A survey of declining palms (Arecaceae) for the 16SrIV-D phytoplasma for evaluating distribution and host range in Florida

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Abstract

The 16SrIV-D phytoplasma was first identified in Florida in 2006. Since its discovery, it has spread throughout most of the state, being most prevalent in the central part of Florida from Hillsborough County on the west coast to St. Lucie County of the east coast. The 16SrIV-D phytoplasma is the causal agent of lethal bronzing disease (LBD), which is also known as Texas Phoenix Palm Decline (TPPD) and affects a variety of common and economically important ornamental palm species as well as the native, and ecologically important species, *Sabal palmetto*. Since the discovery of the disease, it has spread into the southern portions of Florida where palm species diversity is higher. The aim of this survey was to document the spread of the disease in terms of geographic and host range a decade after its introduction into Florida, and also to assess the risk LBD poses to the nursery and landscaping industries. The survey included samples received from stakeholders from throughout the state covering 18 counties, as well as a systematic sampling of palms at the Fort Lauderdale Research and Education Center (FLREC) where the disease is spreading actively. The findings of this survey resulted in the detection of LBD in eight new counties, including Collier, Hernando, Jefferson, Martin, Miami-Dade, Monroe, Seminole, and St. Johns, and expansion of LBD into four new host species, *Cocos nucifera*, *Livistona chinensis*, *Butia capitata*, and *Carpentaria acuminata*. These findings are crucial for stakeholders because it highlights new hosts of 16SrIV-D phytoplasma and geographic expansion of the disease, meaning vigilance is needed when surveying for declining palms.

The 16SrIV-D phytoplasma was first discovered in Hillsborough County, Florida in 2006 from declining Canary Island Date Palms (*Phoenix canariensis* Chabaud), Edible Date Palms (*P. dactylifera* L), Wild Date Palms (*P. sylvestris* L), and Queen Palm (*Syagrus romanzoffiana* Chamisso) (Harrison et al. 2008). Previously the 16SrIV-D phytoplasma had only been known from Texas where it was isolated from *P. canariensis* (Harrison et al. 2002). Subsequently in 2008, the 16SrIV-D phytoplasma was isolated from declining Cabbage Palms (*Sabal palmetto* Walter) in Florida (Hillsborough and Manatee County) (Harrison et al. 2009). In 2011, the 16SrIV-D phytoplasma was also isolated from a declining Pygmy Date Palm (*P. roebelinii* O'Brien) in Florida (Hillsborough County) (Jeyaprakash et al. 2011). The most recent new host record for the 16SrIV-D phytoplasma in Florida was from the Bismarck Palm (*Bismarckia nobilis* Hildebr & Wendl) found in Manatee County (Dey et al. 2018). Besides, Florida and Texas, the only other state within the United States where 16SrIV-D phytoplasma has been detected is Louisiana, where it was detected in declining Chinese Windmill Palms (*Trachycarpus fortunei* Hook) (Singh and Ferguson 2017) . Outside of the United States, the 16SrIV-D has been found only in Mexico from declining *Sabal mexicana* Martius and *Pseudophoenix sargentii* Wendl (Vázquez-Euán et al. 2011), Christmas Palm (*Adonidia merrillii* Beccari) (Lara et al. 2017), and *Pritchardia pacifica* Seeman & Wendl (Narváez et al 2017). Based on published records, this bring the total number of susceptible hosts of the 16SrIV-D phytoplasma to 12 palm species, with seven of the susceptible species confirmed in the state of Florida, United States. In contrast, the 16SrIV-A phytoplasma, the causal agent of lethal yellowing (LY), is known to affect over 30 different species of palm in Florida (Bahder and Helmick 2018a), and was introduced into the southern portion of the state (Corbett 1959) where palm diversity is relatively higher. Many of the host records of LY are due to the introduction of the disease into

Fairchild Tropical Botanic Garden (Howard and Collins 1978) which houses over 100 species of palms, most of which are non-native and tropical in origin and thus cannot survive north of the subtropical climate of south Florida. It is important to note that the hosts associated with LY were established prior to the use of molecular techniques. The diagnostics were based on Scanning Electron microscopy (SEM) that demonstrated the presence of phytoplasma; but could not distinguish between groups or subgroups as they are currently known, thereby casting doubts whether all cases of reported hosts of LY were caused by the 16SrIV-A phytoplasma or other groups. Therefore, there is a need for a systematic study of declining palms in Florida using molecular diagnostics.

