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# A comparision of ghost orchid (*Dendrophylax lindenii*) habitats in Florida and Cuba, with particular reference to seedling recruitment and mycorrhizal fungi

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The ghost orchid, *Dendrophylax lindenii*, is a rare, leafless epiphyte native to southern Florida and Cuba. Populations of *D. lindenii* in southern Florida and Cuba are separated by *c*. 600 km and occur in different habitats. We describe *D. lindenii* in its natural habitats in the Florida Panther National Wildlife Refuge and Guanahacabibes National Park, Cuba. Population size, fungal endophytes and seedling recruitment are also discussed. In total, 116 individuals of *D. lindenii* were recorded in Florida during July 2015, whereas only 16 specimens were known to occur there previously. In Cuba, 241 individuals were counted, nearly one-third (30.3%) of which were seedlings (nearly double the percentage of seedlings documented in Florida; 16.4%). In Florida, *D. lindenii* grew on just two host tree species, *Fraxinus caroliniana* and *Annona glabra*, most (69%) on the former, whereas in Cuba 18 tree species acted as hosts, primarily *Maba crassinervis* (16.2%), *Erythroxylum aerolatum* (15.4%) and *Comocladia dentata* (14.9%). More than half (55.2%) of *D. lindenii* individuals in Florida (55.2%) and Cuba (52.7%) were documented on the north-facing (NW, N, NE) bark of host trees. Significant differences ($P = 0.035$) were detected in directional orientation between the two sites, with Cuban orchids preferring NE, N and E and those in Florida preferring NW, NE and SW surfaces. Roots from mature *D. lindenii* in Florida yielded an endophyte identified as a strain of *Ceratobasidium*. We propose that *D. lindenii* colonizes host trees with moist, corrugated or semi-corrugated bark harbouring *Ceratobasidium* for seed germination.

ADDITIONAL KEYWORDS: *Ceratobasidium* – climate change – epiphytes – Orchidaceae – senile populations.

## INTRODUCTION

The ghost orchid, *Dendrophylax lindenii* (Lindl.) Bentham ex Rolfe (Angraecinae, Orchidaceae), is a rare, leafless epiphyte restricted to forests (hammocks) in southern Florida and Cuba (Brown, 2005). The common name of the species is attributed to its appealing white flowers that appear to hover in mid-air in its dimly lit habitat (Fig. 1). In the Big Cypress Basin Eco-region of southern Florida, *D. lindenii* occurs in cypress domes, sloughs and strand swamps (Fig. 2A) where it grows on trunks and branches of woody trees that overhang standing water. Given that south Florida is subject to occasional sub-zero temperatures during winter months, *D. lindenii* and other cold-sensitive epiphytes are thought to persist from year to year due to the high relative humidity levels in these densely vegetative areas that serve to insulate the plants from

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**Figure 1.** *Dendrophylax lindenii* photographed in Florida Panther National Wildlife Refuge, USA. The long nectar spur and white flower colour provide a striking contrast in its dimly lit, water-filled habitat. Roots of the specimen can be seen radiating outward from the centre of the orchid on corrugated bark of pop ash (*Fraxinus caroliniana*). Photo by Larry W. Richardson.

temperature extremes (Luer, 1972). In western Cuba where sub-zero temperatures do not occur, *D. lindenii* grows on bark of tropical (mixed) semi-deciduous hardwoods rooted in fractured reef limestone (Acevedo, 1992) with little or no standing water (Fig. 2B). Although populations of *D. lindenii* in southern Florida and Cuba are separated by only 600 km, this species appears to occupy two different habitats and colonizes a different set of host trees. This observation seems to counter the prevailing assumption that rare orchids are more restricted in distribution because they are thought to have specific habitat and/or biotic agent needs (Swarts & Dixon, 2009).

Given that populations of *D. lindenii* in the two countries are repeatedly targeted by poachers (Langdon, 1979; Coile & Garland, 2003) and are vulnerable to periodic hurricanes and hydrological changes (Wiegand *et al.*, 2013), documenting its specific habitat needs is crucial to the long-term conservation of this species. Raventós *et al.* (2015), for example, reported that *D. lindenii* could become extinct in Cuba (Guanahacabibes National Park) within 25 years if the annual probability of disturbances, including hurricanes, exceeds 14%. In light of anthropogenically induced climate change, there is a real possibility that the low-lying habitats of *D. lindenii* in Cuba and Florida will succumb to sea level rise this century based on future projections (DeConto & Pollard, 2016). To prepare for this possibility, the specialized needs of *D. lindenii* must be fully understood, including biotic and abiotic factors. For orchids in general, pollinators and mycorrhizal fungi are the two most critical biotic components needed for fruit set and seed germination (seedling recruitment), respectively (Swarts & Dixon, 2009). Little is known about the natural pollinators of *D. lindenii*, but hawkmoths (Sphingidae) are suspected to perform this role. More is known about the mycorrhizal associates of *D. lindenii* in southern Florida (Hoang *et al.*, 2017), where primarily basidiomycetes in Ceratobasidiaceae were confirmed, but mycorrhizal fungi from Cuban orchids have yet to be studied.

**Figure 2.** (A) Typical *Dendrophylax lindenii* habitat in south Florida. The understorey is characterized by stagnant water infiltrated by cypress knees (pneumatophores) of bald cypress (*Taxodium distichum*), and shaded largely by *T. distichum*, red maple (*Acer rubrum*), pop ash (*Fraxinus caroliniana*) and pond apple (*Annona glabra*). During dry episodes, water levels typically drop (pictured). (B) Ghost orchid habitat on Cape San Antonio within the Guahahacabibes National Park, Cuba. Host trees in the Cuban population are rooted on jagged reef limestone, and standing water occasionally collects in pockets in the limestone during rainy episodes.

In this paper, we provide a detailed description of *D. lindenii* in habitats in southern Florida and Cuba, serving as a baseline for future work aimed at its long-term conservation in this 'age of extinction' (Swarts & Dixon, 2009). The number of sites in which the species persists in southern Florida and Cuba is not known, but the two sites sampled appear to be typical of locations in both countries (Luer, 1972; Brown, 2005; Pérez, Pérez & Bocourt, 2014). Population size, fecundity and fungal endophytes of *D. lindenii* are also discussed, and seedlings in early stages of development *in situ* are described for the first time. The goal of this study is to yield fundamental knowledge that may be useful to conservationists and horticulturists and promote additional research collaborations between the USA and Cuba involving other rare species native to both countries.

