to Irma was revealed by the 2018 survey when several host trees at Site C were damaged or submerged in water along with their ribbon orchids. Recently, Borrero et al. [22] also noted marked orchid mortality in *Trichocentrum undulatum* in southern Florida that they attributed to hurricane mortality. Similarly, surveys in western Cuba have revealed long-term negative effects from tropical cyclones on epiphytic orchid populations, including *D. lindenii* [9,23]. Direct damage by Irma, however, did not result in the extirpation of *C. pachyrrhizum* at Site C; however, the ability of the population to recoup such a loss remains to be seen.

There are other factors that could explain the decline in ribbon orchids within the Florida Panther NWR due to the complexity of the ecosystem there. For example, orchids on host trees were likely exposed to higher light and lower relative humidity levels from canopy loss due to Irma. Storm damage to the canopy and other associated vegetation could also adversely impact the community of insect pollinators that the orchids rely on for seed set, as Mújica et al. [23] pointed out for orchids in western Cuba. To our knowledge, the pollinator(s) of *C. pachyrrhizum* has never been documented in Florida nor in other regions where it grows naturally (W Indies, N South America). Based on the flower arrangement (two ranks along a pendant stem, pale flower color, and small size), the inflorescence bears a resemblance to those in the terrestrial genus *Spiranthes* which are often bee-pollinated. Indeed, Cabral and Pansarin [24] reported bee pollination in a related species, *C. micranthum*, raising the possibility that *C. pachyrrhizum* may also rely on bees.

4.2. Mycorrhizal Fungi

It has long been assumed that the leafless orchids (Epidendroideae: Vandae), numbering fewer than 300 species worldwide [25,26], acquire carbon from their photosynthetic roots to compensate for their lack of leaves. Yukawa et al. [27] and Chomicki et al. [28], however, proposed that these orchids associate with mycorrhizal fungi in the Ceratobasidiaceae, raising the possibility that these fungi augment photosynthesis as a supplemental source of carbon. The presence of fungal pelotons in roots of mature *C. pachyrrhizum* (present study) that yielded two *Ceratobasidium* (390, 392) supports their assumption [29]. The identity of both was later confirmed by molecular techniques (L. Corey, pers. com.), but until these isolates are used successfully in vitro to facilitate seed germination, their physiological significance cannot be confirmed. Other species of *Campylocentrum* have yielded *Ceratobasidium*, such as *C. micranthum* in Costa Rica [14] and *C. fasciola* and *C. filiforme* in Puerto Rico [30]. Members of the genus *Dendrophylax* also harbor *Ceratobasidium* fungi, including co-habiting *D. lindenii* at the Florida Panther NWR [8]. One such *D. lindenii* isolate (*Ceratobasidium* 394) facilitated in vitro symbiotic seed germination [31], confirming its status as a mycorrhizal associate. In China, Qin et al. [32] revealed that other leafless genera (*Chiloschista*, *Phalaenopsis*, *Taeniophyllum*) have a highly specialized association with *Ceratobasidiaceae* fungi, several of which facilitated in vitro seed germination. They also concluded that a large portion of their carbon may be supplied by roots and mycorrhizal fungi. Collectively, these studies do seem to support the contention that fungi in the *Ceratobasidiaceae* are associated with the leafless epiphytic orchids, but other types of mycorrhizal fungi should not be ruled out. For example, at least one *Tulasnella* species, isolated from roots of *Dendrophylax porrectus* in the Florida Panther NWR, was effective at facilitating in vitro seed germination of *Encyclia tampensis* (L. Zettler, unpub. Data).

Unruh et al. [33] applied shallow genome sequencing to 32 mycorrhizal fungi isolated from orchids throughout North America, including all three leafless orchids in the Florida Panther NWR. Among the fungi tested were *Ceratobasidium* 392 from *C. pachyrrhizum* (present study), *Ceratobasidium* 394 from *D. lindenii*, and *Tulasnella* 427 from *D. porrectus*. They revealed that the two *Ceratobasidium* isolates were genetically distinct from one another, perhaps comprising different species. Thus, ribbon and ghost orchids may be engaging in ‘niche partitioning’ by targeting different *Ceratobasidium* species within the Florida Panther NWR. This may explain why not a single ribbon orchid was observed growing on the same host tree that harbored a ghost orchid even though pop ash was the preferred host tree for both 80% of ribbon orchids (current study) and 69% of ghost orchids [8]. Moreover, Mújica et al. [5] reported that more ghost orchids were found at one particular location on the Florida Panther NWR that was also the wettest, yet our surveys failed to locate a single ribbon orchid at the same site. Before such a conclusion can be drawn, however, a more thorough analysis is needed by sampling additional orchids throughout southern Florida to determine the extent of genetic diversity in *Ceratobasidium* across the landscape. Given the rarity of both leafless orchids, we advocate that the removal of living roots be restricted to the root tip (3 cm) region where the highest number of pelotons were located. While root detachment is not an ideal practice, determining whether or not these two species are ‘fungal specialists’ [4] engaged in niche partitioning will be crucial to their long-term orchid conservation in the region.