The 16SrIV-D phytoplasma is the causal agent of a disease that was initially called Texas Phoenix Palm Decline (TPPD) due to its original discovery in Texas from declining *P. canariensis*. However, the disease is currently referred to as lethal bronzing disease (LBD) in Florida (Bahder et al. 2018) and Date Palm lethal decline (DPLD) in Texas (Giesbrecht et al. 2014). The name LBD was proposed because it accurately described symptoms observed in affected host species, in which dying leaves display a bronze coloration that varies in hue among species but is consistently different than the color of naturally senescing leaves of palms (Bahder et al. 2019).

The spread of the16SrIV-D phytoplasma in the urban environment of south Florida was shown for a stand of *Sa. palmetto* and *Sy. romanzoffiana* at the UF/IFAS Fort Lauderdale Research and Education Center (FLREC), which were infected after presumed introduction of an infective insect vector (Bahder et al. 2018). This study revealed the decline of approximately 50% of *Sa. palmetto* and 25% of *Sy. romanzoffiana* over an approximately three-year period. Following the termination of this study, further infections were observed in these *Sa. palmetto*

and *P. roebelinii*. At the time, the outbreak of LBD at FLREC was the southernmost record of the 16SrIV-D phytoplasma in Florida (Harrison and Elliott 2016). The FLREC is an ideal location to evaluate the potential of the 16SrIV-D to infect previously unknown hosts, because there is a higher diversity of palms at the research station than surrounding areas (T. Broschat, *personal communication*). Evaluating which palm species are susceptible in a single location can help determine the true host range of the phytoplasma. In parallel, studying the host and geographic distribution of the pathogen throughout the state improves our understanding of the true economic impact of the pathogen in Florida.

The primary objective of this survey was to expand our knowledge of host range and distribution of the 16SrIV-D phytoplasma in the state of Florida by surveying palm species at the FLREC and samples taken from declining palms throughout the state by stakeholders (landscaping and nursery personnel). The results of this study provide valuable data to stakeholders by elucidating the increasing geographical and host range of the 16SrIV-D phytoplasma in Florida and providing further impetus for sampling new potential hosts.

Materials and Methods

Sample Collection and Processing

All samples taken from palms at FLREC (26.084006, 80.237431) consisted of trunk tissue that was obtained according to the protocol outlined by Bahder and Helmick (2018b). Samples were obtained between July 1st, 2016 and March 20th, 2019. Samples were collected from palms that displayed typical symptoms of premature fruit drop/inflorescence necrosis, discolored older leave, and/or spear leaf collapse. In addition to symptomatic palms, adjacent palms of the same species that were asymptomatic were also included in this study. Other palm species that did not display symptoms but were adjacent to symptomatic palms were also sampled. All samples

obtained from stakeholders were collected from trunk tissues according to the protocols outlined by Bahder and Helmick (2018b). These samples were collected during the same time period when samples were also collected from the survey at FLREC. All samples were processed by macerating one gram of tissue in guanidine buffer (guanidine thiocyanate - 4M, 3M sodium acetate – 0.2 M, 0.5 M EDTA - 0.25M, PVP-40 – 0.0006 M) in a BioReba extraction bag using the HOMEX6 tissue homogenizer. Lysate was then extracted using the Plant Mini Kit (Qiagen) as per the manufacturer's instructions. Excess plant tissue was stored at -80˚C.

Screening of Palms by qPCR and Melt Curve Analysis

Eluate obtained from the extraction protocol was diluted to 25 µl when necessary before screening by qPCR. All samples were screened using the qPCR parameters, primers and probe presented by Córdova et al. (2014). Following this TaqMan assay, samples testing positive were screened by a second qPCR assay according to Bahder et al. (2017) to determine if the sample was infected with the 16SrIV-A or 16SrIV-D phytoplasma. Standard PCR, using primers LY16-LSF/LY16-LSR (Córdova *et al*. 2014), was used for amplification of gDNA from sample Spa-12, which had tested positive for LBD. Each PCR contained 5x GoTaq Flexi Buffer, 25 mM $MgCl_2$, 10 mM dNTP's, 10 mM each primer, 10% PVP-40, and 2.5U GoTaq Flexi DNA Polymerase, and sterile dH_20 to a final volume of 25 µL. Thermocycling parameters were as follows: 94°C for 1 min initial denaturation, followed by 35 cycles of 94°C for 30s, 60°C for 30s, 72°C for 30s, and a final extension of 72°C for 5 min. Three µL of each product was electrophoresed on a 1.5% agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA) and visualized using ultraviolet transillumination.

