VARIATION IN THE DENDROPHYLAX PORRECTUS SPECIES COMPLEX (VANDEAE: ORCHIDACEAE) BASED UPON MORPHOLOGICAL AND MOLECULAR DATA

By

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To my family

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LIST OF ABBREVIATIONS

°C	Degrees celcius
AIC	Akaike information criterion
AMES	Harvard University
AMO	Herbario AMO
ANOVA	Analysis of Variance
bps	base pairs
BS	Bootstrap
CICY	Centro de Investigación Científica de Yucatán
cm	centimeter
DELTRAN	Delayed transformation
DNA	Deoxyribonucleic acid
Fig	Figure
FLAS	University of Florida Herbarium
HTF	Partition homogeneity test
intF	internal forward
intR	internal reverse
ITS	Internal Transcribed Spacer gene
JK	Jackknife
M.S	Masters of Science
matK	MaturaseK gene
MEXU	Universidad Nacional Autónoma de México
ml	milliliter
ML	Maximum Likelihood
mm	millimeter

NY	New York Botanical Garden
PCR	Polymerase Chain Reaction
rpm	revolutions per minute
SEL	Marie Selby Botanical Gardens
SPR	Subtree pruning-regrafting
TBR	tree bisection-reconnection
UPRRP	University of Puerto Rico
USF	University of South Florida
UVAL	Universidad del Valle de Guatemala
vs	versus
ycf1	hypothetical chloroplast open reading frame 1
µL	microliter
µm	micrometer

Abstract of Thesis Presented to the Graduate School
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Dendrophylax porrectus (Rchb.f.) Carlsward & Whitten, commonly known as the Jingle Bell orchid, is a leafless epiphytic orchid distributed throughout Florida (USA), the Greater Antilles, Mexico, Guatemala, and El Salvador. Previous phylogenetic analyses based on combined nuclear and plastid sequences (ITS, *matK*, and *trnL-F*) revealed considerable variation across its geographic range. This study aims to resolve the species circumscription and relationships within the *Dendrophylax porrectus* complex, as represented by populations in Florida (USA) and Yucatán (Mexico) through morphometric, statistical, and phylogenetic analyses. Measurements of flower parts and roots were taken for comparative studies, and DNA sequences of ITS and plastid regions *matK* and *ycf1* were used to estimate the phylogenetic relationships within *Dendrophylax* and *D. porrectus*. All morphometric measurements indicate a significance difference between the Florida (thin-rooted) and Yucatán (thick-rooted) entities. Molecular analyses supports the monophyly of *D. porrectus* sensu lato and the hypothesis that the thin and thick-rooted taxa form sister clades. Morphological and

molecular data support the recognition of the thin-rooted populations and the thick-rooted populations as two distinct species.

CHAPTER 1
INTRODUCTION

Taxonomic History

The genus *Dendrophylax* Rchb.f. (13 species) is sister to *Campylocentrum* Benth. (35 species). These two genera are the only Neotropical members of the African subtribe Angraecinae (Carlsward et al., 2003). Although *Campylocentrum* consists of primarily leafy taxa, these two genera both contain leafless species, with 13 leafless species known for *Dendrophylax* and 12 for *Campylocentrum*. The leaflessness of these orchids is exceptional because they have a reduced shoot with non-photosynthetic scales (Carlsward, 2004).

As in all leafless orchids, *Dendrophylax porrectus* (Rchb.f.) Carlsward & Whitten, commonly known as Jingle Bell Orchid or Needle Root Orchid, conducts photosynthesis in its greenish roots. Charles Wright first collected *D. porrectus* in Cuba and Reichenbach (1865) described it as *Aeranthus porrectus* Rchb.f. It was transferred to *Campylocentrum as C. porrectum* by Rolfe (1903) and six years later it was placed in its own genus, *Harrisella* Fawc. & Rendle (1909). The name of this genus was chosen to commemorate William H. Harris, 1860–1920, a British botanist and prolific collector of Jamaican plants (Ackerman, 1995). The molecular analyses of Carlsward (2003) clarified the placement and relationships of *H. porrecta,* and it was transferred to *Dendrophylax,* as *D. porrectus* (Rchb.f.) Carlsward & Whitten (2003). Other synonyms of *D. porrectus* are *Harrisella amesiana* Cogniaux and *H. uniflora* Dietrich (Ackerman, 1995).

Distribution

Based on vouchers and database data acquired from various herbaria: FLAS, USF, SEL, AMES, NY, CICY, MEXU, AMO, and UPRRP (Holmgren et al., 1990), literature (Dix & Dix, 2000; Cansino et al., 1996), personal communications G. Salazar & R. Jiménez Machorro, 2011 (Table 1-1), *Dendrophylax porrectus* is distributed through Florida, the West Indies (Cuba, Cayman Islands, Jamaica, Dominican Republic, and Puerto Rico) and Central America (Mexico, Guatemala, and El Salvador) (Figure1-1). The elevation of *D. porrectus* ranges from 5 m – 1200 m above sea level (a.s.l.). These twig epiphytes do not grow on a specific host. Based upon herbarium records, host plants (phorophytes) include *Brya buxifolia* (Murray) Urb, *Taxodium distichum* (L.) Rich, *Fraxinus caroliniana* Mill, *Juniperus virginiana* var*. silicicola* (Small) A.E. Murray, *Viburnum obovatum* Walter, *Randia aculeata* L, *Gymnopodium floribundum* Rolfe, *Blepharidium guatemalense* Standl., *Citrus sp*., *Psidium sp*., and *Crescentia sp.* Habitats include creek sides, river margins, swamps, cypress domes, hardwood hammocks (Brown, 2002), dry shrub forest, and high forest.

Intraspecific Variation in *D. porrectus*

Dendrophylax porrectus was recognized as a single species until Warford (1997) observed morphological differences in the flowers of populations in Jalisco, Mexico. When compared to populations of the Greater Antilles and Florida, the Jalisco populations have a narrower rostellar entry to the stigma, and the pollinia are flatted and ovoid, not globose (Warford, 1997). These preliminary data suggested that *D. porrectus* may be composed of more than one species. In addition, preliminary phylogenetic analyses based on combined nuclear and plastid data sets (ITS, *matK*, and *trnL-F*) from plants across the distributional range of *D. porrectus*, showed high sequence

divergence among populations (Carlsward et al., 2003). Differences in root thickness, habitat preferences (swamp vs. dry shrub), and flower morphology among sites suggested that *D. porrectus* might be a complex of species that have gone unrecognized by botanists because of their diminutive habit and uniformly inconspicuous flowers.

To test the hypothesis that cryptic species exist within our current concept of *D. porrectus*, I have pursued statistical analyses on morphometric data and estimated the evolutionary relationships within *Dendrophylax* by using phylogenetic analysis of DNA sequence data. The purpose of this study is to estimate the phylogenetic relationships within the *D. porrectus* complex by DNA sequencing of samples across its distributional range and correlating this phylogenetic information with patterns of morphological and anatomical variation, focusing on plants in the Yucatán and Florida. Determination of the appropriate species delimitation within the *D. porrectus* complex will be based on the molecular and phenotype data considered in light of the evolutionary, diagnostic, morphological/phenetic, and apomorphic species concepts (de Queiroz, 2007; Judd et al., 2007; de Queiroz & Good, 1997; Donoghue, 1985; Coyne & Orr, 2004).

Table 1-1. List of herbarium specimens used to generate a distribution map of *Dendrophylax porrectus*. The location from which each specimen was collected are listed. Vouchers are listed by collector and collector number; herbaria are where the voucher is deposited. 1: voucher examined, 2: database data (specimen not examined), 3: literature, 4: photo material, 5: unvouchered DNA sample.

Taxon	Location: Country, State, County	Voucher (Herbaria)/source
D. porrectus	Cuba, Pinar del Río, Sandina	*Ackerman 4514* (UPRRP)[5]
D. porrectus	Dominican Republic, San Juan, Guanito	*Whitten 1950* (FLAS)[1]
D. porrectus	El Salvador, San Salvador, La Libertad	*Hamer 506* (SEL)[1]
D. porrectus	Cayman Islands	*Raymond Tremblay s.n.*[5]
D. porrectus	Guatemala, El Progreso	*Dix 7502* (UVAL)[3]
D. porrectus	Guatemala, Petén	*Dix 7861* (UVAL)[3]
D. porrectus	Jamaica	*Carlsward 184* (FLAS)[1]
D. porrectus	Mexico, Campeche,	*Carnevali 4352* (CICY)[1]
D. porrectus	Mexico, Campeche, Municipio Carmen	*Carnevali 5822* (CICY)[1]
D. porrectus	Mexico, Campeche, Municipio Champoton	*Carnevali 4920* (CICY)[1]
D. porrectus	Mexico, Chiapas, Escuintla	*Matuda 18674* (MEXU)[2]
D. aff. *porrectus*	Mexico, Jalisco, La Huerta	*Salazar 8279* (MEXU)[2]
D. aff. *porrectus*	Mexico, Jalisco, Puerto Vallarta	*Warford 652* (SEL)[1]
D. aff *porrectus*	Mexico, Oaxaca, San Carlos Yautepec	*Salas 32294* (MEXU)[2]
D. porrectus	Mexico, Quintana Roo	*Carnevali 6312* (FLAS)[1]
D. aff. *porrectus*	Mexico, Veracruz, Xalapa	*Salazar 5204* (AMO)[4]
D. aff. *porrectus*	Mexico, Yucatán, Merida	*Carnevali 5907* (FLAS)[1]
D. porrectus	Mexico, Yucatán, Municipio Sotuta	*Ramirez 886* (CICY)[1]
D. porrectus	Puerto Rico, Las Cobanitas	*Ackerman 3340* (UPRRP)[5]
D. porrectus	USA, Florida, DeSoto	*Shuey 1847* (USF)[1]
D. porrectus	USA, Florida, Glades	*Carlsward 329* (FLAS)[1]
D. porrectus	USA, Florida, Highlands	*Luer 100* (SEL)[1]
D. porrectus	USA, Florida, Hillsborough	*Van Hoek 0594* (USF)[1]
D. porrectus	USA, Florida, Lee	*Whitten 3745* (FLAS)[1]
D. porrectus	USA, Florida, Manatee	*Shuey 1714* (USF)[1]
D. porrectus	USA, Florida, Orange	*Stoutamire* s.n.(USF, 85314)[1]
D. porrectus	USA, Florida, Pasco	*Genelle 1582*(USF)[1]
D. porrectus	USA, Florida, Polk	*Wunderlin 9201*(USF)[1]

Table 1-1 (cont.)

Taxon	Location: Country, State, County	Voucher (Herbaria)/source
D. porrectus	USA, Florida, Glades	*Goldman 2271* (FLAS)[1]
D. porrectus	USA, Florida, Hernando	*Lakela 28648* (USF)[1]
D. porrectus	USA, Florida, Lee	*Carlsward 330* (FLAS)[1]
D. porrectus	USA, Florida, Collier	*Woodmansee 1816* (USF)[1]

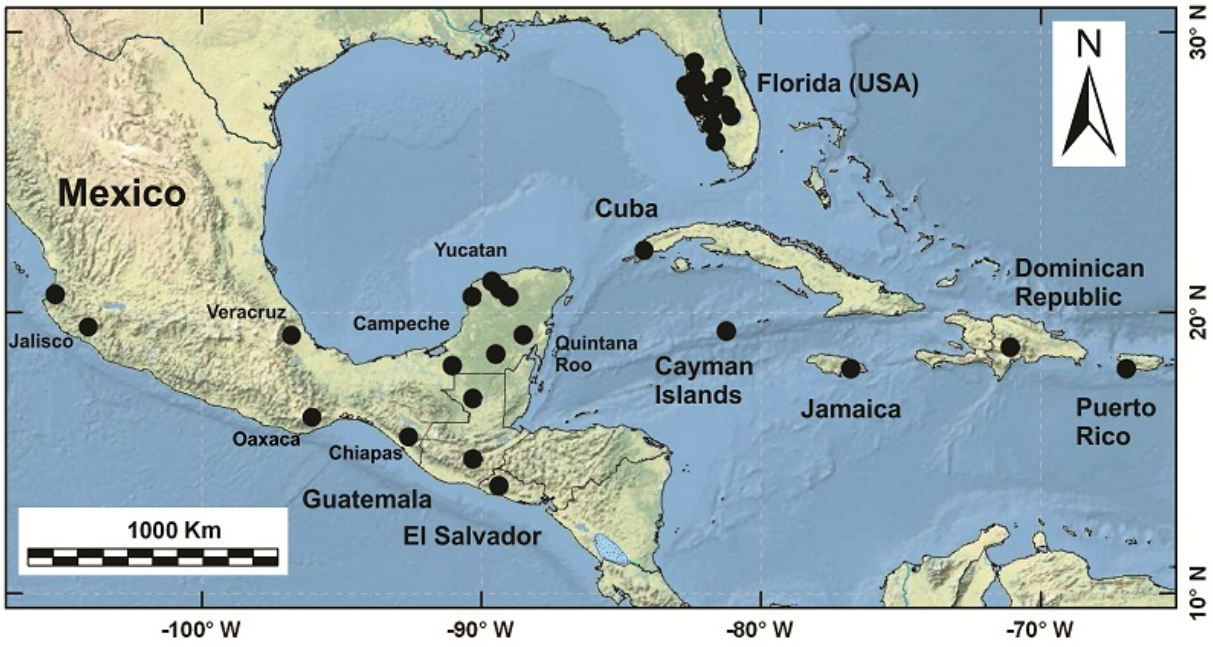

Figure 1-1. Distribution of *Dendrophylax porrectus* sensu lato. Locations are based on herbarium specimens, literature (Dix & Dix, 2000) and photo material (pers. comm. G. Salazar & R. Jiménez Machorro, 2011). *Dendrophylax porrectus* is distributed throughout peninsular Florida, the West Indies (Cuba, Cayman Islands, Jamaica, Dominican Republic, and Puerto Rico) and in Central America (Mexico, Guatemala, and El Salvador).

CHAPTER 2
MATERIALS AND METHODS

Plant Material

Specimens used in this investigation were obtained from wild collected plants and herbarium specimens. Most of the wild collected specimens are from Florida, USA, and Yucatán and Jalisco in Mexico. The roots of the plants were placed in RNAlater™ (Bent & Taylor, 2010) or dried in silica gel (Chase & Hills, 1991). Samples from Florida were collected from private lands in Sarasota and Ft. Myers counties with permission of landowners. Collected plants were cultivated in the greenhouse of the University of Florida Herbarium (FLAS). In collaboration with Centro de Investigación Científica de Yucatán in Mexico (CICY), plants were collected from the Yucatán peninsula. These plants were cultivated, photographed, and measured in Mexico by Germán Carnevali (CICY). Herbarium specimens from USF, SEL, CICY, and FLAS (Holmgren et al., 1990) were examined for this study. Destructive sampling took place on some specimens from CICY. Since this study is an extension of Carlsward' s study of Vandeae (Carlsward, 2004), DNA from her specimens was used to sequence additional DNA regions (Table 2-1).

Morphological Analyses

General Morphology

Morphometric measurements were taken from live or preserved plant material. Detailed measurements and observations of the roots and flowers were performed with a WILD Heerbrugg M3 dissecting stereo microscope. Five roots from five individuals of both thin and thick-rooted plants were drawn with a Camera Lucida attachment (WILD Heerbrugg type 256575).

Within each root, five random parts of the roots were selected for measurements. These recorded measurements were statistically analyzed with a nested ANOVA to test whether there is a significant difference between the thicknesses of the roots of both taxa.