## MATERIAL AND METHODS

### FIELD SITES

The Florida Panther National Wildlife Refuge (FPNWR), part of the Big Cypress-basin Ecoregion, served as the study site in the USA (Table 1; Fig. 2A). This 10 684-ha area is located in remote north central Collier County, FL, and is adjacent to the orchid-rich Fakahatchee Strand Preserve State Park to the south. In the FPNWR, 27 orchid species in 17 genera are known to occur (Stewart & Richardson, 2008), including 16 individuals of *D. lindenii* that were recorded prior to our study. Most of the epiphytic taxa are confined to 'islands' of strand swamps and sloughs shaded by *Taxodium distichum* (L.) Rich. (Cupressaceae), which constitutes the upper canopy. The majority of epiphytic orchids are affixed to bark of trunks and branches of

**Table 1.** General comparison of the Florida Panther National Wildlife Refuge and Guanahacabibes National Park, Cuba

| Characteristic | Florida Panther NWR, USA | Guanahacabibes NP, Cuba |
|---|---|---|
| Latitude, longitude | 26°17′′N, 81°38′W | 21°59′N, 84°50′W |
| Vascular plant associates | *Acer rubrum* L. | *Bromelia peguin* L.* |
| | *Canna flaccida* Salisb. | *Hohenbergia penduliflora* Mez* |
| | *Osmunda regalis* L. | *Tillandsia balbisiana* Schult.f.* |
| | *Polypodium polypodioides* (L.) Watt* | *Tillandsia bulbosa* Hook.* |
| | *Psilotum nudum* (L.) P.Beauv.* | *Tillandsia fasciculata* Sw.* |
| | *Salix caroliniana* Michx. | *Tillandsia flexuosa* Sw.* |
| | *Tillandsia paucifolia* Baker* | *Tillandsia juncea* (Ruiz & Pav.) Poir.* |
| | | *Tillandsia tenuifolia* L.* |
| | | *Tillandsia usneoides* (L.) L.* |
| Orchid associates | *Campylocentrum pachyrrhizum* (Rchb.f.) Rolfe | *Broughtonia* × *cubensis* Cogn. |
| | *Epidendrum amphistomum* A.Rich. | *Encyclia fucata* (Lindl.) Schltr. |
| | *Epidendrum nocturnum* Jacq. | *Encyclia plicata* (Lindl.) Schltr. |
| | *Epidendrum rigidum* Jacq. | *Epidendrum amphistomum* A.Rich. |
| | *Encyclia tampensis* Small | *Epidendrum rigidum* Jacq. |
| | *Harrisella porrecta* (Rchb.f.) Fawc. & Rendle | *Pleurothallis caymanensis* C.D.Adams |
| | *Polystachya concreta* (Jacq.) Garay & H.R.Sweet | *Polystachya concreta* (Jacq.) Garay & H.R.Sweet |
| | *Prosthechea cochleata* (L.) W.E.Higgins | *Prosthechea boothiana* (Lindl.) W.E.Higgins |
| Mean annual temperatures | 24.7 °C | 25.3 °C |
| | Max. (2015) = 35.6 °C (August) | |
| | Min. (2015) = −0.6 °C (February) | |
| Mean annual rainfall | 1399 mm | 1,333 mm |
| Hurricane frequency | 27 in 150 years (Category 1–4) | NA |
| Flowering time of *D. lindenii* | May–August | October–December |
| Fruiting time | January–April | May–June |
| Pollinators | Giant sphinx moth (presumably) | Hawkmoths (Sphingidae)† |

Vascular plant associates are species considered most common in ghost orchid sites; those denoted by an asterisk (*) are epiphytic on host trees that also harbour *D. lindenii*.
†Personal observation (Raventós *et al.*, 2015).

trees in the understorey consisting of pop ash, *Fraxinus caroliniana* Mill. (Oleaceae), and pond apple, *Annona glabra* L. (Annonaceae). These trees are rooted in tannin-rich pools of stagnant water present most of the year except during dry periods. Epiphytic orchid associates of *D. lindenii* include three *Epidendrum* spp. (*E. amphistomum* A.Richard, *E. nocturnum* Jacq., *E. rigidum* Jacq.), *Polystachya concreta* (Jacq.) Garay & Sweet, *Prosthechea cochleata* (L.) W.E.Higgins var. '*triandra*' (Ames) W.E.Higgins and two other leafless species, *Campylocentrum pachyrrhizum* (Rchb.f.) Rolfe and *Dendrophylax porrectus* (Rchb.f.) Carlsward & Whitten [synonym *Harrisella porrecta* (Rchb.f.) Fawc. &Rendle], all of which are listed as 'endangered' in Florida (Coile & Garland, 2003). Images and descriptive information on *D. lindenii* and the associated species can be found at http://goorchids.northamericanorchidcenter.org/.

The study site in Cuba was in Guanahacabibes National Park (part of the Guanahacabibes Peninsula Biosphere Reserve) in Pinar del Río Province, on Cape San Antonio (Table 1; Fig. 2B). Cape San Antonio consists of a narrow, elongated strip of semi-deciduous forest harbouring 18 native orchid species, including *D. lindenii*, and at least one recently described hybrid endemic to the peninsula, *Broughtonia* × *cubensis* (Lindl.) Cogn. (Mújica *et al.* 2015). Prior to our study, 290 individuals were known to occur in Guanahacabibes, dating back to detailed demographic surveys initiated in 2003 (Mújica, 2007; González, 2010). Beginning in December 2006, each previously known individual orchid was assessed yearly for survival, size and fruiting status (Raventós *et al.*, 2015). These data were used to augment the 2016 survey reported here.

### Field sampling

Sampling was carried out in Florida during a 19-day period (10–28 July 2015). In Cuba, sampling took place during 4–8 January 2016. Orchids in five

separate forested wetlands in the Florida Panther Refuge were sampled, each separated by *c*. 1 km from one another. The specific names of these areas have not been disclosed here to minimize potential for poaching and are referred to simply as Sites A, B, C, D and E. These areas were chosen because each was known to harbour existing *D. lindenii* plants (16 in total prior to our study). Quantitative data were collected for individual orchids and their host tree substrates. Host trees were numbered and identified, also noting information pertaining to the bark and the diameter at breast height (DBH) for each individual substrate on which a ghost orchid was found. Data collection for individuals of *D. lindenii* included plant height (= distance above the soil), DBH of host tree, orientation, distance to nearest neighbouring tree species, position in tree (trunk/branch), flowering/fruiting status and information pertaining to the roots. Although ghost orchids are typically found on the main trunk of each host tree and below 3 m, branches higher in the canopy were also visually inspected. Root analysis included dead and living root counts, average living root length and individual root lengths for each individual of *D. lindenii*. For each orchid, the total of all root lengths combined was also calculated and was termed a 'root string'. Individuals having a mass of roots that were unable to be quantified (height, aggregated, etc.) were recorded as outliers, and were considered as mature plants. Seedlings were defined as having one to three living roots with a mean root length of ≤ 3 cm, whereas juveniles had two to six living roots with mean root length ≤ 7.5 cm with one or more dead root, and no signs of an inflorescence in development. Mature plants were those with mean root lengths ≥ 3.5 cm and two or more living roots, with at least one root ≥ 10 cm in length. Flowering individuals were recorded as having an existing flower at the time of sampling, a developing inflorescence or signs of an inflorescence from the previous year. Fruiting individuals were those that had a developing capsule or a capsule from the previous year.