PCR products were cloned using a TOPO® TA Cloning® Kit into vector pCR™2.1-TOPO® (Invitrogen) per the manufacturers protocol. The cloning constructs were transformed

125 into TOPO One Shot® Chemically Competent *E. coli* cells and plated on LB plates containing 50
126 µg/mL Kanamycin. Plates were incubated overnight at 37°C and transformed colonies were
127 chosen for colony PCR using primers LY16-LSF/LY16-LSR (Córdova *et al*. 2014) to verify that
128 they contained the correct insert. Clones with the insert of the correct size were incubated on a
129 shaker overnight in 20 mL LB broth with 50 µg/mL Kanamycin. Plasmids were extracted using
130 a QIAprep Spin Miniprep Kit (Qiagen) per the manufacturer's protocol. Plasmid concentrations
131 were quantified using either a NanoDrop Spectrophotometer (ThermoFisher Scientific, Waltham,
132 MA) or a Qubit® 2.0 Fluorometer (ThermoFisher Scientific, Waltham, MA) using the Qubit
133 dsDNA BR Assay Kit (Invitrogen). Ten-fold serial dilutions were created, 10^8 – 10^3, for use as
134 standards in the qPCR assay that were used to generate the standard curve and quantity
135 estimation (Qty.) for all samples.
136 All qPCR assays were run on a QuantStudio™ Real-Time PCR Software v1.3 (Life
137 Technologies, Inc.). All qPCR assays were run in triplicates per specimen. When a single palm
138 of a species was positive, the average copy number and standard error are calculated based on
139 the replication of the same sample, whereas species with multiple specimens testing positive are
140 presented as the average of the replications of each specimen followed by the average of all
141 specimens.
142 *Nested PCR Reactions and Sequencing*
143 For palm species that were not previous known hosts of the 16SrIV-D phytoplasmas, sequence
144 data was obtained by amplifying a portion of the 16S rDNA using standard and nested PCR
145 protocols as outlined by Bahder et al. (2018). Amplified products from the nested PCR reaction
146 were purified using ExoSAP-IT™ PCR Product Cleanup Reagent (ThermoFisher Scientific,
147 Waltham, MA) per the manufacturers protocol. The cleaned PCR products were sent to Eurofins

Diagnostics for sequencing. Resulting sequences were assembled, visually inspected and corrected for sequencing errors using DNA Baser v. 4.36 (Heracle Biosoft), then aligned using MEGA7 (Kumar et al. 2016). All sequences were identified as 16S rDNA phytoplasma sequences via nucleotide BLAST on the NCBI website (https://blast.ncbi.nlm.nih.gov).

Sequence Analysis and Subgroup Determination

To establish group and subgroup classification for phytoplasmas isolated from new host species, sequences obtained were aligned with 16S sequences from isolates of the A subgroup from each known 16Sr group known to date, and all subgroups from the 16rIV group (Table 1). Subgroup -A was selected because in general, -A subgroups are more common and generally more representative of the disease they are associated with. Additionally, an isolate of *Acholeplasma palmae* (Accession No. NR_029152.1) was included as an outgroup in the analysis. To establish subgroup classification of the isolates obtained in this study, sequences were subjected to the construction of a maximum likelihood tree at 1,000 replicates.

Results

qPCR Screening of Palms

From July 1st, 2016 to March 20th, 2019, 189 palm samples were taken at FLREC that were comprised of 11 different species (Table 2). Of the species sampled at FLREC, *Sa. palmetto*, *Sy. romanzoffiana, P. roebelinii*, and *Adonidia merrillii* were previously described host species of the 16SrIV-D phytoplasma whereas *Carpentaria acuminata* Wendland & Drude, *Phoenix reclinata*, *Sa. mauritiiformis*, *Serenoa repens*, *Sa. etonia*, *Cocos nucifera* L., *Roystonea regia*, and *Wodyetia bifurcata* were not known hosts of the 16SrIV-D. Symptoms were observed in 14 *Sa. palmetto* (Figure 1), one *Sy. romanzoffiana* (Figure 1), two *P. roebelinii* (Figure 1), two *A. merrillii* (Figure 1), one *Carpentaria acuminata* (Figure 1), and two *Cocos nucifera* (Figures 2, 3). All specimens that exhibited symptoms tested positive by the TaqMan qPCR assay (Table 2)