Fifteen flowers of both thin and thick-rooted taxa, fresh or in 70% ethanol, were dissected and drawn with a Camera Lucida. These drawing were used to measure the width and length of all the perianth parts (including the callus), the ovary, and the anther. A Hotelling T^2 test (Johnson & Wichern, 1988) was performed to investigate if the perianth parts of *Dendrophylax porrectus* populations are significantly different. A paired T-test assuming equal variance was also performed on the remaining floral parts of the two putative taxa.

Root Anatomy

Roots were embedded for seven days in FAA (ethyl alcohol-formaldehyde-acetic acid) and vacuumed to remove air from the tissue and to ensure complete penetration of the solution. The roots were transferred to 70% ethanol after seven days. Resin embedding was used to make cross sections of 8 - 10 µm in thickness. The resin-embedded roots were selectively harvested 1-2 cm from the growing tip. This procedure was followed to ensure that the roots are in the same developmental stage, which makes it suitable for comparison studies.

Resin embedding**:** Five roots of different individual plants per taxon were embedded in LR White resin (Electron Microscopy Sciences) using an infiltration process with LR White monomer/ethanol series (see steps in Table 2-1) based on Ruzin's protocol (Ruzin, 1999). After the infiltration process, the roots were placed in Eppendorf tubes with monomer to polymerize at temperature of 60-65 °C for 12-24

hours. The roots were sectioned with a rotary microtome (AO Spencer 820) and sections were stained with 0.1 % aqueous Toluidine Blue O solution in 0.1M Sørensen's phosphate buffer solution, pH 5.8 (Ruzin, 1999). Sections were then air-dried, mounted with Permount™ and photographed with a Zeiss Axiocam HRm camera mounted on a Zeiss Axioplan 2 Imaging microscope. The randomly chosen cell walls of the endodermis in the roots were measured through the photographs with ImageJ (Rasband, 1997-2011). The measurements of both taxa and both protocols were statistically analyzed in a nested ANOVA to investigate if there is a significant difference between the two taxa.

Molecular Analyses

DNA Extraction

DNA material was obtained from wild-collected plants, herbarium dried specimens, silica gel dried material (Chase & Hills, 1991), and cultivated plants (see Table 2-1). Fresh collected roots were field preserved in RNAlater™ (Bent & Taylor, 2010). Genomic DNA was extracted using the cetyl trimethylammonium bromide (CTAB) technique (Doyle & Doyle, 1987), modified and scaled to a 1 ml volume reaction. Approximately 2 cm of root tissue was ground in 1.2 ml CTAB 2X buffer and 5 µL of Proteinase K using a mortar and pestle.

The homogenized mixture was transferred to Eppendorf tubes and incubated at 50°C for 2 hours. After incubation, 500 µL of chloroform/isoamyl alcohol was added to the CTAB tissue mixture and vortexed until milky. The samples were centrifuged at 10,000 rpm for 4 minutes and 750 µL of the supernatant was transferred to a new tube. In order to precipitate the DNA, 30 µL of 3 M sodium acetate and 600 µL of 100% isopropanol was added to the tube and gently mixed. The solution was allowed to chill

overnight in a -20 °C freezer for maximum precipitation of DNA. After chilling, the solution was centrifuged at 13,000 rpm for 20 minutes to obtain a DNA pellet. The alcohol/ sodium acetate solution was poured off without disturbing the pellet and washed 3 times with 1ml of 70% ethanol. In order to resuspend the DNA pellet, 200 µL of 1X Tris-EDTA (ethylenediaminetetraacetic acid) buffer (TE, pH 8.0) was added to the tube. After being incubated for 15 minutes at 65 °C, the samples were stored at - 20°C.

DNA extractions from herbarium material and impure DNA were cleaned with QIAquick PCR purification columns (Qiagen, Valencia, California, USA) and Buffer PE, then eluted with Buffer EB to remove inhibitory secondary compounds. The samples were resuspended in 100 µL of Tris-EDTA (TE) buffer.

Amplification and Sequencing

Amplifications were performed using an Eppendorf Mastercycler EP Gradient S thermocycler and Sigma brand reagents in 25 µL volumes. In this study three regions were amplified: one nuclear region and two plastid regions. The primers for these regions are in Table 2-3. The nuclear region ITS was amplified with the primers 17SE and 26SE (ITS 1 + 5.8S rDNA+ ITS 2) from Sun et al. (1994).

The plastid region *matK-trnK* includes the entire *matK* gene and the flanking 3'*trnK* spacer that is ca. 1800 base-pairs (bps) in length. This region was sequenced with the primers -19F (Molvray et al., 2000) and *trnK* 2R (Johnson & Soltis, 1994). The internal sequencing primers were *matK* intF and *matK* intR. Some samples were amplified using the primers 56F and 1520R (Whitten et al., 2000) that yielded a shorter, but still nearly complete sequence of the *matK* exon (missing the 3' spacer).

The second plastid region used in this study was *ycf1* (Neubig et al., 2009) with ca.1500 bp from the 3' end. The primers used were 3720F and 5500R with the internal

sequencing primers intF and intR. All the components used for the polymerase chain reactions and the programs used for the thermocycler are shown in Table 2-4 and Table 2.5.

The PCR products were sequenced at the Interdisciplinary Center for Biotechnology Research facility (ICBR) at the University of Florida and the sequenced data were edited and assembled using Sequencher ™ 4.10.1(GeneCodes, Inc, Ann Arbor, MI, 2010). All sequences were deposited in GenBank (see Appendix).

Data Analysis

The consensus files of each region for every taxon were used to assemble a manually aligned matrix using Se-Al v2.0a11 (Rambaut, 1996). The matrix was then transferred to PAUP*4.0b10 (Swofford, 2003) for phylogenetic analyses. All nucleotide characters were weighed equally and unordered. The gaps were treated as missing data and indels were not coded as characters. Heuristic searches were performed with 1000 random-addition replicates, saving 10 trees per replicate, with the tree bisection reconnection (TBR) algorithm. Deltran optimization was used for all analyses. Bootstrap analyses utilized 1000 replicates, with 10 random-addition replicates (SPR swapping) per bootstrap replicate.

The partition homogeneity test (HTF) was used to investigate if the data were congruent with one another or if they were significantly different. Combined datasets with missing data were tested with PAUP*4.0b10 (Swofford, 2003; Johnson & Soltis, 1998). With the heuristic search, HTF tests were performed using 100 replicates and a TBR algorithm comparing all four combinations of the three genes (including plastid genes vs. nuclear genes). Ten random-addition replicates were performed per HTF

replicate, holding 10 trees and saving no more than 10 trees per replicate. The probability values were greater than 0.05.

The jModelTest (Posada, 2008) was used to carry out statistical selection of best-fit models of nucleotide substitution. The results were used to perform the Maximum Likelihood analysis in PAUP*4.0b10. Heuristic searches were performed with 100 random-addition replicates, saving 10 trees per replicate, with the tree bisection reconnection (TBR) algorithm. Bootstrap analyses utilized 100 replicates, with 10 random-addition replicates (SPR swapping) per bootstrap replicate.

Carlsward's molecular data (Carlsward, 2004) provided sequence data of ITS and *matK.* The *trnL-F* sequence data were omitted from this study because of its low variability. *Campylocentrum micranthum* (Rchb.f.) Rolfe was used as an outgroup. The sequence data for this taxon were obtained from GenBank. An effort was made to amplify 46 specimens, but not all regions amplified successfully. Five individuals per population were sequenced for four populations using ITS to determine variation within populations. Analyses were conducted with several data sets:(1) ITS data set containing 46 individuals (3 taxa missing data); (2) *matK* data set containing 25 individuals (6 taxa missing data); (3) *ycf1* data set containing 26 individuals (5 taxa missing data); (4) combined plastid data sets (*matK* and *ycf1*) with 27 taxa (with gaps for the missing plastid data); and (5) total combined data sets of nuclear and plastid regions, using 31 taxa (again with gaps for the missing plastid and nuclear data).

Phenology

In order to understand the reproductive mechanisms of *Dendrophylax porrectus*, the plants were monitored during their flowering period. The flowering period was recorded and the flowers were labeled and observed throughout their lifespan (opening

to wilting). All the wild collected plants were collected with young inflorescences developing, which eliminated the possibility that plants were influenced in their reproductive initiation by the greenhouse conditions.

Attempts were made to observe potential pollinators and the plants were also tested to determine self-compatibility. Florida plants with open flowers were completely covered with a piece of mesh to prevent potential pollinators having access to the flowers. The remaining plants were kept outside at the FLAS greenhouse to allow pollinators to visit the flowers. The same procedure was implemented for the Yucatán plants in Mexico.

Table 2-1. Taxa used for molecular phylogenetic study. The specimen numbers are indicated. Gene regions sampled include: I = ITS, M = *matK*, and Y = *ycf1*. The location from which each specimen was collected are listed. Numbers linked to the locations indicate different individuals from one or more population. Vouchers are listed by collector and collector number; Herbaria are the herbarium where the voucher is deposited.

Taxon	Specimen number	Gene region	Location (County)	Voucher (Herbaria)
Campylocentrum micranthum	B346	I, M,	Panama	*Carlsward 315* (FLAS)
C. micranthum	B60	I, M, Y	Mexico	*Carlsward 180* (FLAS)
C. micranthum	B12	I, M,	Puerto Rico	*Ackerman 3341* (UPRRP)
Dendrophylax alcoa Dod	W778	I	Dominican Republic	*Ackerman 2773* (UPRRP)
D. barrettiae Fawc. & Rendle	B36	I, M, Y	Jamaica 1	*Carlsward 199* (FLAS)
D. barrettiae	W714	I, , Y	Jamaica 2	*Whitten 1814* (FLAS)
D. aff. *porrectus*	B14	I, M, Y	Mexico (Yucatán1)	*Carnevali 5907* (FLAS)
D. aff. *porrectus*	M1	I	Mexico (Yucatán2)	*Carnevali 5907* (CICY)
D. aff. *porrectus*	M2	I, M, Y	Mexico (Yucatán3)	*Carnevali 5907* (CICY)
D. aff. *porrectus*	M3	I	Mexico (Yucatán4)	*Carnevali 5907* (CICY)
D. aff. *porrectus*	M4	I	Mexico (Yucatán5)	*Carnevali 5907* (CICY)
D. aff. *porrectus*	M5	I	Mexico (Yucatán6)	*Carnevali 5907* (CICY)
D. aff. *porrectus*	GS8279	I, M, Y	Mexico (Jalisco)	*Salazar 8279* (MEXU)
D. fawcettii Rolfe	W3265	Y	Cayman Islands	*Whitten 3265* (FLAS)
D. funalis (Sw.) Benth. ex Rolfe	B233	I, M, Y	Jamaica 1	*Carlsward 302* (FLAS)
D. funalis	W713	I, Y	Jamaica 2	*Whitten1935* (FLAS)
D. lindenii (Lindl.) Benth. ex Rolfe	W730	I	USA FL (Collier)	*Ward 5365* (FLAS)
D. lindenii	W716	I, M, Y	Cuba	*Claude Hamilton s.n.*
D. porrectus	JDA4514	I, M, Y	Cuba (Sandina)	*Ackerman 4514* (UPRRP)
D. porrectus	B359	I, M, Y	Dominican Republic (Guanito)	*Whitten1950* (FLAS)
D. porrectus	B710	I, M, Y	Cayman Islands	no voucher
D. porrectus	B35	I, M, Y	Jamaica	*Carlsward 184* (FLAS)
D. porrectus	C4468	I, Y	Mexico (Campeche)	*Carnevali 4468* (CICY)
D. porrectus	B212	I, M, Y	Mexico (Quintana Roo)	*Carnevali 6312* (FLAS)
D. porrectus	IR886	M, Y	Mexico (Yucatán7)	*Ramirez 886* (CICY)
D. porrectus	B366	I, M, Y	USA FL 1 (Glades)	*Carlsward 329* (FLAS)
D. porrectus	DP1	I, M, Y	USA FL 2 (Hillsborough1)	no voucher

Table 2-1 (cont.)

Taxon	Specimen number	Gene region	Location (County)	Voucher (Herbaria)
D. porrectus	DP3	I	USA FL 4 (Hillsborough1)	no voucher
D. porrectus	DP4	I	USA FL 5 (Hillsborough2)	*Molgo 221*(FLAS)
D. porrectus	DP5	I	USA FL 6 (Hillsborough2)	*Molgo 221*(FLAS)
D. porrectus	DP30	I	USA FL 7 (Lee)	*Whitten 3745* (FLAS)
D. porrectus	DP31	I	USA FL 8 (Lee)	*Whitten 3745* (FLAS)
D. porrectus	DP32	I	USA FL 9 (Lee)	*Whitten 3745* (FLAS)
D. porrectus	DP33	I	USA FL 10 (Lee)	*Whitten 3745* (FLAS)
D. porrectus	W3745	I, M, Y	USA FL 11 (Lee)	*Whitten 3745* (FLAS)
D. porrectus	DP40	I, M, Y	USA FL 12 (Sarasota)	*Molgo 222* (FLAS)
D. porrectus	DP41	I	USA FL 13 (Sarasota)	*Molgo 222* (FLAS)
D. porrectus	DP42	I	USA FL 14 (Sarasota)	*Molgo 222* (FLAS)
D. porrectus	DP44	I	USA FL 15 (Sarasota)	*Molgo 222* (FLAS)
D. porrectus	DP45	I	USA FL 16 (Sarasota)	*Molgo 222* (FLAS)
D. porrectus	DP 24	I	USA FL 17 (Hillsborough3)	no voucher
D. porrectus	B70	I, M, Y	USA FL 18 (Glades)	Goldman 2271 (FLAS)
D. porrectus	B367	M, Y	USA FL 19 (Ft. Myers))	*Carlsward 330* (FLAS)
D. porrectus	B11	I, M, Y	Puerto Rico	*Ackerman3340* (UPRRP)
D. sallei (Rchb.f.) Benth. ex Rolfe	B360	I, M, Y	Dominican Republic	*Whitten1945* (JBSD)
D. varius (Gmel.) Urb	B153	I	Dominican Republic 1	*Thompson 10683* (SEL)
D. varius (Gmel.) Urb	B362	I, M, Y	Dominican Republic 2	*Whitten1960* (JBSD)
D. varius (Gmel.) Urb	W779	I	Puerto Rico	*Ackerman 2727* (UPRRP)

Table 2-2. Infiltration and polymerization of LR White resin with the root tissue

Step #	Solution	Time
1	100% Ethanol	2 hours
2	50% monomer +50% 100%Ethanol	24 hours
3	70% monomer + 30 % 100% Ethanol	24 hours
4	100% monomer	24 hours
5	100% monomer	24 hours
6	Polymerase in100% monomer at 65°C	12-24 hours

Table 2-3. Primer sequence of one nuclear and two plastid gene regions

Primer	Primer sequence
	ITS
17SE, forward	ACGAATTCATGGTCCGGTGAAGTGTTCG
26SE, reverse	TAGAATTCCCCGGTTCGCTCGCCGTTAC
	matK-trnK
-19F, forward	CGTTCTGACCATATTGCACTATG
1520R, reverse	CGGATAATGTCCAAATACCAAATA
56F, forward	ACTTCCTCTATCCGCTACTCCTT
trnK2R, reverse	ACCTAGTCGGATGGAGTAG
intF, forward	TGAGCGAACACATTTCTATGG
intR, reverse	ATAAGGTTGAAACCAAAAGTG
	ycf1
3720F, forward	TACGTATGTAATGAACGAATGG
5500R, reverse	GCTGTTATTGGCATCAAACCAATAGCG
intF, forward	GATCTGGACCAATGCACATATT
intR, reverse	TTTGATTGGGATGATCCAAGG

Table 2-4. Components of polymerase chain reaction with Sigma brand reagents

PCR components (µL)	ITS	*matK-trnK*	*ycf1*
Water (H_2O)	11.0	14.5-18	14.5-18
10X buffer	2.5	2.5-5.0	2.5-5.0
25 mM $MgCl_2$	2.5	1-3	1-3
Forward primer (10µM)	0.5	0.5	0.5
Reverse primer (10µM)	0.5	0.5	0.5
dNTPs (10µM)	0.5	0.5	0.5
Taq polymerase	0.2	0.2	0.2
DNA template (~10-100 ng) *****	1.0	1.0	1.0

* If the DNA templates are from a herbarium specimen the quantity of template will vary from 2-4 µL.