To isolate and identify fungal endophytes of *D. lindenii* in Florida, 1- to 4-cm segments of mature roots of *D. lindenii* were collected following the general procedure outlined by Yokoya *et al.* (2015). The root samples consisted of actively growing tips that were detached using a sterile scalpel. Care was taken to lift the firmly appressed root tip gently from the host tree substrate. Each root segment was then placed into a sterile bag and refrigerated (4 °C) within 2 h of collection; fungal isolation was carried out 24–48 h thereafter. Roots of *D. lindenii* in Cuba were not collected because legal permits had not been acquired at the time of sampling.

### Fungal isolation and identification

Within 24–48 h of collection, fungal endophytes were isolated in the laboratory from root tips using standard protocols applied to other epiphytic orchids (Richardson, Currah & Hambleton, 1993; Zettler *et al.*, 2013; Yokoya *et al.*, 2015). This consisted of rinsing the roots with sterile deionized water and gentle scraping of the surface to remove surface debris. Each root was placed into a 9-cm-diameter sterile plastic Petri plate, and surface-sterilized for 1 min in a solution of 90 mL sterile deionized water, 5 mL Clorox bleach (8.25% NaOCl) and 5 mL ethanol (95%). After the 1-min rinse, the solution was decanted and each root received two 1-min rinses in sterile deionized water. Roots were then cut into separate 1-cm segments, and each segment was placed into a new sterile Petri dish containing a 5-mL drop of sterile deionized water. By using a sterile scalpel and forceps, each root piece was macerated in the water drop to tease apart clumps of cortical cells harbouring fungal pelotons. Approximately 20 mL of molten (warm) fungal isolation medium containing streptomycin sulphate (FIM; Clements & Ellyard, 1979) was added to each plate, and the agar/cortical cell mixture was gently swirled in a circular motion to disperse pelotons throughout the plate. Plates were allowed to cool and solidify at ambient temperature and then stacked and incubated for 24–28 h. After this time, a dissection microscope was used to inspect plates for the presence of fungal hyphae emerging from pelotons in cortical cells. Hyphae from pelotons were subcultured onto potato dextrose agar (PDA; Difco, Benton, Dickinson and Co., Sparks, MD, USA) using a sterile scalpel and incubated further at ambient temperature until cultures achieved a diameter that would facilitate provisional identification. Morphological characters that distinguished orchid endophytes in the rhizoctonia-like complex included cream to yellowish-orange colonies, rapid growth rates (0.2–0.5 mm/h at ambient temperature), hyphae branching at wide angles and frequently the presence of monilioid cells (Currah *et al.* 1997).

Molecular identification followed the procedures outlined by Zettler *et al.* (2013) and Yokoya *et al.* (2015) involving ribosomal DNA ITS amplification and Sanger sequencing. Liquid broth (FIM; Clements & Ellyard, 1979) without agar or streptomycin was used to grow fungal isolates in flasks placed on a shaker at ambient temperature until harvesting (2–3 weeks after inoculation). An Omega E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-Tek Inc., Norcross, GA, USA) was used for isolation of genomic DNA from mycelia. ITS regions were amplified using primers CeTh1 and CeTh4 (Porras-Alfaro & Bayman, 2007) and an Omega E.Z.N.A. Fungal DNA Mini-Kit. A programmable thermocycler was used to perform reactions for an

initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 47 °C for 30 s and extension at 72 °C for 1 min, with a final extension step at 72 °C for 5 min. Amplification products were verified by electrophoresis on 2% agarose gels containing 0.1 mg/mL ethidium bromide. DNA samples were sent to the University of Illinois UIUC Core Sequencing Facility. Forward and reverse sequences and chromatograms were checked for accuracy and consensus and were compared with database sequences using BLAST (National Center for Biotechnology Information, Bethesda, MD, USA).

## RESULTS

In total, 116 individuals of *D. lindenii* were recorded in the Florida Panther Refuge during July 2015 (Table 2), whereas only 16 specimens were known to occur there previously. The majority (69.0%) were attached to bark of *F. caroliniana* (Table 4). Of the 116 orchids, 41 (35.3%) were present at Site E, and the second largest number (23 or 19.8%) was recorded at Site A (Table 3). Site E also harboured all known seedlings (19), and nearly all juvenile orchids (12 of 14) among the five areas (Table 3) indicating that this location serves as the primary population for seedling recruitment. When means for root number, length per root and root string totals were compared among mature plants from the five locations, those at Site C had the highest values (Table 3). Assuming that root number and lengths indicate plant age, Site C may represent the oldest population of *D. lindenii* in the Florida Panther Refuge. In contrast, Sites E and D had the lowest values among the five populations, suggesting that these two populations may be the youngest (Table 3).

In Cuba, 241 *D. lindenii* individuals were documented spanning all three growth stages, almost one-third (30.3%) of which were seedlings [nearly double (16.4%) the percentage of seedlings documented in Florida] (Table 2). Approximately two-thirds (67%) of the orchids located in Cuba were affixed to bark of five host tree species with 13 additional species also serving this role (Table 4). Just two of the 18 host tree species in Cuba harboured the great majority of seedlings (*Maba crassinervis* Urb. and *Erythroxylum areolatum* Vell.), whereas half (50%) of all mature plants were affixed to three different species [*Comocladia dentata* Jacq., *Mastichodendron foetidissimum* (Jacq.) H.J.Lam., *Tabebuia angustata* Britton; Table 4]. Moreover, many (65.4%) of the ghost orchids in Cuba were solitary individuals, not in clusters or groups unlike those in Florida which were mostly aggregated (79.3%; Table 5). Mean height of the orchids on host trees in Florida (1.9 m) was not significantly different ($P = 0.121$) from those in Cuba (1.2 m; Table 5). In Florida, *D. lindenii* was affixed to just two host tree species, *F. caroliniana* and *A. glabra*, the vast majority (69%) on the former (Table 4). The majority (89.4%) of all individuals of *D. lindenii* in Cuba grew on host trees that had corrugated or semi-corrugated bark (Table 4). More than half (55.2%) of all *D. lindenii* documented in both Florida (55.2%) and Cuba (52.7%) were attached to the north-facing (NW, N, NE) surfaces of host trees (Table 6). Significant differences ($P = 0.035$) were detected in directional orientation between the two sites, with Cuban orchids preferring NE, N and E surfaces, but those in Florida preferring NW, NE and SW surfaces. In Florida, both host tree species (*F. caroliniana*, *A. glabra*) had bark characterized as corrugated. In Cuba, 15 of the 18