and yielded a melting temperature signature (Tm) that matched positive controls for the 16SrIV-D phytoplasma in the melt curve analysis (Table 2). In addition to these samples, a single *Cocos nucifera* that did not exhibit symptoms at the time of sampling tested positive for the 16SrIV-D phytoplasma by qPCR and was confirmed by melt curve analysis. All other palms sampled that did not exhibit symptoms tested negative for phytoplasma. Asymptomatic palms included *Phoenix reclinata* Jacquin, *Roystonea regia* Kunth, *Sabal etonia* Swingle, *Sabal mauritiioformis* Karsten, *Serenoa repens* Bartram, and *Wodeytia bifurcata* Irvine.

From July 1st, 2016 to March 20th, 2019, a total of 302 samples were received from throughout Florida (Table 3). Samples were submitted by landscape/nursery personnel as well as county extension agents and private homeowners. Of the samples received, the majority were taken from *P. sylvestris* (61.3% of total samples) with 56.2% testing positive (Table 3). The next two most abundant species sampled were *P. dactylifera* (9.6% of total samples) and *P. canariensis* (8.6% of total samples) with 51.7% and 34.6%, respectively, testing positive (Table 3). Eleven samples of *Sa. palmetto* and seven samples of *Sy. romanzoffiana* were received with 54.5% and 57.1% testing positive for phytoplasma, respectively (Table 3). Other species that tested positive but are not included in Table 3 are *Butia capitata* Martius (2/5 samples positive) (Figure 4) and *Livistona chinensis* Jacquin (1/5 samples positive) (Figure 5). Other palm samples that were submitted but tested negative (No Ct) are *Cocos nucifera* (nine samples), *Bismarckia nobilis* (three samples), *Washingtonia robusta* Wendland (seven samples), *Adonidia merrillii* (two samples), *Wodyetia bifurcata* (seven samples), *Livistona nitida* Rodd (one sample), and *Coccothrinax saxicola* León (one sample). All samples testing positive for phytoplasma yielded a Tm product that matched the Tm product for the 16SrIV-D positive control (Table 4). Of the palms included in this sample, *Butia capitata*, *Carpentaria acuminata*, *Cocos nucifera*,

and *L. chinensis* represented new host records for the 16SrIV-D phytoplasma and were thus sequenced for further analysis and confirmation. In addition, the isolate from *Adonidia merrillii* was sequenced because, while a known host of the 16SrIV-D phytoplasma in Mexico, this is the first known case of this species being affected in the United States.

DNA Sequence Analysis

The 16S sequences obtained for the isolates from *Adonidia merrillii*, *Butia capitata* (GenBank Accession No. MK421966), *Carpentaria acuminata* (GenBank Accession No. MH577010), *Cocos nucifera* (GenBank Accession Nos. MK421150, MK421151, MK421152), and *Livistona chinensis*, were placed within the 16SrIV phytoplasmas based on the maximum likelihood analysis (Figure 6). Within this group, they demonstrated >99% identity with the 16SrIV-D subgroup, confirming that isolates obtained from these hosts was the 16SrIV-D phytoplasma (Figure 6).

Discussion

This survey expands the known palm hosts of the 16Sr1V phytoplasma from 12 to 16. The new susceptible host species are the Pindo palm (*Butia capitata*), Carpentaria palm (*Carpentaria acuminata*), Coconut palm (*Cocos nucifera*), and Chinese fan palm (*Livistona chinensis*). All of these species are common ornamental palms found throughout southern and coastal central region of Florida. The knowledge of new susceptible palm hosts to the 16SrIV-D phytoplasma is essential from a management perspective, because it allows stakeholders to carry out more inclusive sampling of declining palms. Interestingly, the titer measured in *C. nucifera* and *L. chinensis* were substantially lower than the other hosts documented. This difference could be due to sampling error, however, could also be related to phloem density variation among species. While quantifying the vascular tissue densities among the species presented herein is beyond the

scope of the study, variation in phloem density among palm species is well documented (Rich 1987) and could influence detectable levels of phytoplas in infected palms, where palms with lest vascular tissue yield lower titers and palms with greater amounts of vascular bundles could yield higher titers. An additional explanation, is that because these rare host species are not well categorized, the titers presented in this study are not accurate or representative of the true titers present if additional specimens could be analyzed.