Table 2-5. Thermocycler programs for polymerase chain reactions

Steps #	Temperature (°C)	Time	Notes
		ITS	
1	94	2 minutes	
2	94	1 minute	Step 2 -4,15 times
3	76	1 minute	Reducing 1°C per cycle in step 3
4	72	1 minute	
5	94	1 minute	Step 5-7, 21 times
6	59	1 minute	
7	72	1 minute	
8	72	3 minutes	
		matK-trnK	
1	94	3 minutes	
2	94	45 seconds	Step 2-4, 33 times
3	60	45 seconds	
4	72	2 minutes	
5	72	3 minutes	
		ycf1	
1	94	3 minutes	
2	94	30 seconds	Step 2-4, 8 times
3	60	1 minute	
4	72	3 minutes	
5	94	30 seconds	Steps 5-7, 30 times
6	50	1 minute	
7	72	3 minutes	
8	72	3 minutes	

CHAPTER 3
RESULTS

General Morphology

The Florida and Yucatán populations differ significantly in numerous subtle morphological traits, including root thickness, ovary length, labellum width, callus length, anther size, pollinium shape, and rostellum shape. Figure 3-1 presents photographs of representative individuals from Florida and Yucatán populations and highlights their differences. These differences are summarized in Figures 3-2 to 3-9.

Root Thickness

A root thickness analysis was performed with 250 measurements and 125 per taxa. The roots from the Yucatán population were thicker (mean diameter 1.90mm) than those of the Florida populations (mean diameter 1.23 mm). The nested ANOVA analysis tested whether there is a plant location effect present and if there was a variation among root thickness within locations.

There is no location effect if all plants are equal and there is a location effect if not all of the plants are equal. The test resulted in a test statistic: $F_{observed}$= 20.814, P-value: ($F_{1,8} \geq 20.814$) = 0.00185. These results imply that the plants are significantly different between the Florida and Yucatán locations.

There is no variation among root thickness within location if all thicknesses are equal and there is an effect if they are not equal. The test resulted in a test statistic: $F_{observed}$= 9.164, P-value: ($F_{8,40} \geq 9.164$)≈ 0.000. These results imply that the root thickness variation is significantly different in both locations. In Figure 3-2, there is a box plot that represents the data of the root thickness. The boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median, a dashed line

the mean, and the boundary of the box farthest from zero indicates the 75th percentile. The error bars above and below the box indicate the 90th and 10th percentiles. (The symbols used in the box plots in Figures 3-3 and 3-8 are the same.)

Ovary Length

Fifteen ovaries were measured of each population in Florida and Yucatán. The mean length of the ovary in Florida plants was 2.00 mm versus 1.50 mm for Yucatán plants. A two-sample T test was performed to test whether a significant difference was present. The test resulted in t = 4.088 with 28 degrees of freedom, P-value = <0.001. These results imply that the ovary length is significantly different in the two populations. The box plot in Figure 3-3 shows the distribution of the ovary length data.

Sepal Measurements

The flowers of the Florida and Yucatán populations have both a width of 3 mm (Fig. 3-1), but when the perianth parts are uncurled, it is seen that they are significantly different. The length and width of the dorsal sepal were measured in 15 flowers of the Florida and Yucatán populations. A paired Hotelling T^2 test was performed to investigate if the length and width of the dorsal sepal of both populations are equal or not. The mean dorsal sepal length and width for Florida and Yucatán populations were, 1.88 x 1.13 mm. vs. 2.27 x 1.15 mm. The test resulted in T^2=70.267, test statistic: $F_{observed}$=33.878, P-value :($F_{2,27} \geq 33.878$) ≈ 0.000. These results imply that the dorsal sepal is significantly different in the two populations. The scatter plot in Figure 3-4 depicts the length of the dorsal sepal on the X-axis and the width on the Y-axis, and visually demonstrates the difference between the Florida and Yucatán populations.

The Hotelling T^2 test was also performed with the lateral sepal data to test whether the mean length and width of the lateral sepal are equal or not equal. The mean lateral

sepal length and width for Florida and Yucatán were, 2.16 x 1.03 mm. vs. 2.37 x 1.12 mm. The test resulted in T^2=10.75, test statistic: $F_{observed}$=5.18, P-value: ($F_{2,27} \geq$ 5.18)=0.012. These results imply that the lateral sepal is significantly different in both populations. The scatter plot in Figure 3-5 depicts the length of the lateral sepal on the X-axis and the width on the Y-axis, and indicated that there is much overlap in the pattern of variation, although the populations are differentiated.

Petal and Labellum Measurements

The average petal measurements were analyzed with the Hotelling T^2 test to investigate if the petals in the Florida and Yucatán populations were equal. The mean petal length and width for Florida and Yucatán were1.99 x 0.85 mm. vs. 2.36 x 0.86 mm. The test resulted in T^2=27.29, test statistic: $F_{observed}$= 13.16, P-value: ($F_{2,27} \geq 13.16$) = 0.00103. These results imply that the petals are significantly different in both populations. The scatter plot in Figure 3-6 depicts the length of the petal on the X-axis and the width on the Y-axis, and illustrates the differences between these two populations.

The data gathered from the labellum was also tested with the Hotelling T^2 to test whether the labella from the two populations are equal. The mean labellum length and width for Florida and Yucatán populations were, 1.99 x 0.85 mm vs. 2.36 x 0.86 mm.

The test resulted in T^2=40.05, test statistic: $F_{observed}$=19.31, P-value: ($F_{2,27} \geq 13.16$) $\approx$ 0.000. These results imply that the labellum is significantly different in both populations. The scatter plot in Figure 3-7 depicts the variation in the labellum shape. The calli on the labella were measured for Florida and Yucatán plants. The mean length of the callus of the Florida plants was 0.37 mm versus 0.15 mm for that of Yucatán plants. A two-sample T test was performed to investigate whether a significant

difference was present. The test resulted in t = 7.843 with 24 degrees of freedom, P-value = <0.001. These results imply that the callus is significantly different in Florida and Yucatán populations. The box plot in Figure 3-8 depicts the distribution of the callus length data.

Anther Measurements

The Hotelling T^2 test was also performed with the anther data to test whether the mean length and width of the anther are equal or not equal. The mean anther length and width for Florida and Yucatán were, 0.49 x 0.34 mm. vs. 0.59 x 0.43 mm. The test resulted in T^2=82.99, test statistic: $F_{observed}$=3.976, P-value: ($F_{2,23} \geq 3.976$) ≈ 0.000. These results imply that the anther is significantly different in both populations. The scatter plot in Figure 3-9 depicts the length of the anther on the X-axis and the width on the Y-axis, and shows a clear separation of the Florida and Yucatán populations.

In Figure 3-1 the pollinia of the Yucatán individuals are slightly flattened and ovoid and the pollinia of the Florida individuals are globose. The stipes of the Florida pollinia appear as a single structure in Figure 3-1, but they are not. In fact, the stipes represent two single structures attached to one another as they appear when attached to the rostellum. Even though the rostellum is the same in size, the rostellar entry is not. The entry in the Florida individuals is 0.06 mm wider than the Yucatán entities.

Root Anatomy.

Differences in the root sections were observed as comparisons were made of resin embedded roots of the Florida and Yucatán populations (Fig. 3-10). The endodermis wall of the Yucatán entities is smaller versus the wall of the Florida entities.

The data was normally distributed and equal in variance (Fig 3-11). The mean of the Florida cell wall was 3.645 µm and the mean of the Yucatán was 2.004 µm. The t

test of comparison resulted in a t = 9.329 with 48 degrees of freedom, (P-value = <0.001). These results imply that the endodermis wall of the roots is significantly different in the two populations.

Molecular Analysis

In all analyses, *Dendrophylax* is monophyletic, which concurs with the results of Carlsward et al. (2003). Three outgroups were used, all *Campylocentrum micranthum,* but with three accessions from different countries. The populations of *Dendrophylax porrectus* form a monophyletic group, which is comprised of two diagnosable subclades, here called the thick-rooted clade and the thin-rooted clade. Within the thin-rooted clade there are two sister groups, which are identified as thin-rooted clades 1 and 2. Statistical comparisons of analyses for each gene region and combined gene regions are given in Table 3-1.

ITS Analyses of *D. porrectus*

In the ITS analysis 46 accessions were used and the analysis supported the monophyly of *Dendrophylax* (95% BS; 92% JK; Fig. 3-12). The *D. porrectus* clade is well supported (BS 98%; JK 93%), with a thin-rooted clade (BS 98%; JK 93%). The thin-rooted *D. porrectus* includes two subclades, clade 1 (BS 86%; JK 75 %) and clade 2 (BS 99%; JK 96%). *Dendrophylax porrectus* clade 2 shows a polytomy of all the various Florida populations and their replicates. A polytomy is also seen in the thick-rooted *D. porrectus* clade. This indicates that all the replicates in the populations have no sequence divergence and are genetically very similar. The thick-rooted clade is strongly supported (BS 100%; JK 100%), indicating that the Yucatán and Jalisco plants are sister to the Florida populations.

The other *Dendrophylax* species have the same topology as reported by Carlsward et al. (2003). *Dendrophylax barrettiae* is sister to the rest of the *Dendrophylax* clade. The subclade with *D. funalis* and *D. fawcettii* is strongly supported (BS 99%; JK 97%). The subclade with *D. varius*, *D. sallei*, *D. lindenii,* and *D. alcoa* is moderately supported (BS 81%; JK 77%) but within this monophyletic group there is strong support for groupings among the different species.

matK* Analyses of *D. porrectus

The *matK* matrix had 25 taxa with only one representative per population. The analysis supported the monophyly of the *Dendrophylax* clade (BS 88%; JK 85%; Fig. 3-13). The branch between *D. barrettiae* and the rest of the *Dendrophylax* clade is poorly supported (BS 60%; JK 61%). The *D. porrectus* clade is strongly supported (BS 99%; JK 96%). The *D. porrectus* clade has the same topology as in the ITS analysis. The support for the thick-rooted clade is still high (BS 98%; JK 92%), but the thin-rooted *D. porrectus* clade is only weakly supported (BS 63 %). This does not mean that there is a conflict in the data, but merely that a well supported clade in ITS is poorly supported in *matK* due to lack of phylogenetic signal.

The *D. funalis* + *D. fawcettii* subclade is strongly supported (BS 100%; JK 99%). The *D. lindenii* + *D. sallei, + D. varius* subclade has moderate support (BS 87%; JK 76%).

ycf1* Analyses of *D. porrectus

There were 26 accessions in the *ycf1* matrix and the analysis generated the same topology as the ITS and *matK* analyses (Fig. 3-14). There is strong support for the monophyly of *D. porrectus* (BS 100%; JK 100%). The thin-rooted clade 1 is supported by BS 94%; JK 89%. For both BS and JK there is 100% support for thin-rooted clade 2

and the thick-rooted clade is supported by BS 100%; JK 99%. The other *Dendrophylax* species show a similar topology as in the *matK* analysis. *Dendrophylax barrettiae* is strongly supported (BS; JK 100%). *Dendrophylax funalis* and *D. fawcettii* are a clade (BS 99%; JK 96%), which was also reported in Carlsward et al. (2003), and *D. lindenii*, *D sallei,* and *D. varius* form a well supported clade (BS 90%; JK 87%).

Combined Plastid Analyses of *D. porrectus*

In the combined plastid analyses, there were 27 accessions and the analysis supported the monophyly of the *Dendrophylax* clade (BS 92%; JK 87%; Fig. 3-15). The *D. porrectus* clade is also strongly supported (BS 100%; JK 100%), as is the thick-rooted clade (BS 100%; JK 100%), but the thin-rooted *D. porrectus* clade is only moderately supported (BS 79%; JK 67%). The thin-rooted *D. porrectus* clade 1 is well supported (BS 95%; JK 88%) and the thin-rooted clade 2, also shows strong support (BS 100%; JK 100%). In this tree, *D. barrettiae* is sister to the rest of the *Dendrophylax* taxa. These results are congruent with the previously presented trees.

Combined ITS and Plastid Analyses of *D. porrectus*

The combined ITS, *matK,* and *ycf1* matrix included 31 taxa. Most clades have strong BS support in this analysis and there were no conflicts between the regions (Fig. 3-16). A partition homogeneity test with heuristic search supported the congruence between these three datasets: for ITS and plastid (p-value = 0.99), for *ycf1* and *matK* (p-value = 1.00), for ITS and *matK* (p-value = 1.00), and for *ycf1* and ITS (p-value = 1.00).

The *Dendrophylax* clade is strongly supported with BS 100%; JK 99%. The support for the *D. porrectus* clade is 99% for BS and JK and both thin-rooted and thick-rooted clades also are strongly supported. The thin-rooted clade divides into two subclades: thin-rooted 1 and 2. These subclades are also strongly supported (BS; JK

higher than 90%). *Dendrophylax barrettiae* is sister to the rest of the taxa of *Dendrophylax,* and the other species and species groups are well supported, which agree with the results of Carlsward et al. (2003).

The results of the combined plastid and ITS analysis are congruent with the trees of the separate gene regions, and there were no contradictions when the combined plastid tree was compared with the three-gene tree.

Maximum Likelihood

Only the three-gene matrix with 31 accessions was used for the Maximum Likelihood (ML) analyses. The jModelTest selected the GTR+G model (Lanave et al., 1984) as the best fit statistical selection of nucleotide substitution with the following criteria: a negative log likelihood (-lnL) of 7547.2470, number of estimated parameters (K) = 69, Akaike Information Criterion (AIC): 15232.4940, AIC difference weight: 0.0000 and a cumulative AIC weight of 0.5742. The ML tree has the same topology as the three-gene tree generated by parsimony analysis (Fig. 3-17). The *Dendrophylax* clade is 100% supported as well as the *D. porrectus* clade. Strong support (BS 100%) was also recorded for the thin-rooted and thick-rooted clades. However, slight differences are noticeable in the trees. The bootstrap supports in the ML tree are slightly higher, which led to support of branches that were not supported under parsimony.

Distribution of *D. porrectus*

The three most important monophyletic groups are placed on a distribution map to clarify biogeographical relationships among the different populations of *Dendrophylax porrectus* (Fig. 3-18). Black circles indicate the thin-rooted clade 1, blue circles indicate the thin-rooted clade 2, and red circles show the location of collections of the thick-rooted clade. The red squares indicate voucher specimens/ photo material that are

considered to represent the thick-rooted clade (pers. comm. G. Salazar & R. Jiménez Machorro, 2011). The black squares are specimens that were examined with photo material, but a definite decision could not be made if they belong to the thin-rooted or thick-rooted clade due to limitations of the images. The white squares indicate populations that were not determined a clade membership, due to the fact that specimens of these populations were not examined.

Phenology

Several plants from the Florida populations and the Yucatán populations were observed in cultivation during their flowering period. When the members of these populations where collected, inflorescences were already developing. The flowering period of the Florida entity started on August 30th and lasted until September 24th. The Yucatán entity started flowering on September 24th and lasted until January 20th.