**Table 2.** *Dendrophylax lindenii* population size and fecundity documented in south Florida and Cuba

| | Mature | Juvenile | Seedling | Total |
|---|---|---|---|---|
| Florida Panther NWR | | | | |
| Number of plants | 83 (71.5%) | 14 (12.1%) | 19 (16.4%) | 116 |
| Number flowering | 26 (31.3%) | — | — | 26 (22.4%) |
| Number fruiting | 3 | — | — | 3 (2.3%) |
| Guanahacabibes NP | | | | |
| Number of plants | 150 (62.2%) | 18 (7.5%) | 73 (30.3%) | 241 |
| Number flowering | 26 (17.3%) | — | — | 26 (10.8) |
| Number fruiting | 1 | — | — | 1 (0.4%) |

**Table 3.** Comparison of *Dendrophylax lindenii* root growth per growth stage at five different sites within the Florida Panther National Wildlife Refuge, and throughout Guanahacabibes National Park

| Florida Panther NWR | | | | | |
|---|---|---|---|---|---|
| | Site A | Site B | Site C | Site D | Site E |
| Growth stage | M J S | M J S | M J S | M J S | M J S |
| Mean root number | 8.1 – – | 7.5 3.0 – | 13.8 6.0 – | 12.0 – – | 8.2 4.4 1.9 |
| Mean length/root (cm) | 14.6 – – | 17.2 3.7 – | 18.3 5.8 – | 10.3 – – | 14.3 3.8 1.6 |
| Mean root string (cm) | 127.9 – – | 127.5 11.0 – | 263.8 33.0 – | 67.5 – – | 107.2 16.0 3.0 |
| Guanahacabibes NP | | | | | |
| Growth stage | Mature | Juvenile | Seedling | Total | |
| Mean root number | 9.5 | 6.1 | 3.1 | 6.8 | |
| Mean length per root (cm) | 20.9 | 10.1 | 3.1 | 13.6 | |

M, mature; J, juvenile; S, seedling.

**Table 4.** Tree species in south Florida and Cuba that serve as hosts for *Dendrophylax lindenii* spanning three different orchid growth stages (mature plants, seedlings and juveniles)

| Tree species | Bark type | Mature | Juvenile | Seedling | Total |
|---|---|---|---|---|---|
| Florida Panther NWR | | | | | |
| *Fraxinus caroliniana* | C | 58 | 12 | 10 | 80 (69.0) |
| *Annona glabra* | C | 25 | 2 | 9 | 36 (31.0) |
| Guanahacabibes NP | | | | | |
| *Maba crassinervis* | C | 11 | 0 | 28 | 39 (16.2) |
| *Erythroxylum aerolatum* | C | 11 | 0 | 26 | 37 (15.4) |
| *Comocladia dentata* | C | 32 | 2 | 2 | 36 (14.9) |
| *Mastichodendron foetidissimum* | C | 23 | 1 | 1 | 25 (10.4) |
| *Tabebuia angustata* | SC | 20 | 0 | 4 | 24 (10.0) |
| *Cedrela odorata* | C | 8 | 3 | 6 | 17 (7.1) |
| *Gymnanthes lucida* | SC | 5 | 10 | 0 | 15 (6.2) |
| *Plumeria taberculata* | S | 13 | 0 | 0 | 13 (5.4) |
| *Chascoteca neopeltandra* | C | 10 | 0 | 1 | 11 (4.6) |
| *Stigmatophyllum sagraeanum* | S | 5 | 0 | 1 | 6 (2.5) |
| *Ficus laevigata* | S | 3 | 0 | 3 | 6 (2.5) |
| *Sideroxylon fruticosum* | C | 1 | 2 | 0 | 3 (1.2) |
| *Drypetes alba* | SC | 2 | 0 | 0 | 2 (0.8) |
| *Celtis trinervia* | SC | 2 | 0 | 0 | 2 (0.8) |
| *Catalpa pubescens* | C | 1 | 0 | 0 | 1 (0.4) |
| *Pichrodendrum macrocarpum* | C | 0 | 0 | 1 | 1 (0.4) |
| *Schaferia frutescens* | SC | 1 | 0 | 0 | 1 (0.4) |
| *Adelia ricinella* | SC | 1 | 0 | 0 | 1 (0.4) |
| Dead trees (unknown taxon) | | 1 | 0 | 0 | 1 (0.4) |

Numbers of *D. lindenii* at different life history stages found on different host tree species. Numbers in parentheses are percentages. For bark types listed, C = corrugated, SC = semi-corrugated, and S = smooth.

tree species that served as hosts to *D. lindenii* had corrugated or semi-corrugated bark (Table 4).

Fungal endophytes resembling members of the rhizoctonia complex were isolated from pelotons of mature *D. lindenii* roots at Site B in the Florida Panther Refuge, and one strain (422) was retained for further study (e.g. genetic sequencing, symbiotic germination trials). On PDA, this fungus yielded colonies that were initially cream-coloured, turning yellowish-orange with age (>7 days). Mycelial growth was most evident at the surface of the agar, becoming slightly more raised as a white fluffy appearance in the centre of the colony after > 14 days. The most distinguishing morphological features were: (1)

**Table 5.** Height of *Dendrophylax lindenii* on host trees, and the number of solitary orchids (one orchid counted per tree) for the three growth stages (mature plants, seedlings and juveniles)

| | Mature | Juvenile | Seedling | Total |
|---|---|---|---|---|
| Florida Panther NWR | | | | |
| Mean height (m) | 2.0 | 2.0 | 1.8 | 1.9 |
| Number solitary | 19 | 3 | 2 | 24 |
| | (16.4) | (2.6) | (1.7) | (20.7) |
| Guanahacabibes NP | | | | |
| Mean height (m) | 1.4 | 1.9 | 1.1 | 1.2 |
| Number solitary | 117 | 18 | 23 | 158 |
| | (48.5) | (7.5) | (9.5) | (65.5) |

Numbers in parentheses are percentages of all individuals that were solitary. No significant differences were detected in orchid height on host trees between Florida and Cuba ($P = 0.121$).