4.3. Seedling Observations—Living Dangerously?

In addition to assessing ribbon orchid numbers spanning different growth stages, detailed sampling provided a glimpse of the species’ early seedling growth, development, and survival in situ. Like the ghost orchid, *C. pachyrrhizum* produces a fleshy, leaf-like dorsal crest shortly after germination that eventually falls off once roots are established. In the ribbon orchid, however, one to three small leaves were observed originating from the dorsal crest, perhaps preceding the formation of roots (Figure 2), whereas, in the ghost orchid, no such leaves have been observed in situ (present study) nor in vitro culture [31] to our knowledge. In Cuba, the dorsal crest of *D. lindenii* appears swollen during wetter

months, perhaps attributed to water imbibition, but gradually becomes wrinkled during the dry months (L. Zettler, pers. obser.). Assuming the same is true for ghost orchids in Florida, the dorsal crest in *D. lindenii* may essentially function as a ‘water bottle’ supplying critical moisture to the seedling during dry periods. In contrast, the dorsal crest in *C. pachyrrhizum* was comparable in size but noticeably thinner than those of *D. lindenii* and would presumably hold less water. Given that leaves are also prone to water loss, it seems plausible that the ribbon orchid may be adapted to live on moist substrates compared to the ghost orchid, which apparently lacks leaves altogether. This assumption is supported by our survey that documented 54% of ribbon orchids rooted closer to the high water mark (100–150 cm) on host trees. While seedlings rooted closer to water may be more likely to survive during dry years, they would conceivably be more vulnerable to periodic flooding associated with tropical cyclone activity, which is what we observed after the 2017 survey (Figure 3). Thus, *C. pachyrrhizum* may be a ‘risk taker’, staking its fecundity on seedling numbers gained when flood waters remain stable. As climatic models project, however, this strategy could prove detrimental to long-term ribbon orchid numbers in a region where tropical cyclone activity is expected to increase in frequency and severity.

Although this study was limited to six years of data (2016–2018, 2020–2022) collected at one location (Florida Panther NWR), the survey took place in the northern portion of the Fakahatchee Strand before and after the passage of a major hurricane (Ivan), under challenging circumstances exacerbated by the easily overlooked (leafless) growth form of this orchid. As preliminary as this survey may be, our study helps to fill a sizeable knowledge gap in an age undergoing rapid environmental change. The most surprising and potentially troubling finding was just how few ribbon orchids are now (2022) thought to exist (44) in a habitat where the species was assumed to be thriving or at least stable, free from direct human contact (e.g., poaching). Equally surprising and concerning was the apparent vulnerability of this species to periodic disturbances (flooding) and the fact that most (80%) were rooted on the bark of one host tree species (pop ash).

4.4. Recommendations for Immediate Conservation

As a preliminary study that was limited to one protected region (Florida Panther NWR), more data spanning additional years and other sites are needed before generalizations can be made regarding the current status of *C. pachyrrhizum* in south Florida. Nevertheless, the stark decline in the number of ribbon orchids revealed by our study does call attention to the importance of obtaining additional data in a timely manner. At the same time, we recommend that conservation strategies be prioritized and implemented, beginning with safeguarding existing orchids from poaching on protected lands, if possible. Although *C. pachyrrhizum* lacks the appealing floral display compared to that of the ghost orchid (*D. lindenii*), both species remain the target of poaching, perhaps because both are leafless and may be mistaken for one another when not in flower. Additionally, more studies are needed to fully investigate the environmental conditions at each site down to the level of the host tree. For instance, knowing more about illumination intensity, average temperature, humidity levels, and associated vegetation might explain fluctuations in ribbon orchid numbers. Special attention should also be given to documenting the microhabitat where seedling stages occur, especially associated mosses and lichens on host tree bark. Propagating *C. pachyrrhizum* from seed in the laboratory represents another step that could be aimed at the orchid’s conservation. Seedlings generated in vitro, for example, could potentially be released into existing sites or new habitats, augmenting existing numbers. Testing the efficacy of *Ceratobasidium* fungi on seed germination in vitro would also reveal more about the mycorrhizal fungi that the ribbon orchid depends on for survival.

While all of the conservation strategies mentioned above are, to some extent, achievable, we recommend that special attention be directed at efforts that take sea level rise and increased cyclone activity into account. Considering that the greater Fakahatchee Strand is highly vulnerable to both climate-related threats given the region’s low topography and

close proximity to coastal areas, relocating *C. pachyrrhizum* into habitats on higher ground may ultimately become necessary.

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