The wilted Florida flowers fell off after a day if not pollinated and the Yucatán flowers remained attached to the inflorescence up to 5 days. The dried up flowers then turned black on the inflorescence. In the populations of Ft. Myers (FL), I observed that the plants flowered twice in one year. Plants flowered from August until September and flowered for the second time from December until January. This observation was not seen in the Sarasota (FL) population. The same patterns were seen in cultivation and in plants in their natural habitats. If the flowers of both populations were not pollinated, the life span of the flower of Florida populations was between 10 and 15 days and that of the Yucatán population was between 12 and 19 days.

The flowers of both entities had a fragrance at night. The Florida entity had a strong, sweet, honey-like fragrance but the Yucatán entity had a much milder fragrance that is difficult to describe.

Table 3-1. Comparison of tree statistics for each gene region and combinations of these gene regions for parsimony analyses.

Statistical inference	ITS	*matK*	*ycf1*	*matK & ycf1*	*ITS & matK & ycf1*
Aligned characters	702	1355	1689	3044	3746
Uninformative characters	30	28	67	95	123
Informative characters	85	42	74	116	201
Number of trees saved	4	3	1	1	18
Tree length	161	78	168	239	397
Consistency index (CI) with uninformative characters	0.850	0.923	0.875	0.916	0.889
CI without uninformative characters	0.812	0.88	0.792	0.861	0.838
Retention index (RI)	0.952	0.949	0.936	0.955	0.942
Rescaled consistency index (RC)	0.810	0.876	0.819	0.875	0.837

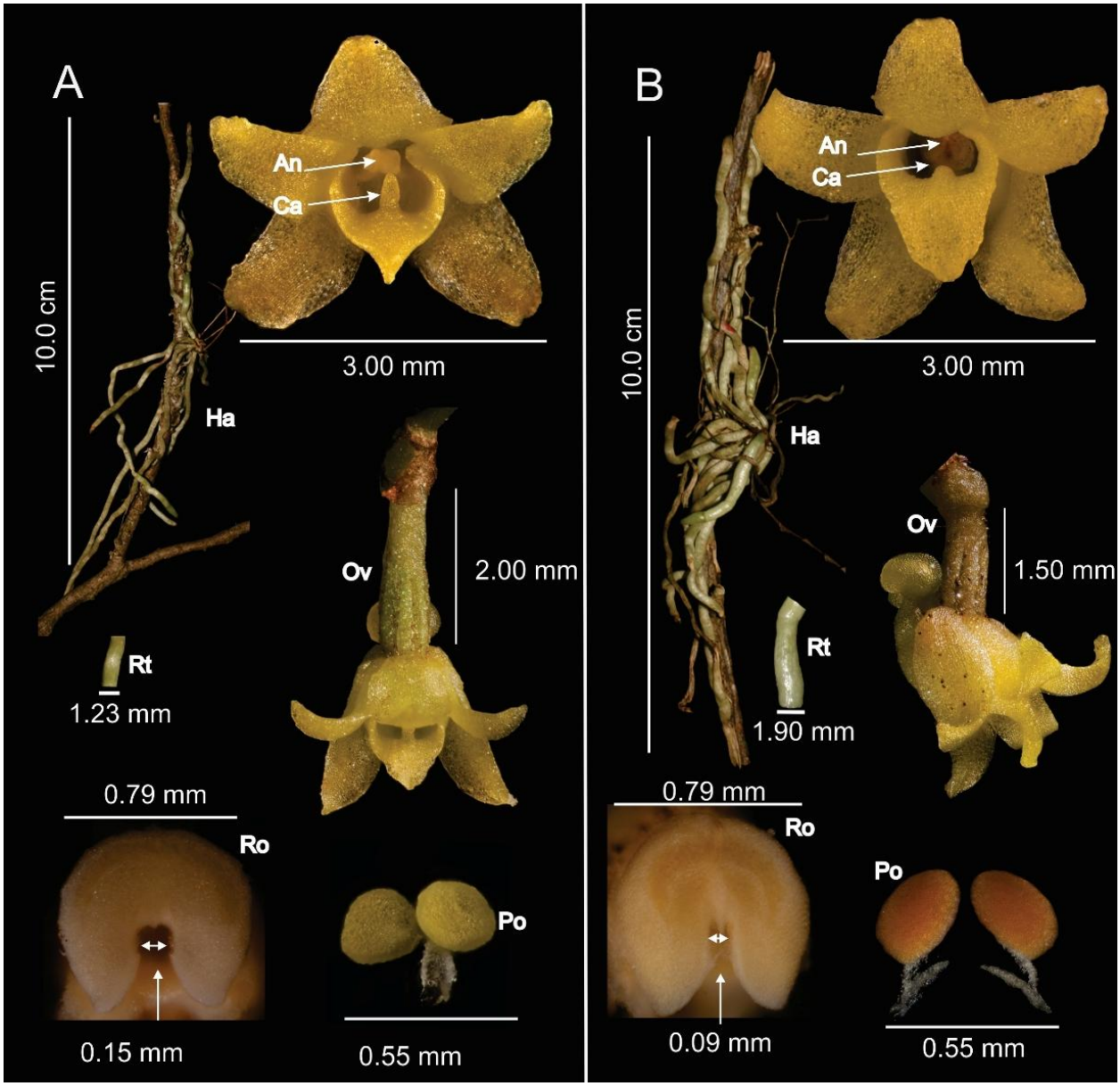

Figure 3-1. Comparison of two representative individuals (Florida, A and Yucatán, B) of *Dendrophylax porrectus* illustrating measurements of different plant and floral parts. Ha = habit, Rt = root, An = anther, Ca = callus, Ov = ovary, Ro = rostellum, and Po = pollinaria.

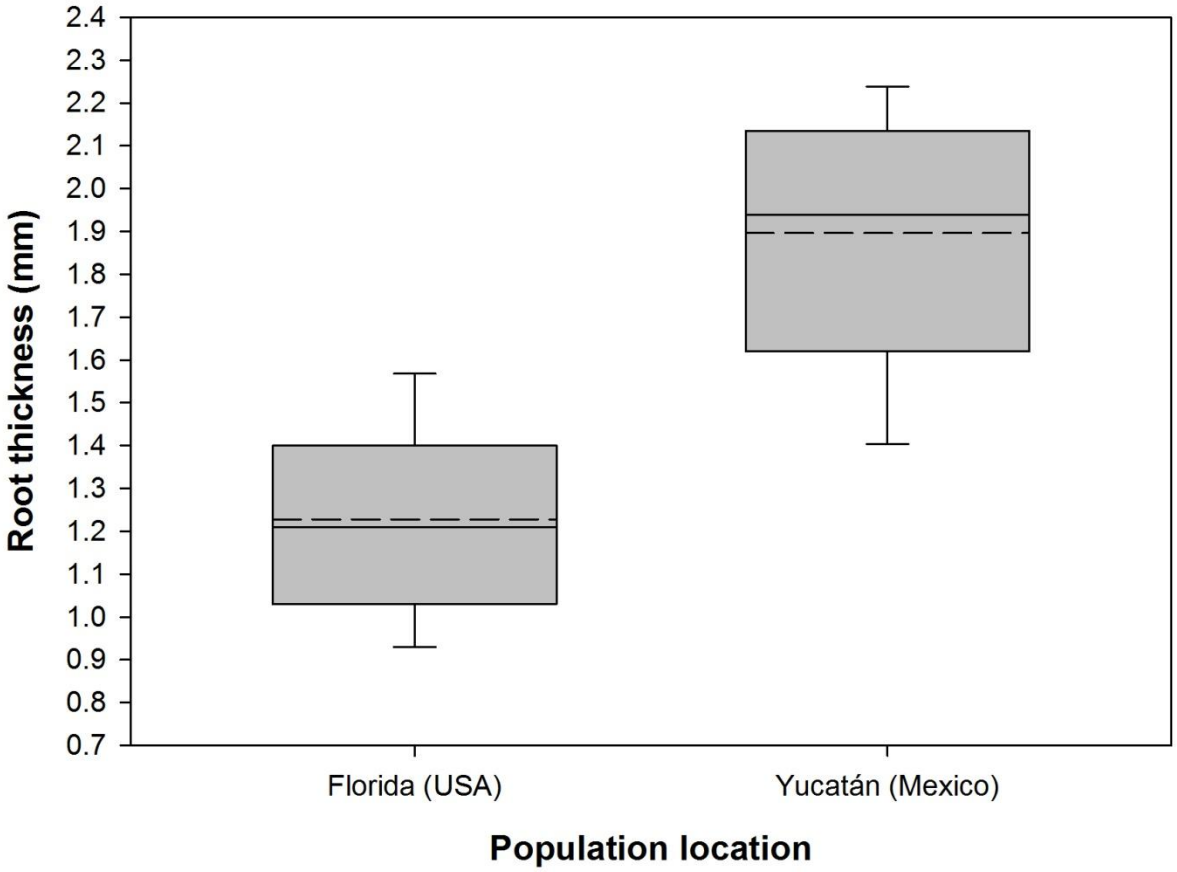

Figure 3-2. The difference in root thickness within two populations of *D. porrectus* in Florida and Yucatán.

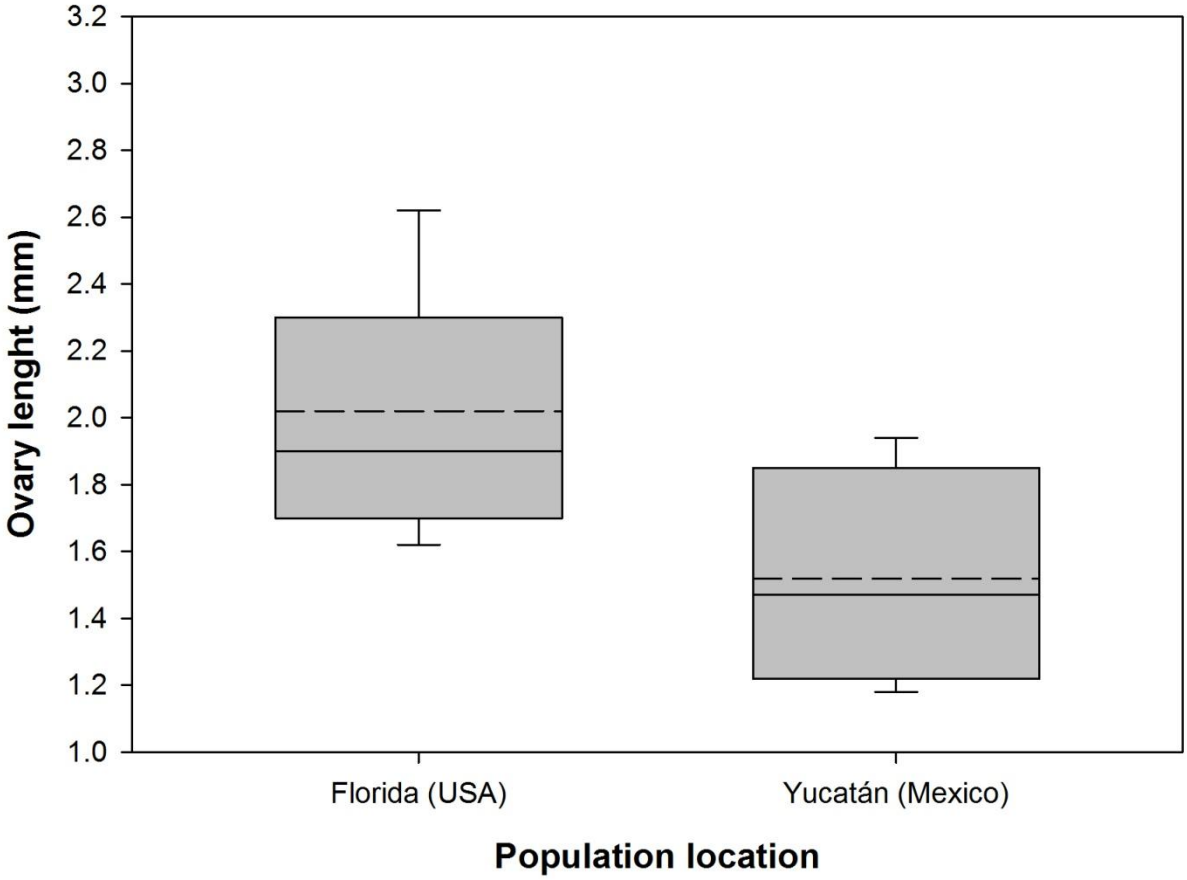

Figure 3-3. The difference in ovary length within two populations of *D. porrectus* in Florida and Yucatán.

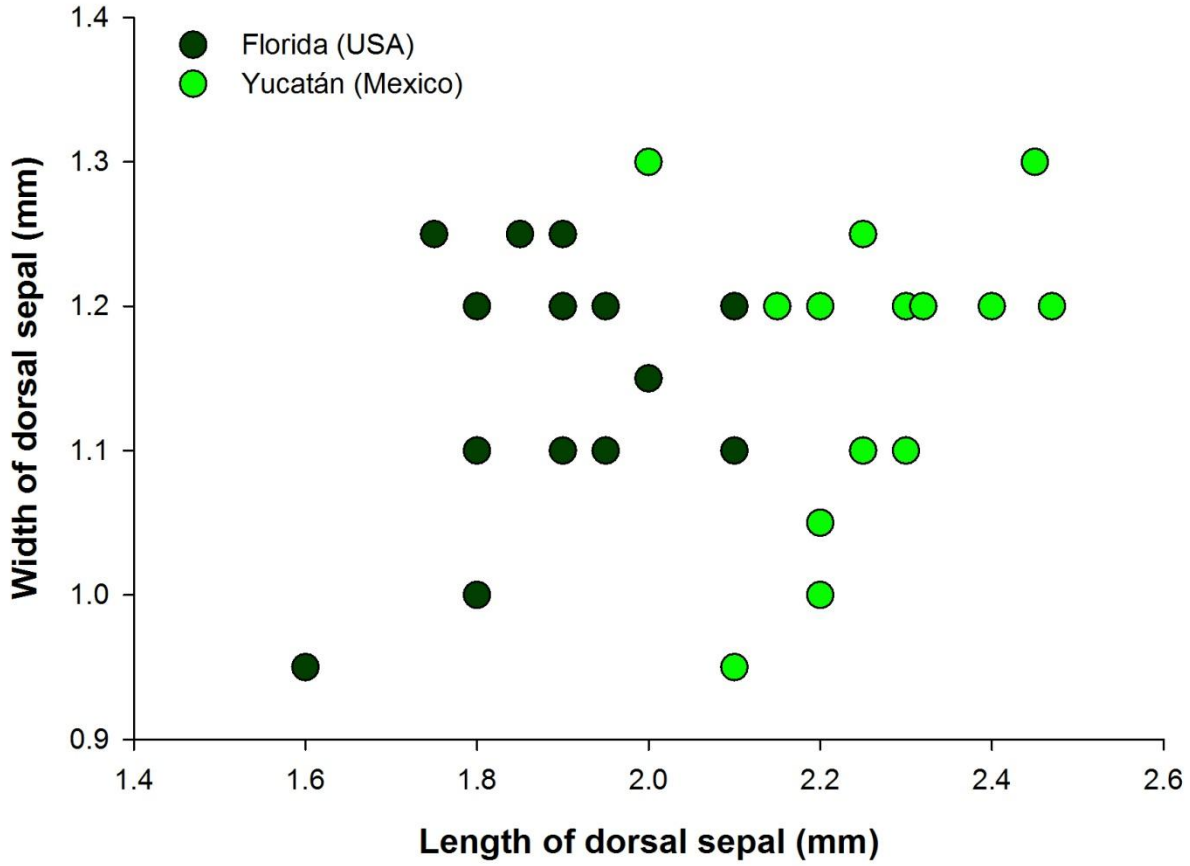

Figure 3-4. Differences in the dorsal sepal of *D. porrectus* within the populations of Florida and Yucatán. The dark green represent Florida and the light green represent Yucatán. The length of the dorsal sepal is plotted on the X-axis and the width on the Y-axis