**Table 6.** Directional orientation of *Dendrophylax lindenii* on host trees for the three growth stages (mature plants, seedlings and juveniles) in south Florida and Cuba

| | Mature | Juvenile | Seedling | Total |
|---|---|---|---|---|
| Florida Panther NWR | | | | |
| North (N) | 11 | 3 | 3 | 17 (14.6) |
| North-east (NE) | 17 | 3 | 2 | 22 (19.0) |
| East (E) | 2 | 0 | 2 | 4 (3.4) |
| South-east (SE) | 6 | 2 | 2 | 10 (8.6) |
| South (S) | 6 | 0 | 1 | 7 (6.0) |
| South-west (SW) | 19 | 0 | 2 | 21 (18.1) |
| West (W) | 7 | 1 | 1 | 9 (7.7) |
| North-west (NW) | 15 | 5 | 5 | 25 (21.5) |
| Total N-facing | 43 | 11 | 10 | 64 (55.2) |
| Total S-facing | 31 | 2 | 5 | 38 (32.7) |
| Guanahacabibes NP | | | | |
| North (N) | 19 | 8 | 13 | 40 (16.6) |
| North-east (NE) | 30 | 4 | 22 | 56 (23.2) |
| East (E) | 27 | 1 | 10 | 38 (15.8) |
| South-east (SE) | 16 | 0 | 5 | 21 (8.7) |
| South (S) | 14 | 0 | 0 | 14 (5.8) |
| South-west (SW) | 4 | 3 | 13 | 20 (8.3) |
| West (W) | 18 | 0 | 3 | 21 (8.7) |
| North-west (NW) | 22 | 2 | 7 | 31 (12.9) |
| Total N-facing | 71 | 14 | 42 | 127 (52.7) |
| Total S-facing | 34 | 3 | 18 | 55 (22.8) |

Numbers in parentheses are percentages. Significant differences were detected in directional orientation between the two sites, with Cuban orchids preferring NE, N and E but those in Florida preferring NW, NE and SW surfaces ($P = 0.035$).

the presence of mycelial arms or straight branches that radiated outward from the centre of the colony (Fig. 3); (2) lack of visible concentric zonation; and (3) absence of sclerotia on aged (> 3 months) cultures. Hyphal growth rates at 25 °C were rapid (0.3–0.4 mm/h). Microscopic examination of hyphae revealed mostly barrel-shaped, broadly attached monolioid cells in short chains. Collectively, these cultural characteristics suggest that fungus 422 may be linked to Ceratobasiaceae (Cantherellales), in particular *Ceratobasidium* D.P.Rogers. Subsequent use of molecular analysis confirmed the identity (93% match in BLAST) of this fungus as belonging to *Ceratobasidium*, based on amplification and sequencing of the ITS region of ribosomal DNA. This fungus was deposited in the UAMH Centre for Global Microfungal Biodiversity, Toronto, Ontario, Canada, as UAMH 11954.

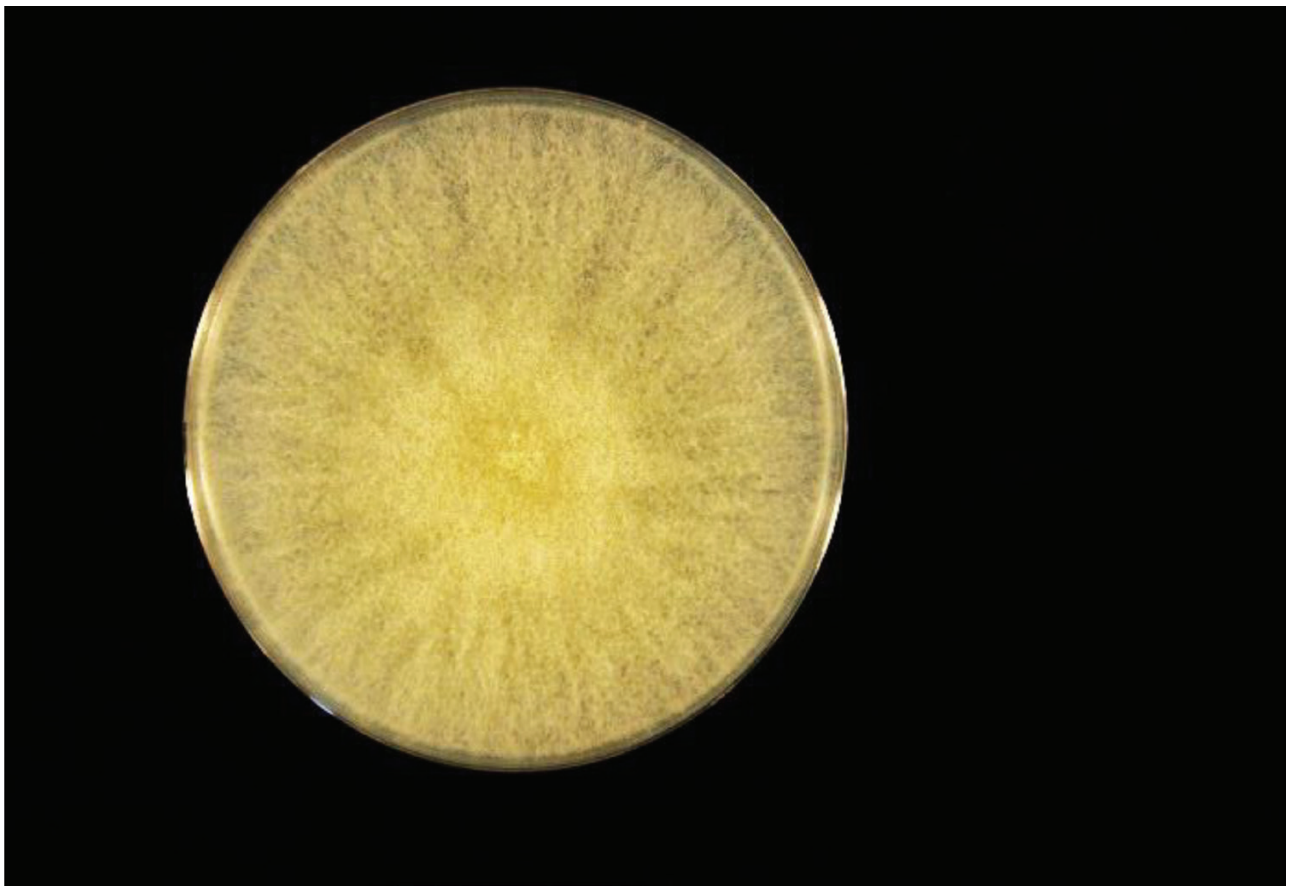

**Figure 3.** Fungus 422, assignable to the Ceratobasidiaceae, in culture on half-strength potato dextrose agar (PDA) within a 9-cm-diameter Petri dish after 7 days of incubation at ambient temperature. Mycelial arms can be seen radiating outward from the centre of the colony. Concentric zones – often typical of *Ceratobasidium* – are noticeably absent. Photo courtesy of Michael E. Kane.