Although the majority of samples received belonged to the genus *Phoenix*, it is unclear if this is due to higher susceptibility of the genus or if this is due to sampling bias. The bias in sampling might be due to the difference in value among the palms. Phoenix palms are highly prized and therefore the stakeholders might have a tendency to sample *Phoenix* palms more often than *Sabal palmetto* and *Syagrus romanzoffiana,* which are considered cheap and aesthetically less pleasing. Antibiotic treatment is expensive; therefore, less valuable palms may be perceived as not worth the cost of sampling, testing, and treatment. In contrast, systematic sampling at FLREC, where the disease is spreading naturally, showed that the amount of declining *Sabal palmetto* was comparable to infection rates of *Phoenix* spp. observed in some nursery settings (B.W. Bahder, *unpublished data*). However, until a reliable vector assay is developed, it cannot be determined with certainty which palm species are more susceptible.

Another question of epidemiological significance is the detection of the 16SrIV-D phytoplasma in Miami-Dade and Monroe Counties, where palm diversity is especially high. It is unclear if the phytoplasma has naturally spread into these areas or if the samples represented palms that were infected in a different area with more disease pressure and were subsequently transported to the location where they were finally sampled. Regardless, these infections pose a huge risk to other susceptible palms in the extreme southern portion of the state. It is highly

likely more new host species will emerge in the coming years if 16SIV-D becomes established in this region of Florida. Prior to this study, the 16SrIV-D phytoplasma was recorded from 22 different counties in Florida (Harrison and Elliott 2016) (Figure 7). With eight new counties emerging from this survey, this number is now 30 (Table 3) (Figure 7). The samples received from stakeholders are mostly from urban areas and nurseries within those areas and represent only a small fraction of the total palms declining from the 16SrIV-D phytoplasma. While no formal assessment has been made of the economic impact of the 16SrIV-D phytoplasma on the nursery and landscaping industry, it is likely the loss incurred will be tens of millions of dollars, potentially as high as ranging into the hundreds of millions. In one instance, 100% crop loss was documented from *P. sylvestris* which amounted to $4.5 million dollars loss reported by a single grower (*personal communication from anonymous grower*). Palms have an approximate sales value of $404 million for the nursery and landscape industries in Florida as of 2010 (Khachatryan and Hodges 2017). Moreover, the recent initiatives to plant more palms along the major highways in Florida by the Florida Department of Transportation is likely to exacerbate losses due to this disease. Based on the impact seen in a single nursery plot as well as a preliminary assessment through samples received by stakeholders, it is apparent that LBD is widespread in Florida and poses a significant threat to the sustainability of palm production in the state.

The findings of this survey are important both from biological and applied standpoints. It demonstrates that this pathogen is actively spreading in time and space throughout the state as well as expanding into new palm hosts. Future efforts are required to survey declining palms in both urban environments and natural areas of Florida to have a clear understanding of the disease incidence and broader impact.

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Figure Legend

Figure 1. Symptomatic *Sabal palmetto* (A), *Syagrus romanzoffiana* (B), *Phoenix roebelinii* (C), *Adonidia merrillii* (D), and *Carpentaria acuminata* infected with the 16SrIV-D phytoplasma at the Fort Lauderdale Research and Education Center.

Figure 2. Necrotic inflorescence (A) and collapsed spear leaf (B) from *Cocos nucifera* infected with the 16SrIV-D phytoplasma**.**

Figure 3. Symptom progression of lethal bronzing disease in *Cocos nucifera* Cnu-3200: September 2018 (A), November 2018 (B), January 2019 (C), February 2019 (D).

Figure 4. Symptomatic *Butia capitata* (A) with a close-up of symptomatic, bronzed leaves (B), collapsed spear leaf (C), and necrotic inflorescence (D).

Figure 5. Symptomatic *Livistona chinensis* (A) with a close-up of symptomatic, bronzing leaf (B), and dying spear leaf (C).

Figure 6. Maximum likelihood tree generated from the 16S gene sequences for all known subgroups of the 16SrIV phytoplasmas and the A subgroup from all known phytoplasma taxonomic 16Sr group with *Acholeplasma palmae* as an outgroup. Values on branches indicate bootstrap support based on 1000 samples. Sequences from new hosts *Butia capitata*, *Cocos nucifera*, *Livistona chinensis* and *Carpentaria acuminata* are identical to the 16SrIV-D reference sequence.