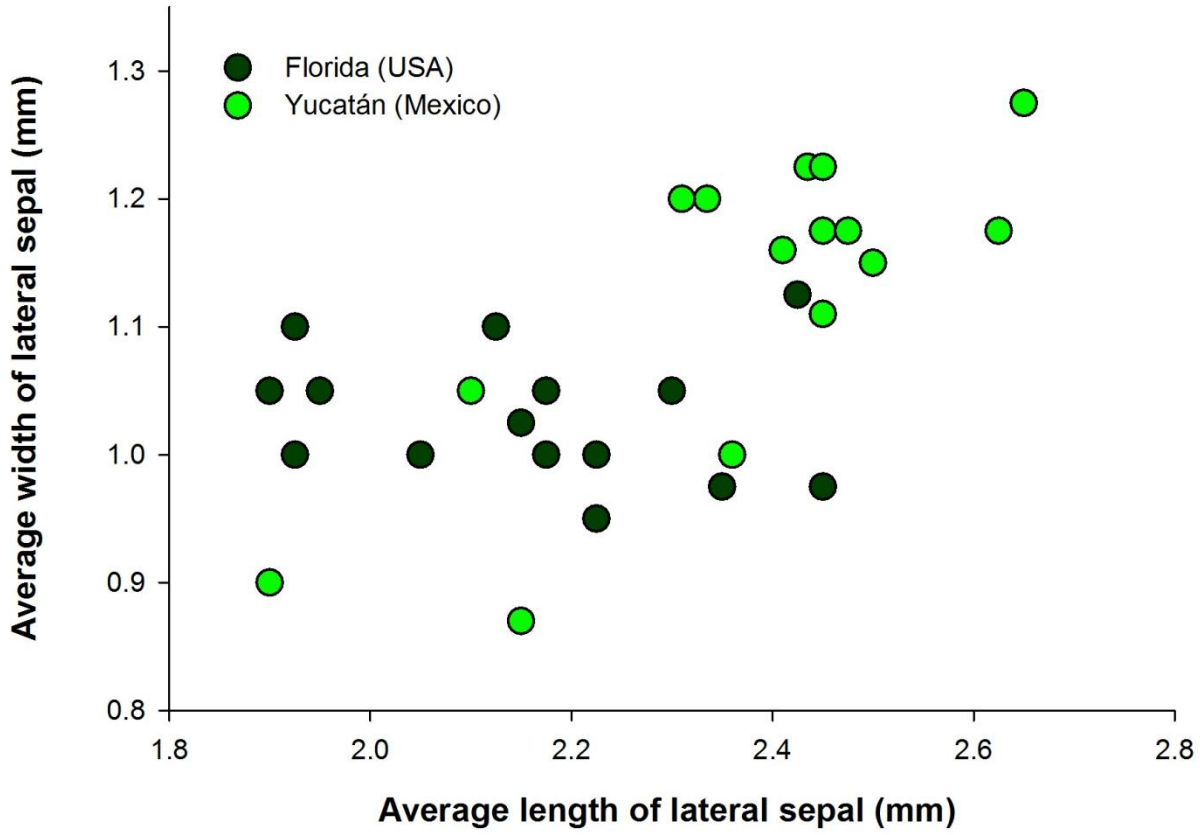

Figure 3-5. Differences in the lateral sepal of *D. porrectus* within the populations of Florida and Yucatán. The dark green represent Florida and the light green represent Yucatán. The average lengths of the lateral sepals are plotted on the X-axis and the average width on the Y-axis.

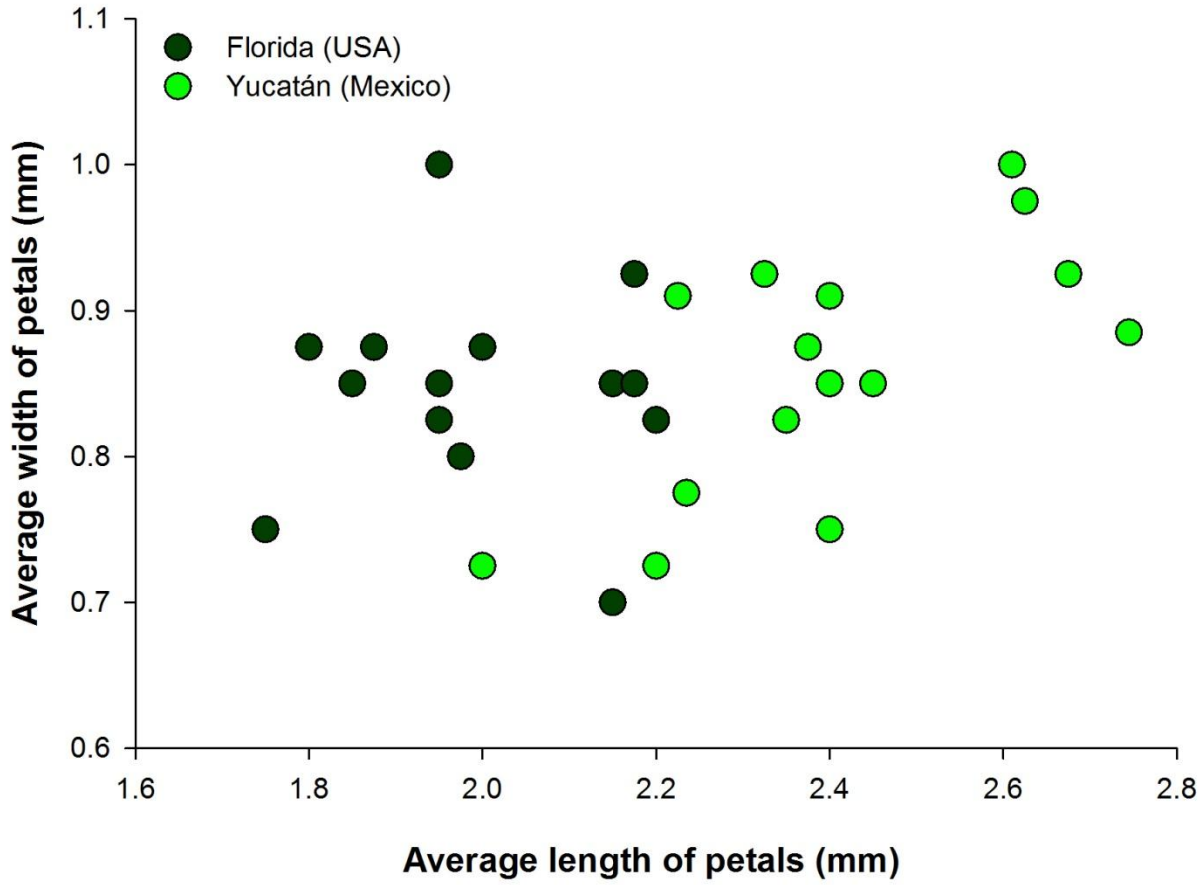

Figure 3-6. Differences in the petals of *D. porrectus* within the populations of Florida and Yucatán. The dark green represent Florida and the light green represent Yucatán. The average lengths of the lateral sepals are plotted on the X-axis and the average width on the Y-axis.

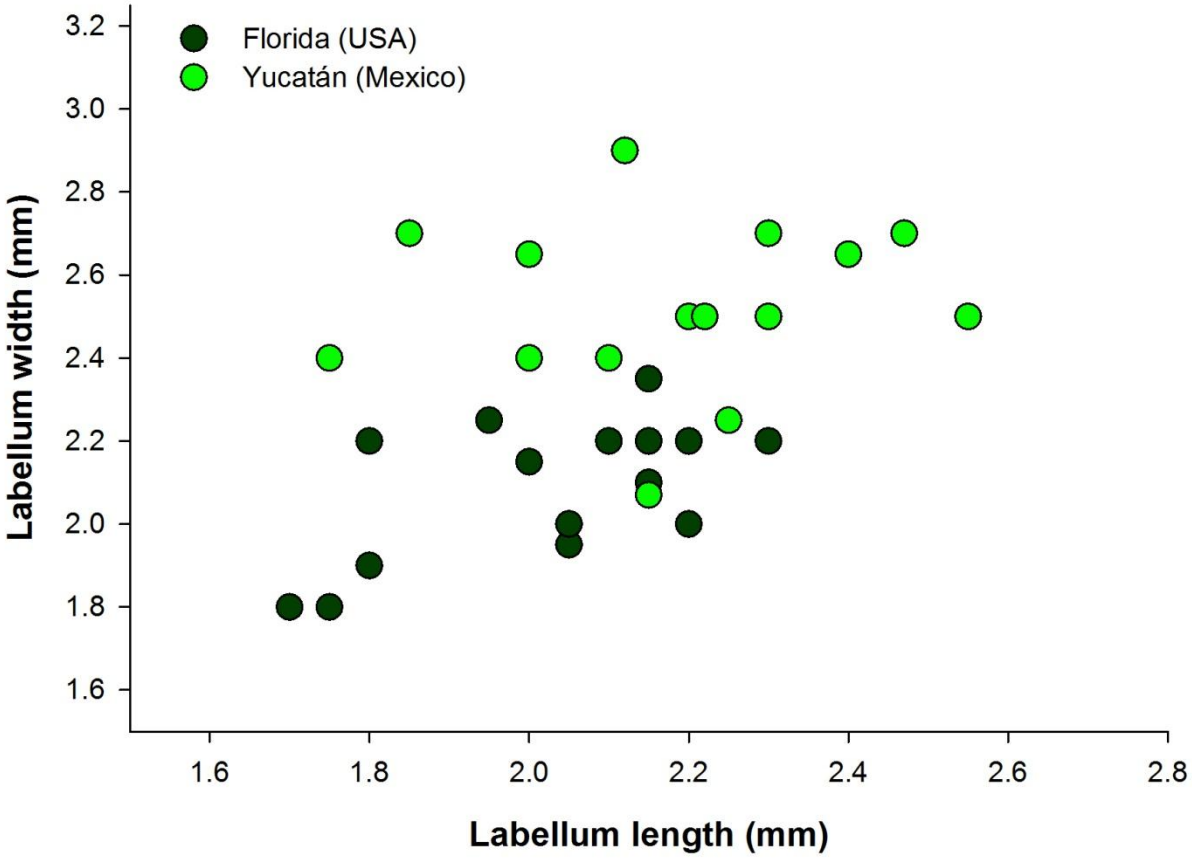

Figure 3-7. Differences in the labellum of *D. porrectus* within the populations of Florida and Yucatán. The dark green represent Florida and the light green represent Yucatán. The length of the labellum is plotted on the X-axis and the width on the Y-axis.

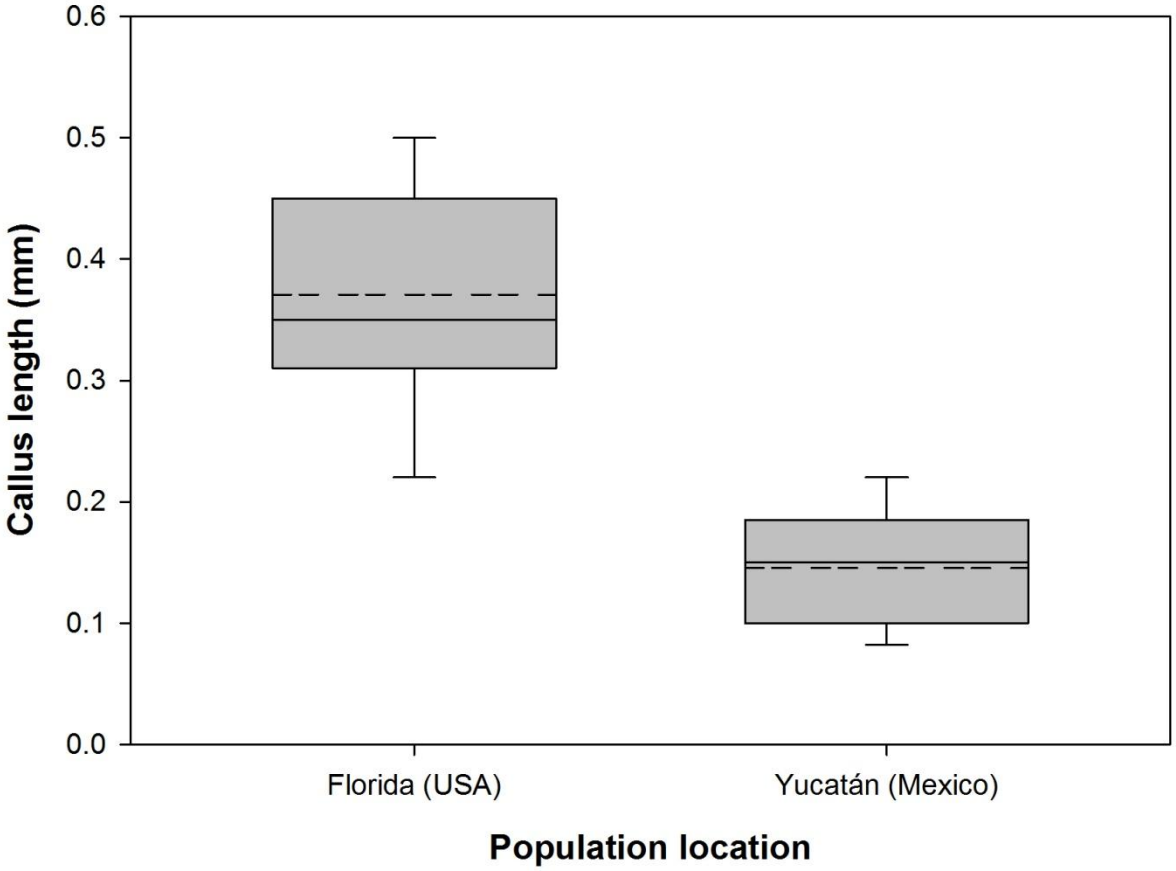

Figure 3-8. The difference in callus length within two populations of *D. porrectus* in Florida and Yucatán.