## DISCUSSION

Seedling recruitment of *D. lindenii* was more widespread in Cuba, as evidenced by more solitary individuals, multiple host trees, a higher percentage (30.3%) of plants in seedling stages and seedling clusters on trees that lacked mature plants. In Florida, the opposite was true, i.e. that seedling recruitment was much more limited. Why more seedlings were not observed in Florida is a perplexing question, but could be attributed to stem density and/or spacing of trees with appropriate bark characteristics. It is also conceivable that young seedlings in Florida are more prone to occasional cold periods compared to established plants. Rasmussen *et al.* (2015) characterized ageing orchid populations as 'senile' when opportunities for local seedling recruitment have all but disappeared such as in habitats that are degraded, altered or reduced in extent. Given that many individual host trees (*A. glabra*, *F. caroliniana*) of *D. lindenii* in Florida were fully mature, perhaps dating back to widespread cypress logging in the 1940s, *D. lindenii* may persist in these areas year to year in a slow and subtle decline with one exception (Site E). Thus, it is conceivable that some of the populations of *D. lindenii* in Florida are 'senile', perhaps attributed to habitat degradation linked to host tree ageing. To address this concern fully and to gain confidence in predicting *D. lindenii* population trends in the Florida Panther Refuge, continued monitoring of these populations is being planned for at least the next 3 years, including the monitoring of existing seedlings. Comparing the populations in the Florida Panther Refuge to other sites for *D. lindenii* in southern Florida and elsewhere (e.g. Cuba) will be necessary to determine if *D. lindenii* is threatened, or if it merely lacks the components needed for seedling recruitment. The use of seed packets adapted for epiphytic orchids (e.g. Zettler *et al.*, 2011), along with continued monitoring to assess the potential for seedling establishment, could also be applied to further assess the specific components needed for recruitment.

Even if orchid population models project reasons for optimism, there is an underlying concern that habitats of *D. lindenii* in southern Florida and Cuba are undergoing rapid, irreversible change imposed by climate change and other factors (e.g. alteration of the landscape and its hydrology, invasive/exotic species). Both regions, for example, are vulnerable to sea-level rise this century (3.2 mm/year; https://climate.nasa.gov, accessed 23 January 2018) given their low elevation (< 15 m; https://www.freemaptools.com/elevation-finder.htm, accessed 23 January 2018) and the severity and frequency of tropical cyclone activity is another concern (Mujíca *et al.*, 2013). In North America, the devastation inflicted on native species of *Fraxinus* L. by an introduced Asian beetle (emerald ash tree borer, *Agrilus planipennis*) is another reason for concern. This beetle has recently been discovered as far south as Atlanta, Georgia, raising the possibility that it will ultimately spread to Florida. It is conceivable, therefore, that this beetle could infect the mature stands of *F. caroliniana* in southern Florida. Should that occur, it would have a devastating effect on populations of *D. lindenii* in the Florida Panther Refuge, given that 69% of the orchids were affixed to the bark of this tree species.

A similarity shared by host trees in both Florida and Cuba was the flaky corrugated bark (with crevices *c.* 0.5–2.0 cm deep) especially on the older trees. Indeed, all 116 specimens of *D. lindenii* in Florida were attached to corrugated or semi-corrugated bark, none being present on smoother bark of other associated trees (e.g. *Acer rubrum* L., *Taxodium distichum*). Thus, wind-dispersed seeds released from capsules might be more likely to lodge in crevices of trees having rough bark as opposed to smooth bark. Once lodged there, seeds would have an opportunity to germinate after the embryo is physically infected by a suitable mycorrhizal fungus present on the substrate. This assumption was supported by our direct observation in Cuba where we noted 26 small seedlings in bark crevices of one host tree, *E. aerolatum* (Fig. 4). The identity of these early-stage seedlings as *D. lindenii* was verified by the presence of a dorsal crest (= modified first leaf) observed on protocorms, and an emerging root on protocorms transitioning to seedlings (Fig. 4); these characteristics closely match published descriptions of *D. lindenii* seedlings cultivated *in vitro* (e.g. Hoang *et al.*, 2017). To our knowledge this is the first report to have documented early-stage seedlings of *D. lindenii in situ*. Assuming that the developmental time of these seedlings is comparable to those observed by Hoang *et al.* (2017) *in vitro*, we estimate that these seedlings germinated *c.* 100 days earlier, i.e. in late August or early September. This time frame agrees with the May–June fruiting times observed on the Guanahacabibes Peninsula (Table 1). Seed dispersal from capsules during late summer/early autumn also coincides with peak hurricane frequency for the region. Thus, we speculate that seeds of *D. lindenii* may be dispersed over longer distances and into corrugated bark crevices by tropical cyclone activity.

Another similarity we noted between the two sites was the mean height of the orchids on host trees, i.e. 1–2 m above the ground. Although no significant differences were detected in mean orchid height on host trees, those in Florida were slightly higher off the ground (1.9 m) than those in Cuba (1.2 m), which might be attributed to the higher water levels. In other words, the persistence of stagnant water in southern Florida during the rainy season may serve as a barrier to seed germination and seedling establishment on bark surfaces that remain submerged for prolonged periods of time. Indeed, during our surveys in Florida, we did not observe *D. lindenii* or any other orchid species affixed to bark below the water line, even during dryer periods. Seasonal flooding may also explain why fewer tree species (two) served as hosts for *D. lindenii* in Florida compared to Cuba (18) (Table 4), as only a select few tree species might be adapted to survive while rooted in stagnant water for long periods of time. In Cuba, it is possible that more tree species serve as hosts simply because a rocky substrate affords their roots with more aeration.