Figure 7. Distribution of the 16SrIV-D phytoplasma in Florida by county around the time of discovery in 2008, five years post discovery, around 2013, and a decade post discovery, around late 2018.

354 **Table 1.** Phytoplasma isolates used for construction of the maximum likelihood tree to establish
355 relationship of isolates from new palm host identified in Florida.

16Sr Classification	Disease	GenBank Accession No.
I-A	Aster yellows witches' broom	NC_007716
II-A	Peanut witches' broom	L33765
III-A	Peach X-disease	JQ044392
IV-A	Lethal yellowing	AF498309.1
IV-B	Yucatan coconut lethal decline	U18753.2
IV-C	Tanzanian coconut lethal decline	X80117.1
IV-D	Lethal Bronzing	MG993140.1
IV-E	Dominican Republic coconut lethal decline	DQ631639.1
IV-F	Washingtonia Robusta lethal decline	EU241512.1
V-A	Elm yellows	AY197655
VI-A	Clover proliferation	AY390261
VII-A	Ash yellows	AF092209
VIII-A	Loofah Witches' broom	AF086621
IX-A	Pigeon pea witches' broom	AF248957
X-A	Apple proliferation	AJ542541
XI-A	Rice yellow dwarf	AB052873
XII-A	Stolbur	AF248959
XIII-A	Mexican periwinkle virescence	AF248960
XIV-A	Bermuda white leaf	AJ550984
XV-A	Hibiscus witches' broom	AF147708

XVI-A	Sugarcane yellow leaf syndrome	AY725228
XVII-A	Papaya bunchy top	AY725234
XVIII-A	American potato purple top wilt	DQ174122
XIX-A	Chestnut witches' broom	AB054986
XX-A	Rhamnus witches' broom	AJ583009
XXI-A	Pine phytoplasma	AJ310849
XXII-A	Lethal yellow disease Mozambique	KF751387
XXIII-A	Buckland valley grapevine yellows	AY083605
XXIV-A	Sorghum bunchy shoot	AF509322
XXV-A	Weeping tea witches' broom	AF521672
XXVI-A	Sugarcane phytoplasma D3T1	AJ539179
XXVII-A	Sugarcane phytoplasma D3T2	AJ539180
XXVIII-A	Derbid phytoplasma	AY744945
XXIX-A	Cassia witches' broom	EF666051
XXX-A	Salt Cedars witches' broom	FJ432664
XXXI-A	Soybean stunt	HQ225630
XXXII-A	Malaysian p. virescence	EU371934
XXXIII-A	Allocasuarina phytoplasma	AY135523

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358 **Table 2.** Palm species sampled and tested by qPCR at the Fort Lauderdale Research and

359 Education Center for the presence of the 16SrIV-D phytoplasma

Species	**No. Infected**	**No. Tested**	**Avg. Ct[1]**	**Avg. Qty.[2]**	**Avg. Tm[3]**
Adonidia merrillii	1	5	25.4±0.1	30,199±433	80.03±0.0
Carpentaria acuminata	1	4	24.2±0.1	156,777±100	80.03±0.0
Cocos nucifera	3	100	28.1±0.2	9,222±678	80.03±0.0
Phoenix reclinata	0	5	No Ct	0	60.1±0.0
Phoenix roebelinii	2	11	23.1±0.2	221,334±2,344	80.01±0.0
Roystonea regia	0	10	No Ct	0	60.2±0.0
Sabal etonia	0	1	No Ct	0	62.3±0.0
Sabal palmetto	10	21	20.9±0.4	856,799±20,998	80.01±0.0
Sabal mauritiioformis	0	5	No Ct	0	N/A
Serenoa repens	0	20	No Ct	0	N/A
Syagrus romanzoffiana	2	2	25.4±0.4	31,201±3,566	80.01±0.0
Wodeytia bifurcata	0	10	No Ct	0	N/A
(+) Control (16SrIV-D)	N/A	N/A	22.3±0.1	324,000±889	80.03±0.0
(+) Control (16SrIV-A)	N/A	N/A	24.6±0.1	115,444±431	80.54±0.0
Healthy Control	N/A	N/A	No Ct	0	60.1±0.0
Water Control	N/A	N/A	No Ct	0	60.2±0.0

360 [1]Ct=cycle threshold for measuring dye florescence relative to reference dye

361 [2]Qty.=estimated copy number per microliter

362 [3]Tm=melting temperature of the amplicon in degrees Celsius

363

364 **Table 3.** Samples testing positive out of total samples received for common palms surveyed by
365 stakeholders throughout the state of Florida.