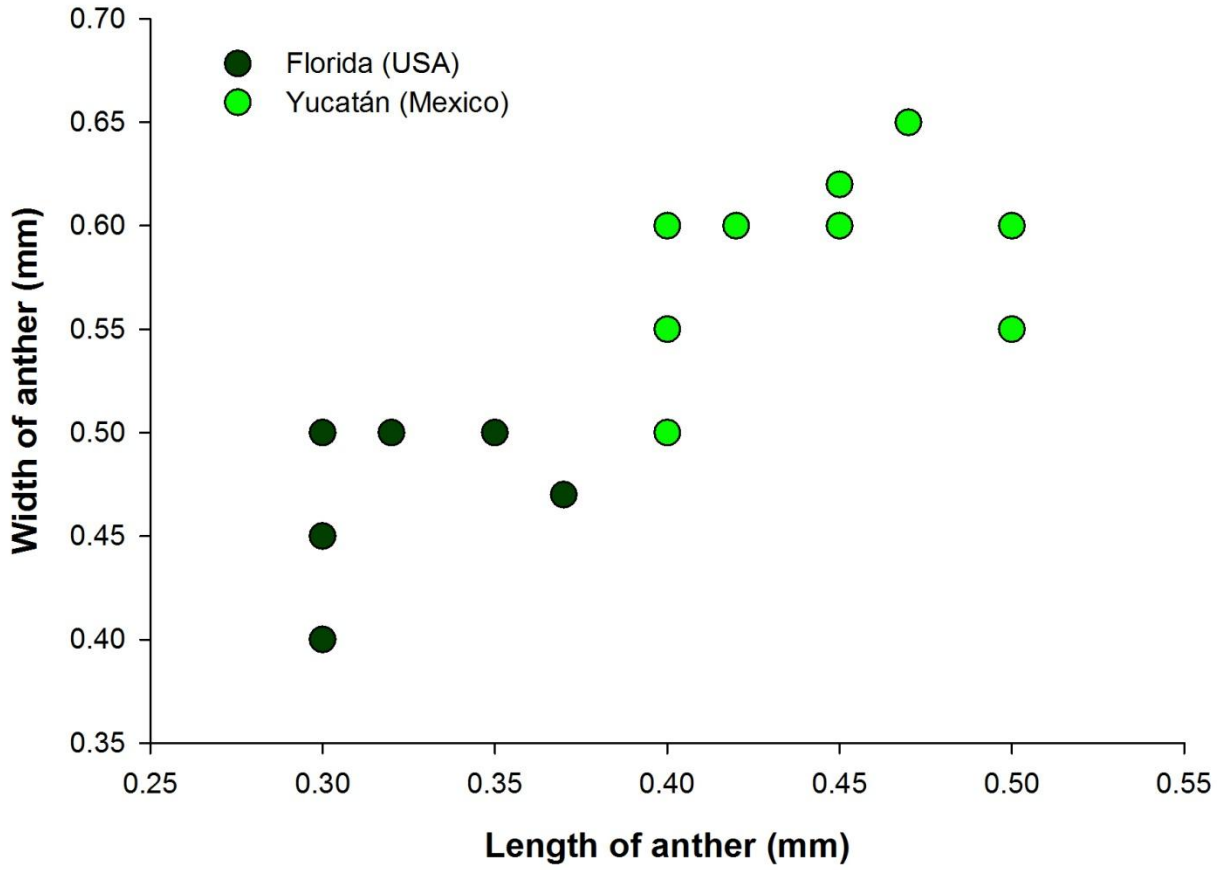

Figure 3-9. Differences in the anther of *D. porrectus* within the populations of Florida and Yucatán. The dark green represent Florida and the light green represent Yucatán. The length of the labellum is plotted on the X-axis and the width on the Y-axis.

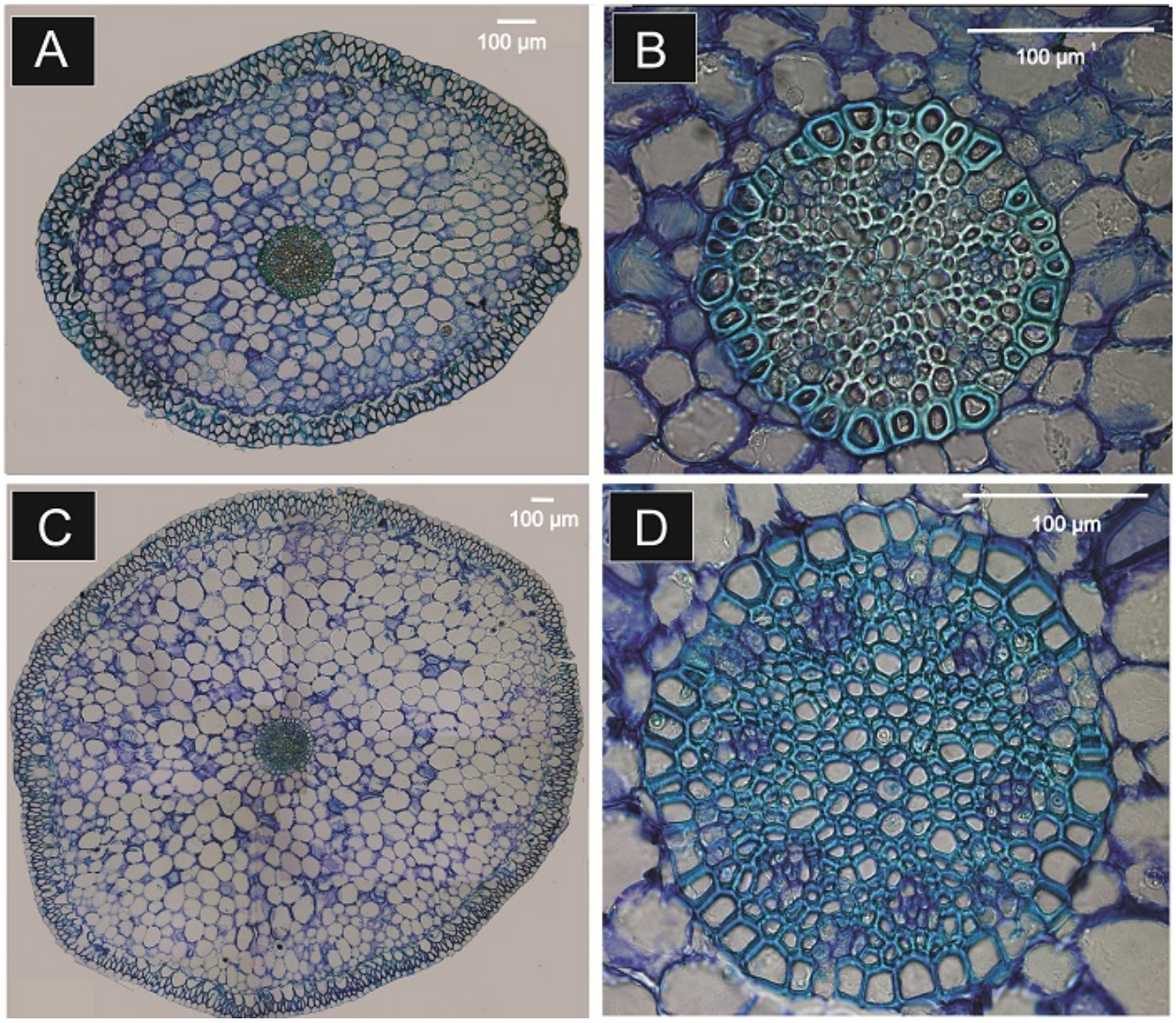

Figure 3-10. Resin embedded root sections of *D. porrectus* representing Florida and Yucatán populations. A) cross section showing a Florida root. B) close up of the Florida entity showing the stele with the thin-walled endodermis cells. C.) cross section of the Yucatán root. D) close up of the Yucatán entity showing the stele with the thick-walled endodermis cells.

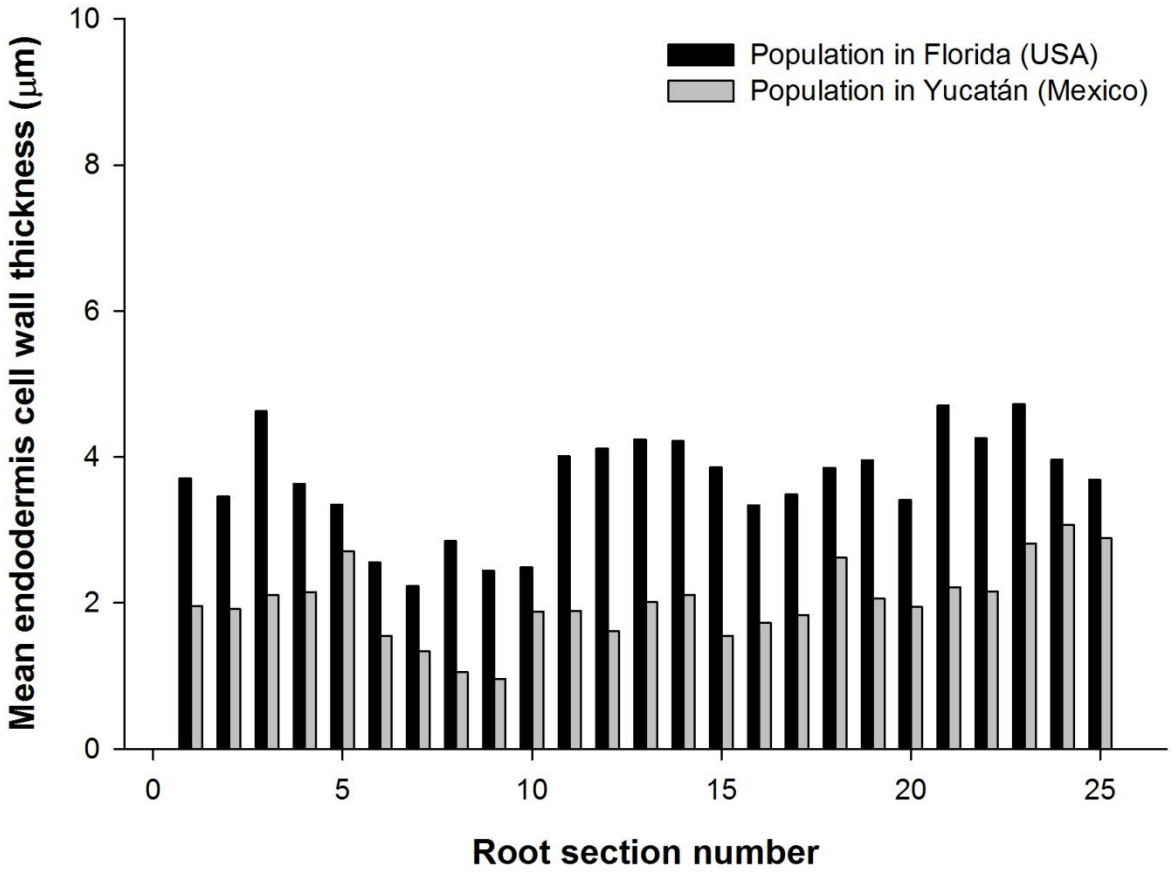

Figure 3-11. Resin embedded comparisons of cell wall thickness in the endodermis of two *D. porrectus* populations. The black bars represent Florida and the gray bars Yucatán. Every five root-section numbers on the X-axis represent one root of one plant

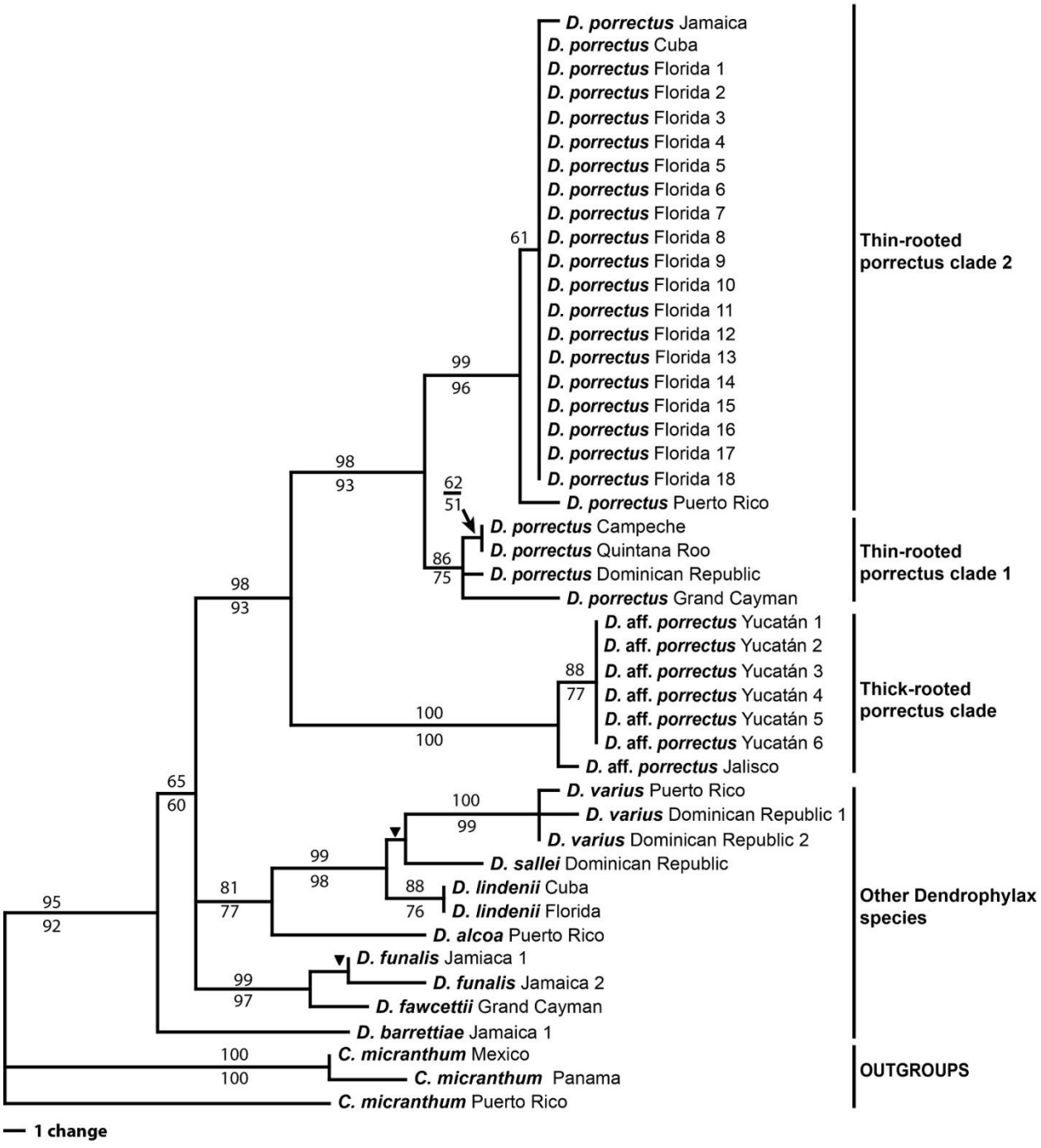

Figure 3-12. One of the 4 equally parsimonious trees of ITS. The bootstrap values are listed above the branches and the jackknife percentages are listed below the branches. The triangles indicate the nodes that collapsed in strict consensus.