### Fungal endophytes and substrates

Yukawa *et al.* (2009) proposed that members of Ceratobasidiaceae are linked to orchids in tribe Vandae, and especially in the Angraecinae subtribe (e.g. *D. lindenii*). Similarly, Chomicki, Bidel & Jay-Allemand (2014) noted that 'the type and fungal structures observed in *Dendrophylax* and the phylogenic signal in Angraecinae altogether suggest that *D. lindenii* forms mycorrhiza with a basidiomycete fungus of the Ceratobasidiaceae, although this needs to be confirmed by a molecular study'. In the present study, fungal isolate 422 displayed cultural characteristics generally typical of Ceratobasidiaceae, and *Ceratobasidium* in particular (Currah *et al.*, 1997). However, our isolate lacked concentric zones (rings) of mycelial growth along the agar surface (Zettler *et al.*, 2001), a feature common to several *Ceratobasidium* strains recovered from orchids in North America, including *D. lindenii* (Hoang *et al.*, 2017). Amplification and sequencing of the ITS region confirmed the identity of isolate 422 as *Ceratobasidium*. Hoang *et al.* (2017) recovered two strains of *Ceratobasidium* (379, 394) from *D. lindenii* at Site E during 2013 and 2014, respectively, and the identity of one (394) was confirmed by ITS sequences in the NCBI database. Moreover, isolate 394 did not match any specific accession in the database,

suggesting that it may be a unique, previously unreported fungus (Hoang *et al.*, 2017). Subsequent molecular analysis has confirmed that strains 379 and 394 are genetically identical (L. L. Corey, unpubl. data), indicating that this fungus is present in different ghost orchids at Site E. Recently, isolate 394 was also shown to be identical to the predominant sequences recovered directly from mature roots of *D. lindenii* in a population > 30 km from Site E (D. L. Taylor, pers. comm.). Based on our initial sequencing results, isolate 422 appears to be different from isolates 379/394, suggesting that *D. lindenii* has the potential to harbour different strains of *Ceratobasidium* in roots of mature plants. Whether isolate 422 is a mycorrhizal associate of *D. lindenii* is not yet known. Taking the above evidence together, it is conceivable that *D. lindenii* in southern Florida is a specialist orchid, targeting a narrow group of *Ceratobasidium* strains to meet its mycotrophic needs. To what extent *D. lindenii* harbours other types of peloton-forming fungi in the *Rhizoctonia* DC. complex (e.g. *Tulasnella* J.Schröt.) is not yet known, but this possibility should be explored more fully. Studies are also needed to determine if *Tulasnella* is present in other epiphytic orchids that coexist on the same substrate as *D. lindenii*. Plans are under way to isolate additional endophytes from Orchidaceae in southern Florida and to expand our work into Cuba, assuming that appropriate collection and export/import permits can be secured. Isolation, identification and preservation of fungi from young seedlings of *D. lindenii* on the Guanahacabibes Peninsula (Fig. 4) is of particular interest. Subsequent use of these endophytes to germinate seeds *in vitro* would be likely to improve conservation of *D. lindenii* in both countries, if our efforts are successful.

Although fungal isolation attempts involving Cuban orchids are being planned, roots of epiphytic orchids in the Florida Panther Refuge have yielded rhizoctonia-like fungi from several cohabiting species in the past decade. For example, several leaf-bearing taxa have yielded fungi assignable to *Tulasnella* (e.g. *Encyclia tampensis* Small; Zettler *et al.*, 2011) and *Sebacina* (e.g. *Prosthechea cochleata*; L. W. Zettler, unpubl. data), whereas two other cohabiting leafless species


**Figure 4.** One of 26 early-stage seedlings of *Dendrophylax lindenii* observed on the corrugated bark of a single individual host tree, *Erythtoxylum aerolatum*, in Guanahacabibes National Park, Cuba. The dorsal crest and emerging root are visible on the right and left of the seedling, respectively. Based on *in vitro* germination studies by Hoang *et al.* (2017), this seedling appears to be *c*. 100 days old (post-germination). Another smaller seedling is visible left of the ruler.

(*Campylocentrum pachyrrhizum*, *Dendrophylax porrectus*) have yielded mostly *Ceratobasidium* strains (Radcliffe *et al.*, 2015; L. W. Zettler, unpubl. data). Recently (2016), an endophyte provisionally identified as *Tulasnella* was isolated from mature roots of *D. porrectus*, and this fungus facilitated seed germination of *E. tampensis in vitro* (L. W. Zettler, unpubl. data). Six strains of *Cetatobasidium* have been acquired from *C. pachyrrhizum*, and two are being tested in seed germination trials at the University of Florida by our collaborators. Preliminary identification using ITS amplification and sequencing revealed that the *Ceratobasidium* strain in *D. lindenii* was different from those in *C. pachyrrhizum* and that *C. pachyrrhizum* utilized the same *Ceratobasidium* strain in different parts of the Florida Panther Refuge (Sites C and E; Radcliffe *et al.*, 2015; L. L. Corey, unpubl. data). In Costa Rica, Richardson et al. (1993) isolated *Ceratorhiza* R.T.Moore (= *Ceratobasidium*) from *Campylocentrum micranthum* (Lindl.) Rolfe. Similarly, in nearby Puerto Rico, Otero *et al.* (2002) documented *Ceratobasidium* from *Campylocentrum fasciola* (Lindl.) Cogn. and *C. filiforme* (Sw.) Cogn. ex Kuntze and concluded that many epiphytic orchids there associate with this fungal genus. They also reported that specificity of the orchid mycorrhizal association varies dramatically, even among closely related species. Swarts *et al.* (2010) provided evidence for orchid rarity as a cause and consequence of high mycorrhizal specialization. Consequently, it is possible that the distribution of *D. lindenii* was also limited by its mycorrhizal associate, in this case specific strains of *Ceratobasidium*.

Why *D. lindenii* appears to associate with *Ceratobasidium* in southern Florida remains unresolved, but the answer, in part, may be linked to nutrition *in situ*. For example, rhizoctonia-like fungi are known to produce enzymes for decomposition, e.g. cellulase (Zelmer, Cuthbertson & Currah, 1996). *Ceratobasidium*, however, is also known to produce polyphenoloxidases that are involved with lignin breakdown (Rasmussen, 1995). Thus, it is conceivable that *D. lindenii* utilizes *Ceratobasidium* to gain a selective advantage in colonizing corrugated bark surfaces where woody debris and moisture would accumulate. Indeed, our observation of 26 small seedlings of *D. lindenii* on bark crevices in Cuba (Fig. 4) lends support for this hypothesis. As opposed to smooth surfaces, corrugated bark probably served as a physical anchor point for wind-blown seeds, and the presence of *Ceratobasidium* in the substrate itself may have facilitated the early nutritional needs of the orchid. This may also explain why *D. lindenii* was absent from the surfaces of jagged reef limestone in Cuba because such surfaces were generally devoid of visible organic matter.