County	***P. sylvestris***	***P. dactylifera***	***P. canariensis***	***Sa. palmetto***	***Sy. romanzoffiana***
Alachua	1/1	N/A	N/A	N/A	N/A
Bay	0/7	N/A	N/A	N/A	N/A
Broward	0/1	N/A	N/A	0/1	N/A
Charlotte	0/1	N/A	0/1	N/A	0/1
Collier*	6/8	0/1	N/A	N/A	N/A
Duval	2/2	N/A	N/A	N/A	N/A
Gadsden	0/1	N/A	N/A	N/A	N/A
Hardee	4/4	N/A	N/A	N/A	N/A
Hernando*	1/1	N/A	N/A	N/A	N/A
Highlands	4/4	N/A	N/A	N/A	N/A
Hillsborough	4/6	5/7	1/1	4/6	1/2
Indian River	21/31	2/2	0/1	N/A	N/A
Jefferson*	N/A	1/1	N/A	N/A	N/A
Lake	13/13	N/A	0/1	N/A	N/A
Lee	2/14	N/A	N/A	N/A	N/A
Manatee	3/3	7/11	0/1	N/A	N/A
Martin*	1/1	N/A	0/1	N/A	N/A
Miami-Dade*	3/6	0/3	0/4	N/A	N/A
Monroe*	2/3	N/A	0/2	N/A	N/A
Orange	7/17	0/3	4/5	0/1	1/1

Palm Beach	2/12	0/1	1/1	1/2	N/A
Polk	8/8	N/A	N/A	N/A	N/A
Sarasota	7/8	N/A	2/4	N/A	0/1
Seminole*	1/7	N/A	N/A	N/A	N/A
St. Johns*	6/20	N/A	N/A	N/A	N/A
St. Lucie	5/5	N/A	1/3	0/1	1/1
Sumter	2/2	N/A	0/1	1/1	N/A
Total	104/185	15/29	9/26	6/11	4/7

366 *New county record

367

368 **Table 4.** qPCR and melt curve results for all samples tested on behalf of stakeholders for
369 verification of phytoplasma identity.

Species	**N**	**Avg. Ct[1]**	**Avg. Qty[2]**	**Avg. Tm[3]**	**Tm Range**
B. capitata	2	20.3±0.2	921,710±602	80.3±0.0	N/A
L. chinensis	1	27.9±0.0	6,333±200	80.0±0.0	N/A
P. canariensis	9	21.1±3.1	309,277±9,001	80.1±0.3	79.4—80.4
P. dactylifera	15	24.1±5.6	64,435±2,332	80.1±0.1	79.4—80.1
P. sylvestris	104	22.5±9.2	180,430±3,444	80.1±0.3	79.4—80.4
Sa. palmetto	6	22.3±6.7	204,271±8,445	80.2±0.0	80.1—80.3
Sy. romanzoffiana	4	23.5±5.4	100,508±3,499	80.3±0.2	80.1—80.3
IV-D (+) control	N/A	23.4±0.2	117,428±1,222	80.1±0.3	80.0—80.4
IV-A (+) control	N/A	25.1±0.1	56,777±677	80.8±0.1	80.6—80.9
(-) water control	N/A	No Ct	0	60.1±0.1	58.7—64.9
(-) healthy control	N/A	No Ct	0	65.4±0.1	61.1—70.2

370 [1]Ct=cycle threshold for measuring dye florescence relative to reference dye