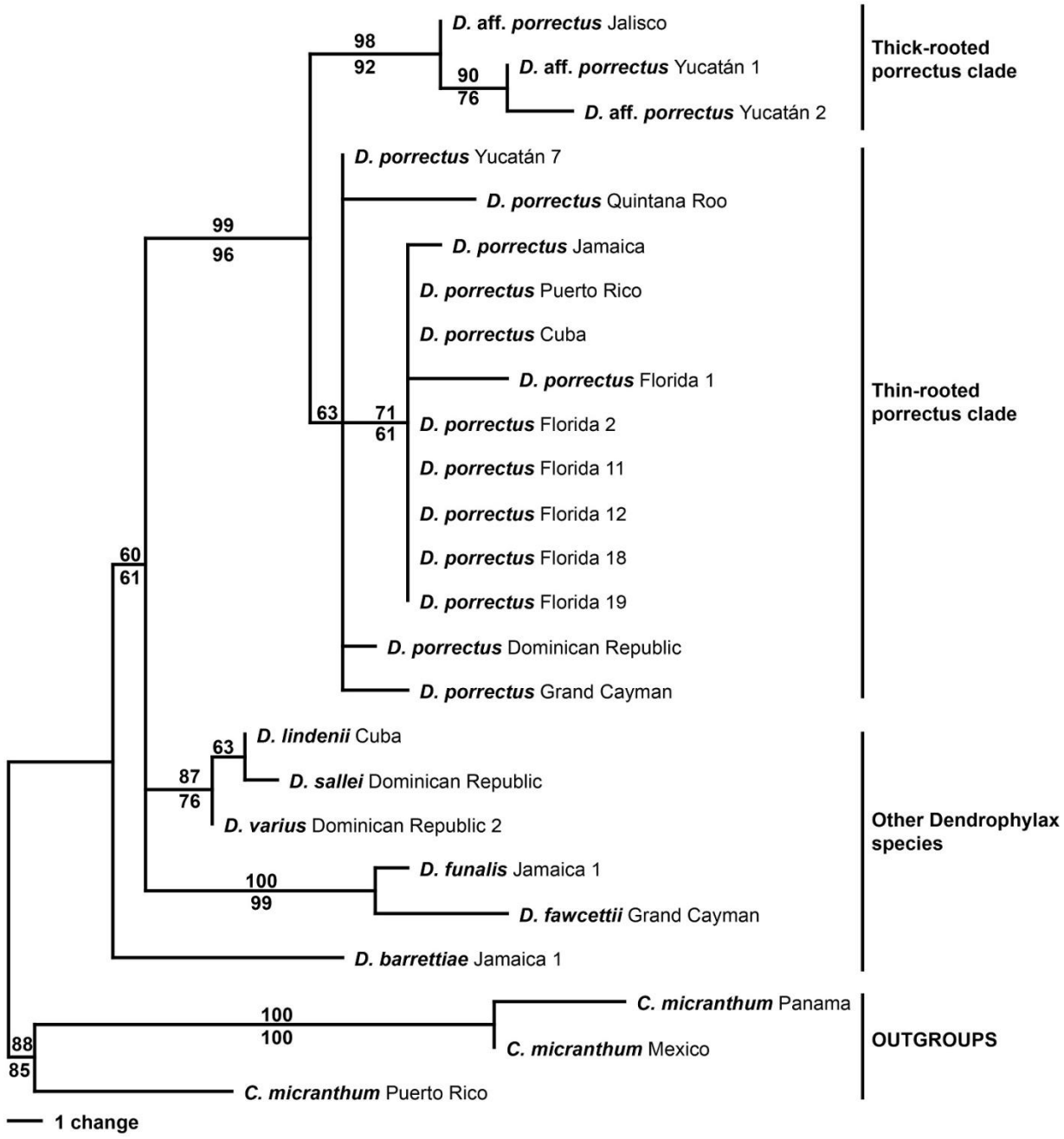

Figure 3-13. One of the 3 equally parsimonious trees of *matK*. Bootstrap values are listed above the branches and the jackknife values are listed below the branches. The topology of the three trees are identical as none of the nodes collapsed in a strict consensus.

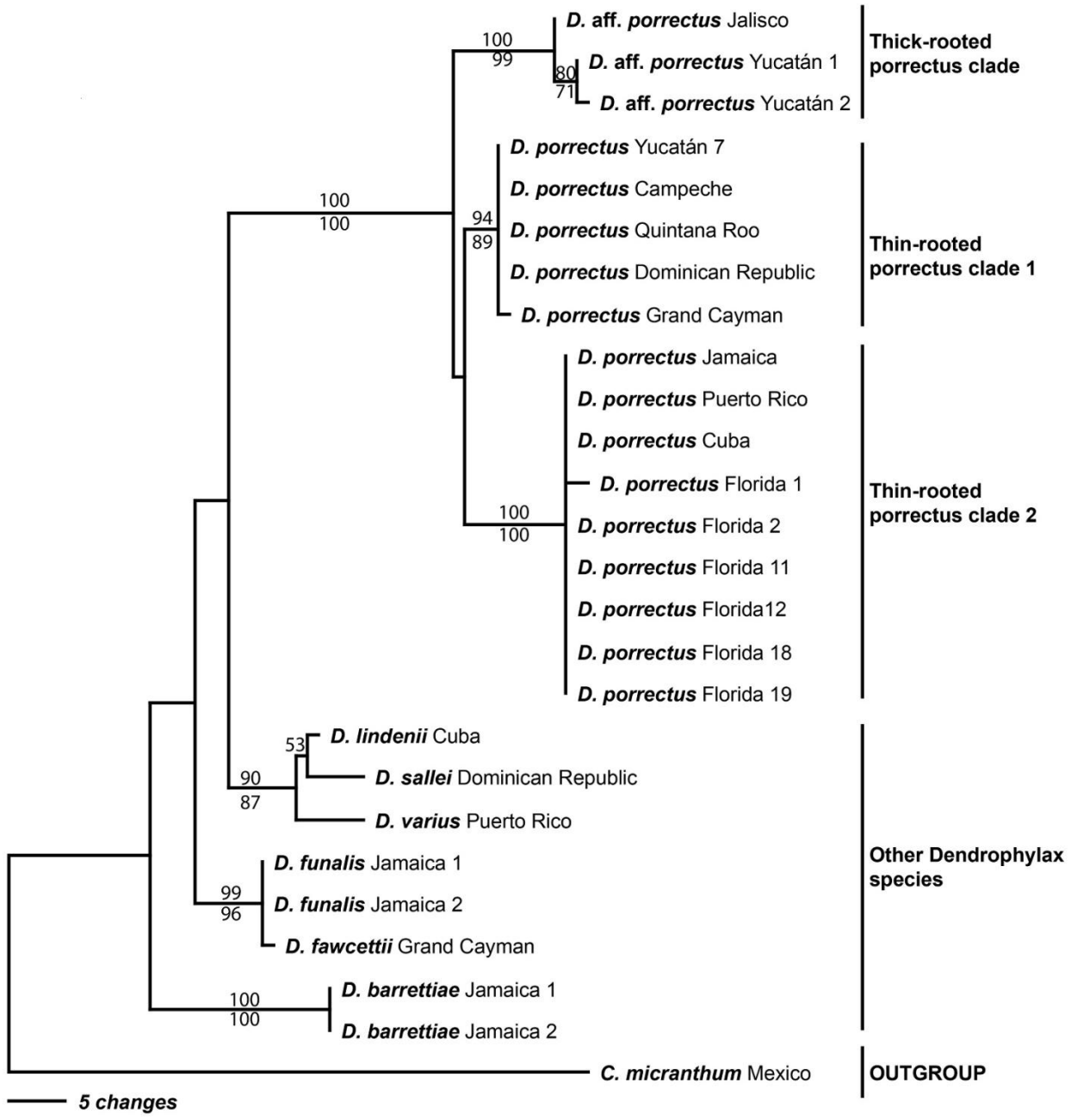

Figure 3-14. The most parsimonious tree of *ycf1*. Bootstrap values are above branches; jackknife values below branches.

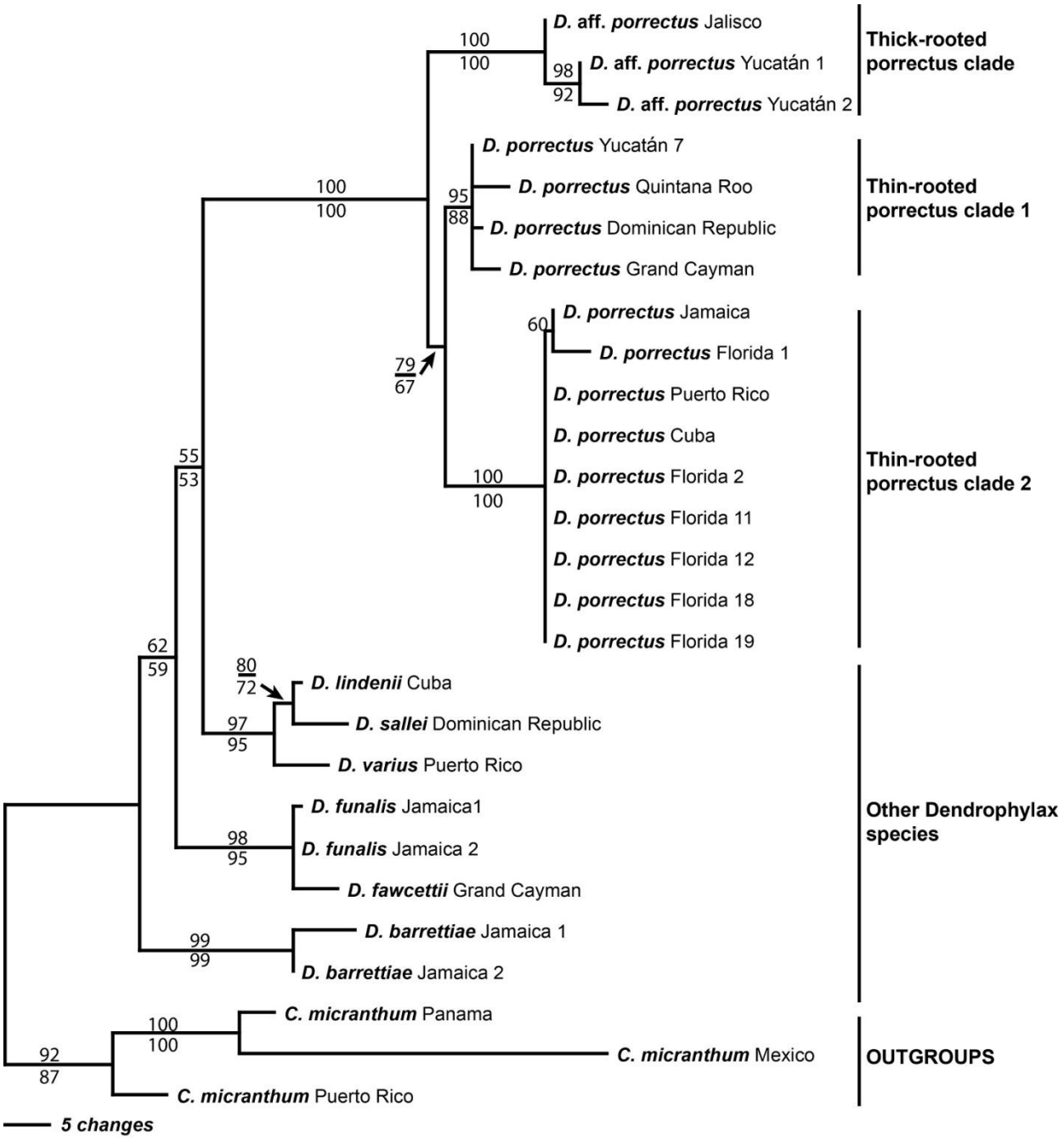

Figure 3-15. The most parsimonious tree of combined *matK* and *ycf1* analysis. Bootstrap values are above branches; jackknife values below branches.

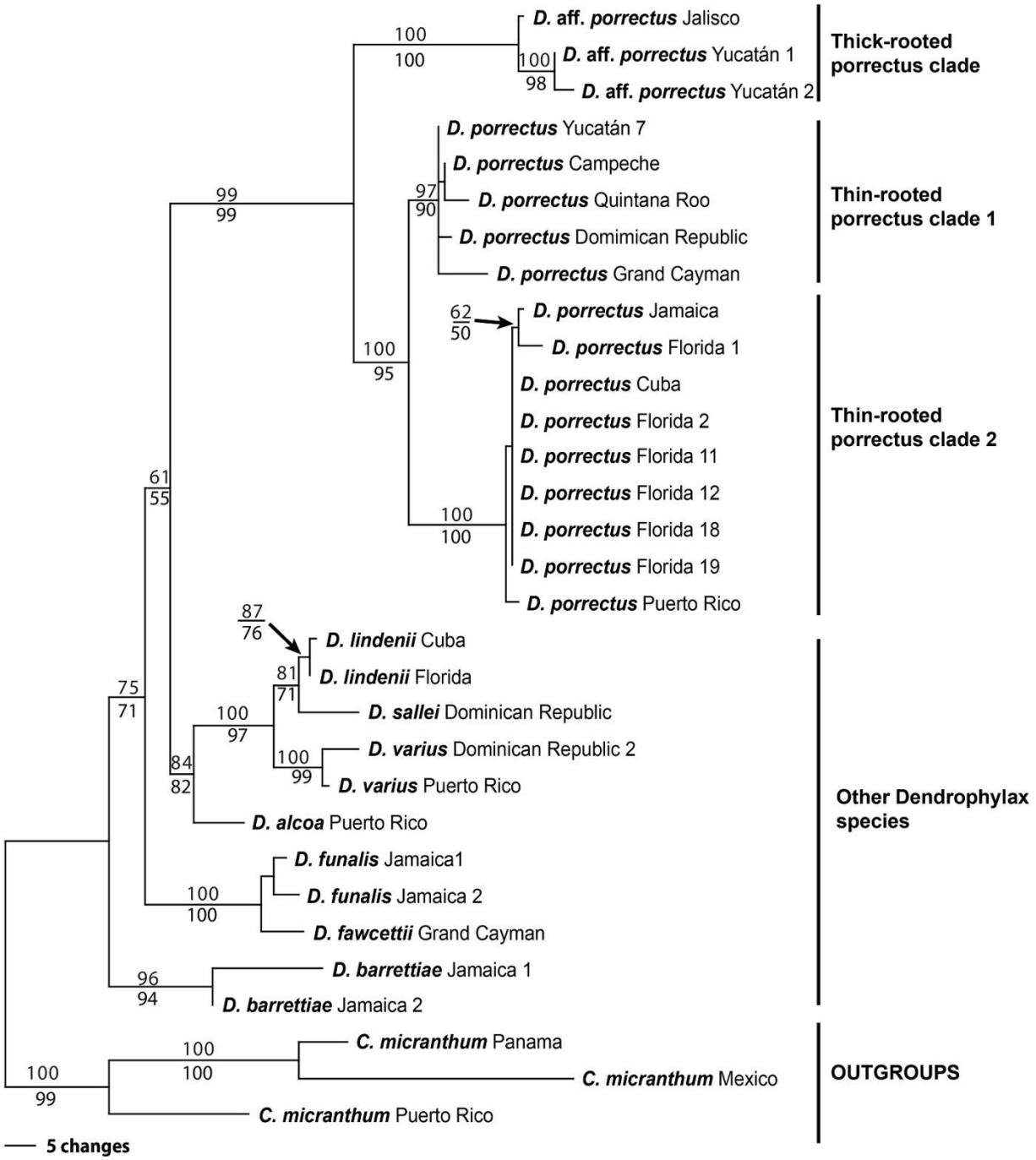

Figure 3-16. The most parsimonious tree of combined ITS, *matK,* and *ycf1* analysis. Bootstrap values are above branches; jackknife values below branches.