Moisture could be another factor linked to the preference of *D. lindenii* for trees with corrugated bark. For example, rough bark surfaces would have a higher surface area compared to smooth surfaces. Following rains, corrugated bark is likely to become spongy from water absorption and to have better moisture retention capacity during dry periods. Although corrugated bark often contains superficial cork layers that are water repellent, it is conceivable that natural weathering would eventually render such layers in crevices more hydrophilic. Vertical grooves could then potentially serve as a conduit for channelling water and nutrients from the upper canopy down the host tree and into orchid roots present in the grooves. Indeed, roots of juvenile *D. lindenii* were frequently observed growing in such a manner (Fig. 5). As the orchid matures, an increase in root length would give individual plants the ability to tap new moisture-rich zones on all sides of the host tree (wrap-around effect), as we observed (Fig. 1). In this manner, orchids affixed higher in the tree would then have access to water closer to the ground. Moist corrugated bark may also benefit seed germination directly by providing the mycoflora of the substrate with moisture. In southern Florida, both primary host trees (*A. glabra*, *F. caroliniana*) displayed corrugated bark features (Fig. 5), as did *E. aerolatum* in Cuba. The presence of mosses would also be likely to impart a survival advantage to young developing orchids. Osorio-Gil, Forero-Montaña & Otero (2008), for example, noted that roots of *Ionopsis utricularioides* (Sw.) Lindl. in Costa Rica had a higher percentage of cortical cells harbouring pelotons for roots in contact with mosses. They proposed that these fungi may be parasitic on the mosses, or that the presence of moss favours mycorrhizal formation through increased humidity. Yoder, Zettler & Stewart (2000) proposed that epiphytic orchids utilize mycotrophy as a source of free water, not just carbon, because their arboreal habitat renders them more prone to desiccation compared to terrestrial orchids. Thus, the presence of moisture linked to mosses and corrugated bark alike would be conducive to fungal growth, which in turn would benefit seedling hydration through mycotrophy. Indeed, more than half (55.2%) of all *D. lindenii* individuals documented in Florida (55.2%) and Cuba (52.7%) were attached to the north-facing (NW, N, NE) surfaces of host trees (Table 6) where water retention would be higher due to less direct light exposure. Taken together, a combination of biotic and abiotic factors present in southern Florida and Cuba best explain the presence of *D. lindenii* in the two habitats (corrugated bark, probable presence of *Ceratobasidium* and moisture), all of which would presumably facilitate seedling recruitment.

**Figure 5.** Two ghost orchid individuals photographed on the corrugated bark of *Annona glabra* in Florida Panther National Wildlife Refuge. Note that many roots appear to be growing into vertical gaps along the bark surface. Both plants would be categorized as 'juveniles' given that they each harbor 2–6 living roots ≤7.5 cm in length. Roots of this species are characterized by small white marks (pneumatodes) that are visible on the longest root pictured. Both individuals were marked by two thumb tacks to facilitate documentation during the survey.

## FUTURE DIRECTIONS – A REASON FOR OPTIMISM

According to Swarts & Dixon (2009), the ability to conserve terrestrial orchids in this age of extinction will depend on three actions that seem equally applicable to epiphytes such as *D. lindenii*: (1) design and management of natural reserves, taking into account the specialized needs of orchids; (2) establishment of *ex situ* seed and mycorrhiza banks for orchids under immediate threat; and (3) development of techniques for orchid restoration. In action one, the 'specialized needs' of *D. lindenii* encompass abiotic and biotic factors, the latter of which includes pollinators and mycorrhizal fungi to ensure fruit set and seed germination, respectively. We know surprisingly little about the pollinators of *D. lindenii*. Sadler *et al.* (2011) analysed floral fragrance and concluded that the blend of chemicals, coupled with the floral structure (long nectar spur, white colour), were indicative of hawkmoth (Sphingidae) pollination. In southern Florida, the only native moth with a proboscis long enough to probe flowers of *D. lindenii* for nectar is the giant sphinx moth, *Cocytius antaeus*. To our knowledge there are no published reports that document the capture of this moth with *D. lindenii* pollinia affixed to its body. However, it is reasonable to assume that this is indeed the natural pollinator given that the flight time of the moth coincides with ghost orchid flowering and its larval food source is *A. glabra*, the same host tree species used by *D. lindenii*. In Cuba, Raventós *et al.* (2015) observed hawkmoth pollination of *D. lindenii*, but the moth species was not identified. Given that *A. glabra* and another member of Annonaceae [*Oxandra lanceolata* (Sw.) Baill.] were both present in Guanahacabibes National Park, the giant sphinx moth could potentially serve as a pollinator of *D. lindenii* in both countries. In Florida, the application of pesticides for mosquito control in nearby urban areas and in agricultural lands bordering the habitat of *D. lindenii* could potentially have a detrimental effect on hawkmoth numbers, resulting in reduced pollination and fruit set. On the remote, isolated Guanahacabibes

Peninsula, pesticides do not (currently) pose a threat, but severe hurricanes have been implicated in reducing fruit set in *D. lindenii*, a species already known for yielding low percentages of capsules (Mujíca *et al.* 2012), which we have also noted (Table 2). Why both flowering and fruiting time differ greatly between the two populations (Table 1) is not known, but might be attributed to differences in climate and/or genetic differences in the populations.

Unlike the pollination biology of *D. lindenii*, more is known about the mycorrhizal association, with members of Ceratobasidiaceae apparently serving this role. This hypothesis is supported by repeated isolation of *Ceratobasidium* from root pelotons spanning several years, coupled with *in vitro* confirmation through symbiotic germination (Hoang *et al.*, 2017). What remains to be determined is whether *D. lindenii* also utilizes *Ceratobasidium* in Cuba, and if the orchid is specific for certain strains as proposed by Hoang *et al.* (2017). If confirmed, this would represent a significant step for the long-term conservation of *D. lindenii*, as this fungus (394) might be used to augment the propagation of the orchid from seed leading to reintroduction. Efforts are under way to safeguard seed and mycorrhizal associates of *D. lindenii* in *ex situ* banks at Soroa Orchid Garden, Cuba, and in the United States coordinated through the North American Orchid Conservation Center (Whigham & Zettler, 2016), in fulfilment of the second action item proposed by Swarts & Dixon (2009).

The recent success by Hoang *et al.* (2017) in propagating *D. lindenii* from seed has led to its reintroduction spearheaded by Dr Mike Kane's group at the University of Florida. To date, laboratory-generated seedlings have been reintroduced in the FPNWR and at the Naples Botanical Garden with low mortality after 1 year (M. Kane, pers. comm.). Assuming that modest survivorship is achieved followed by anthesis, seed set and subsequent appearance of spontaneous seedlings, the long-term conservation of *D. lindenii* may actually be possible.

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