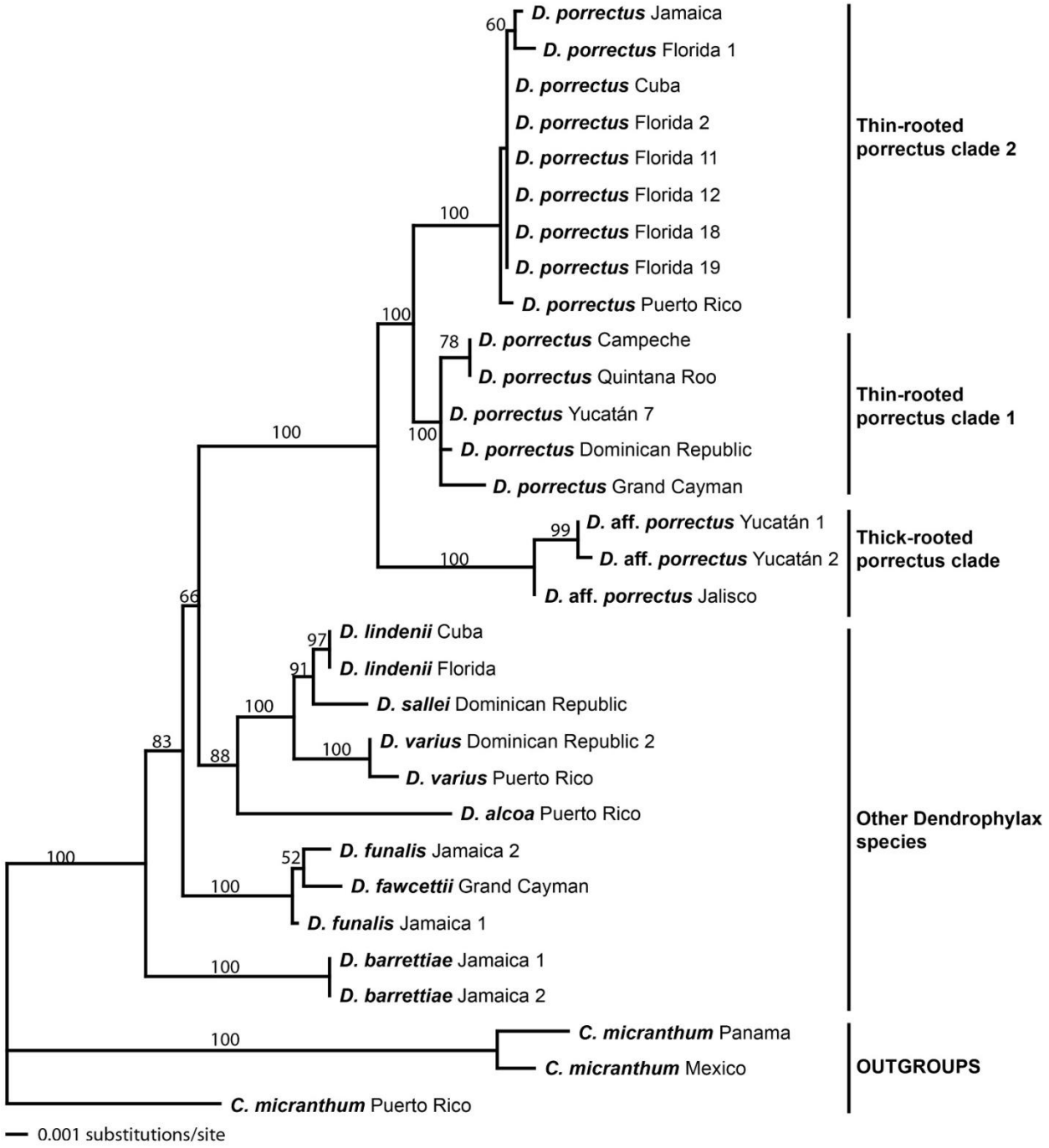

Figure 3-17. Maximum Likelihood tree of combined nuclear and plastid data assuming the GTR+G model, -log L = 7547.2470 based on100 replicates. Bootstrap support values are indicated above the branches.

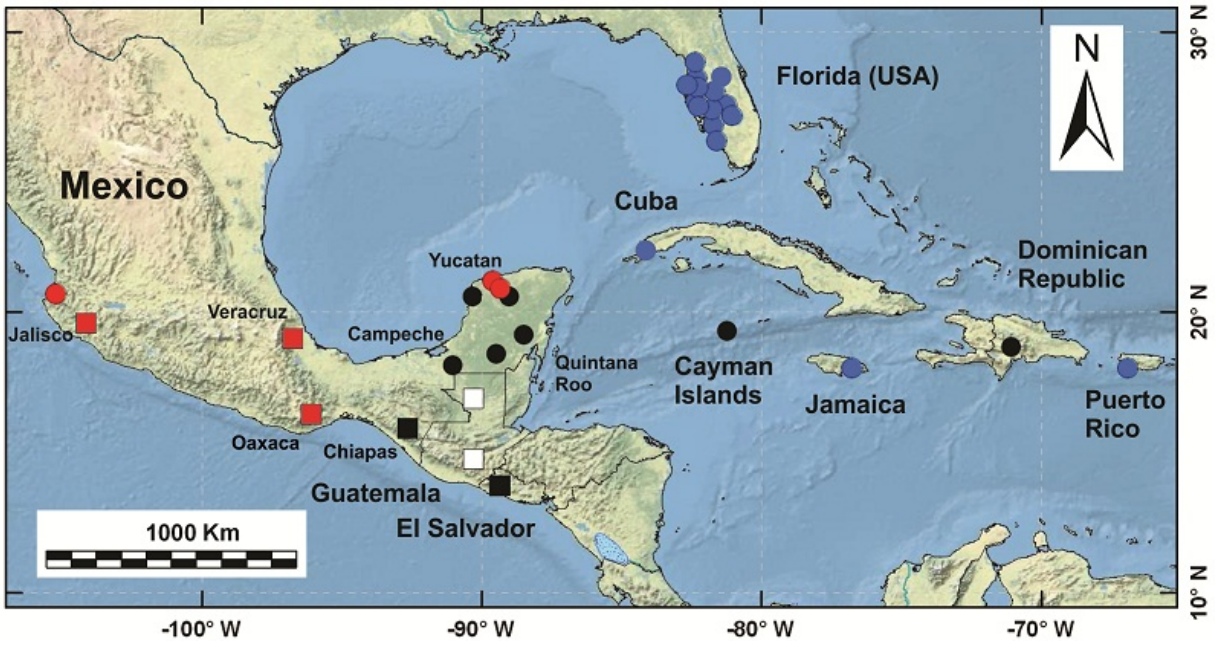

Figure 3-18. Distribution of *D. porrectus* with the three major clades shown in three different colors. Black circles indicates the thin-rooted clade 1, blue circles indicates the thin-rooted clade 2, and red circles shows the location of the thick-rooted clade. The red squares indicate voucher specimens/ photo material that are morphologically similar to the thick-rooted clade (pers. comm. G. Salazar & R. Jiménez Machorro, 2011). The black squares are specimens which were photographically examined, but a definite decision could not be made if they belong to the thin-rooted or thick-rooted clade. The white squares indicate herbarium records not seen or unverified and literature reports (Dix & Dix, 2000).

CHAPTER 4
DISCUSSION AND CONCLUSION

Discussion

The Florida populations differ significantly from the Yucatán populations in many morphological characters, including root thickness, ovary length, perianth shape, anther dimensions, callus length, rostellar entry opening, and pollinium shape. Although subtle, the difference in root thickness is readily apparent, especially if plants or herbarium specimens are observed side-by-side. For plants in flower, the difference in length of the callus is the most reliable feature that distinguishes the thick-rooted Yucatán populations (thick-rooted clade) from the thin-rooted populations (thin-rooted clades 1 and 2).

The anatomical root results in Figure 3-10 shows that the endodermis cell walls of the Florida plants are thicker than the Yucatán plants, but these differences may be an artifact of the age of the root tissue. As the roots age, the endodermis cell wall thickens (Dickison, 2000). Since root growth and development are dependent on environmental factors (Dycus & Knudson, 1957), it is difficult to obtain roots that are in the same developmental stage, which then can be compared. I suggest that the root endodermis cell wall thickness it not a good character to distinguish these two entities.

The Yucatán populations are the thick-rooted plants and the results of this study revealed that the Yucatán entities are morphologically similar to the populations from Jalisco and Veracruz. The thin-rooted plants (Florida entity) are similar to the populations in the Greater Antilles (Cayman Islands, Cuba, Dominican Republic, Jamaica, and Puerto Rico), Campeche, and Quintana Roo.

The differences between the thin and thick- rooted entities based on morphology correlate perfectly with the DNA based clades as the two entities show high divergence in both ITS and plastid molecular data. Together, these results suggest that these major clades should be recognized as two distinct species, based on the phenetic/morphological, apomorphic, and evolutionary species concepts (de Queiroz & Good, 1997; de Queiroz, 2007; Coyne & Orr, 2004; Donoghue, 1985; Judd et al., 2007).

Dendrophylax porrectus sensu stricto is comprised of the thin-rooted clade 1 which occurs in Mexico, the Cayman Islands, and the Dominican Republic, and the members of the thin-rooted clade 2, which occurs in Jamaica, Puerto Rico, Cuba, and Florida. Although the molecular data reveal two distinct clades of *D. porrectus* sensu stricto, I am unable to find any morphological differences that would warrant their recognition as distinct species. They are thus retained in *D. porrectus* sensu stricto, at least until that clade is studied in more detail. These thin- rooted-plants retain the name *D. porrectus* because the type specimen is thin-rooted. The thick-rooted entity/clade, therefore, represents an undescribed species.

The distribution map (Fig. 3-18) depicts the three major clades and indicates that the Yucatán thick-rooted populations grows in sympatry with the thin-rooted population in this region. These data imply that *D. porrectus* sensu stricto and the new species (*D.* aff. *porrectus)* are not interbreeding which suggests that the biological species concept (Mayr, 2000) could also be applied, justifying their recognition as distinct species.

The disjunction between Yucatán and Jalisco populations (thick-rooted clade) is not novel as similar distributions are also seen between sister taxa of *Enriquebeltrania* in the *Euphorbiaceae* (De-Nova et al., 2006). *Dendrophylax porrectus* has been

reported from Oaxaca and Veracruz (Table 1-1) and photographs of herbarium specimens and living plants from the Veracruz and Oaxaca populations, showed features similar to those of the Yucatán-Jalisco entity (thick-rooted clade) and these are considered here as additional populations of this clade. There is still a gap between the Veracruz-Oaxaca populations and the Jalisco populations, but the distributional gaps could be an artifact of the few available collections.

According to Brown (2002), the flowering period of *D. porrectus* in Florida is from August to November. In this study, members of the Ft. Myers population flowered twice in a year with a gap of two months. This twice a year flowering pattern was not observed in the Sarasota populations. Plants in the greenhouse and in their natural habitat had the same flowering pattern. The flowering dates also match with the collected specimens deposited in various herbaria. It seems that there is a variation in the phenology of *D. porrectus* in Florida, and that flowering plants have been collected from August until January.

Members of the thin and thick-rooted populations occurring in the Yucatán flowered from September until January, which means that interbreeding could take place between the *D. porrectus* and the new species, as the plants grow in sympatry. Since we do not have any evidence of hybrids, I suggest that there is an interbreeding barrier present. This barrier could be the result of the difference in rostellar entry opening and the length of callus, or there could be genetic or chromosomal barriers to hybridization. Nothing is known about chromosome numbers in these species.

I was not successful in determining the pollinator but, according to the flower morphology (apron-like rostellum) the pollinator was suggested of a lepidopteran (Dressler, 1993).

Conclusion

The results of this study clearly support recognition of two distinct species within *Dendrophylax porrectus*; these two species differ most conspicuously in root thickness, but also in various floral characters and in molecular sequence divergence. Since the type of *D. porrectus* is from Cuba where only the thin-rooted are known to occur, the thick-rooted clade needs to be described as a new species. Within *D. porrectus* sensu stricto (the thin-rooted clade), molecular data reveal two subclades, but at present I am unable to find morphological characters that correlate with these molecular clades. Additional studies, especially focused on the morphology of the entities within *D. porrectus* sensu stricto are needed in order to resolve their taxonomic status.

APPENDIX
SPECIMENS USED IN THIS STUDY WITH THEIR GENBANK ACCESSION NUMBER

Table A-1. Specimens used in this study with their GenBank accession number

Taxon	Voucher	Locality	ITS	*matK*	*ycf1*
C. micranthum	*Carlsward 315* (FLAS)	Panama	AY147220*	AY147235*	N/A
C. micranthum	*Carlsward 180* (FLAS)	Mexico	AF506298*	AF506347*	EU490725*
C. micranthum	*Ackerman 3341* (UPRRP)	Puerto Rico	AY147219*	AF506346*	N/A
D. alcoa	*Ackerman 2773* (UPRRP)	Dominican Republic	AF506307*	N/A	N/A
D. barrettiae	*Carlsward 199* (FLAS)	Jamaica	AF506308*	AF506353*	JN176133
D. barrettiae	*Whitten 1814* (FLAS)	Jamaica	N/A	N/A	JN176134
D. aff *porrectus*	*Salazar 8279* (MEXU)	Mexico (Puerto Vallarta)	JN176093	JN176103	JN176111
D. aff *porrectus*	*Carnevali 5907* (CICY)	Mexico (Yucatán)	AF506314*	AF506357*	JN176112
D. aff *porrectus*	*Carnevali 5907a* (CICY)	Mexico (Yucatán)	JN176094	JN176104	JN176113
D. aff *porrectus*	*Carnevali 5907b* (CICY)	Mexico (Yucatán)	JN176095	N/A	N/A
D. porrectus	*Ramirez 886* (CICY)	Mexico (Yucatán)	N/A	JN176105	JN176114
D. fawcettii	*Whitten 3265* (FLAS)	Cayman Islands	AF506309*	AF506354*	JN176135
D. funalis	*Carlsward 302* (FLAS)	Jamaica	AY147221*	AY147229*	JN176131
D. funalis	*Whitten 1935* (FLAS)	Jamaica	AF506310*	N/A	JN176132
D. lindenii	*Ward 5365* (FLAS)	USA (Florida, Collier)	AF506319*	N/A	N/A
D. lindenii	*Claude Hamilton s.n.*	Cuba	AF506318*	AF506362*	JN176130
D. porrectus	*Ackerman 4514* (UPRRP)	Cuba	JN176098	JN176106	JN176121
D. porrectus	*Whitten 1950* (FLAS)	Dominican Republic	AY147224*	AY147238*	JN176119
D. porrectus	no voucher	Cayman Islands	JN176097	AF506361*	JN176118
D. porrectus	*Carlsward 184* (FLAS)	Jamaica	AF506315*	AF506358*	JN176117
D. porrectus	*Carnevali 4468* (CICY)	Mexico (Campeche)	JN176096	N/A	JN176115
D. porrectus	*Carnevali 6312* (FLAS)	Mexico (Quintana Roo)	AF506316*	AF506360*	JN176116
D. porrectus	*Goldman 2271* (FLAS)	USA (Florida, Glades)	JN176102	AF506359*	JN176122
D. porrectus	*Carlsward 330* (FLAS)	USA (Florida, Ft. Myers)	N/A	JN176110	JN176124
D. porrectus	Molgo 221 (FLAS)	USA (Florida, Hillsborough)	JN176099	JN176107	JN176127
D. porrectus	*Molgo 222* (FLAS)	USA (Florida, Sarasota)	JN176101	JN176109	JN176126
D. porrectus	*Carlsward 329* (FLAS)	USA (Florida, Glades)	AY147223*	AY147237*	JN176123
D. porrectus	*Ackerman 3340* (UPRRP)	USA (Puerto Rico)	AF506313*	AF506356*	JN176120

Table A-1 (cont.)

Taxon	Voucher	Locality	ITS	*matK*	*ycf1*
D. sallei	*Whitten 1945* (JBSD)	Dominican Republic	AY147225*	AY147239*	JN176128
D. varius	*Whitten 1960* (JBSD)	Dominican Republic	AY147222*	AY147236*	JN176129
D. varius	*Thompson 10683* (SEL)	Dominican Republic	AF506312	N/A	N/A
D. varius	*Ackerman 2727* (UPRRP)	Puerto Rico	AF506311*	N/A	N/A

* Indicates sequences previously published by Carlsward (2004) and Neubig et al. (2009).
Note. N/A = not sequenced

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BIOGRAPHICAL SKETCH

Iwan Eduard Molgo was born in Paramaribo, Suriname, to Eduard Stuart Molgo and Jacqueline Molgo in 1974. He graduated from the Henri Dalhberg High School in 1993 and started his undergraduate career in 1994 at the Anton de Kom University of Suriname (AdeKUS). In 2002, he graduated from AdeKUS with a Bachelor of Science degree in agriculture and later that year he started working at the Foundation of Nature Conservation of Suriname as Research Coordinator and Wildlife Supervisor. In 2006, he was hired as Assistant Researcher at the National Herbarium of Suriname and was responsible for all orchid related research and the orchid collections. After working for three years, Iwan decided to begin his graduate career at the University of Florida under the supervision of Dr. Norris Williams where he studied the evolutionary relationships of *Dendrophylax porrectus*. Iwan graduated with a M.S. in botany in August 2011 and hopes to continue his graduate career at the University of Florida in order to support his country after he has finished his studies.