371 [2]Qty.=estimated copy number per microliter

372 [3]Tm=melting temperature of the amplicon in degrees Celsius

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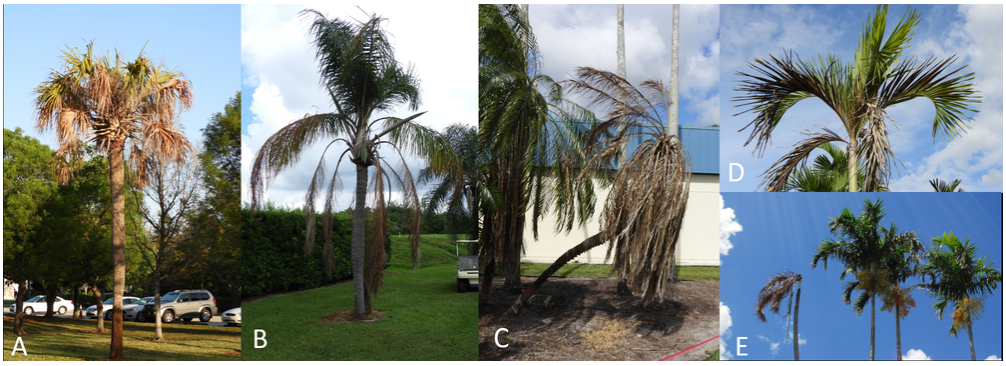

Symptomatic Sabal palmetto (A), Syagrus romanzoffiana (B), Phoenix roebelinii (C), Adonidia merrillii (D), and Carpentaria acuminata infected with the 16SrIV-D phytoplasma at the Fort Lauderdale Research and Education Center.

84x30mm (300 x 300 DPI)

Figure 2. Necrotic inflorescence (A) and collapsed spear leaf (B) from Cocos nucifera infected with the 16SrIV-D phytoplasma.

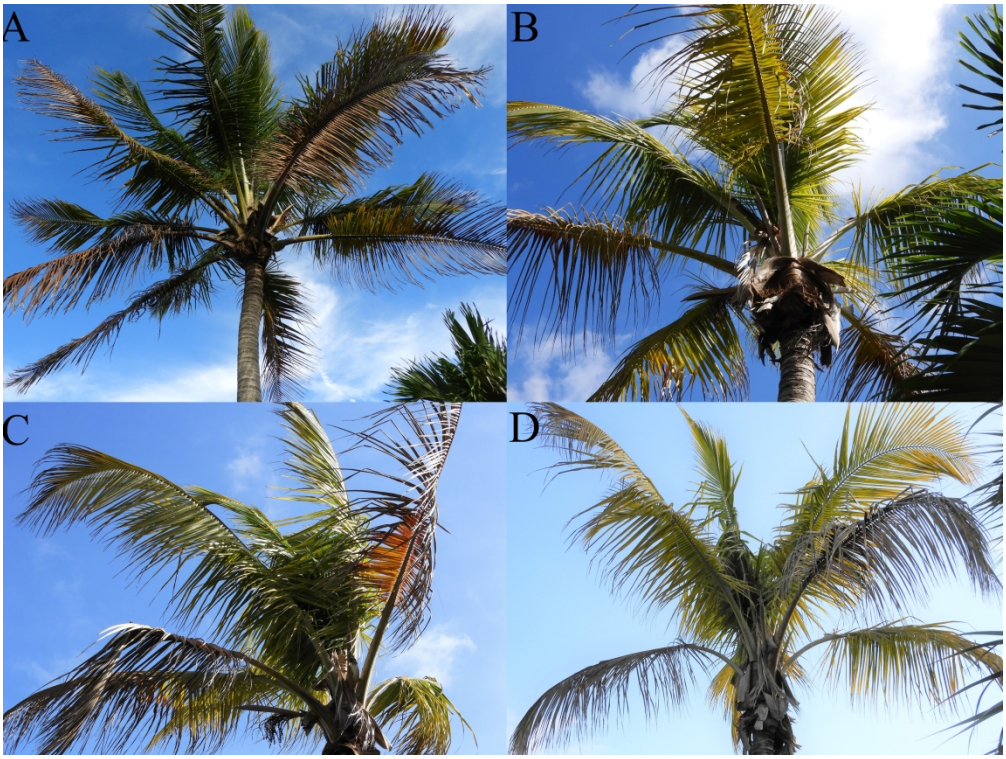

Figure 3. Symptom progression of lethal bronzing disease in Cocos nucifera Cnu-3200: September 2018 (A), November 2018 (B), January 2019 (C), February 2019 (D).

Figure 4. Symptomatic Butia capitata (A) with a close-up of symptomatic, bronzed leaves (B), collapsed spear leaf (C), and necrotic inflorescence (D).

Figure 5. Symptomatic Livistona chinensis (A) with a close-up of symptomatic, bronzing leaf (B), and dying spear leaf (C).

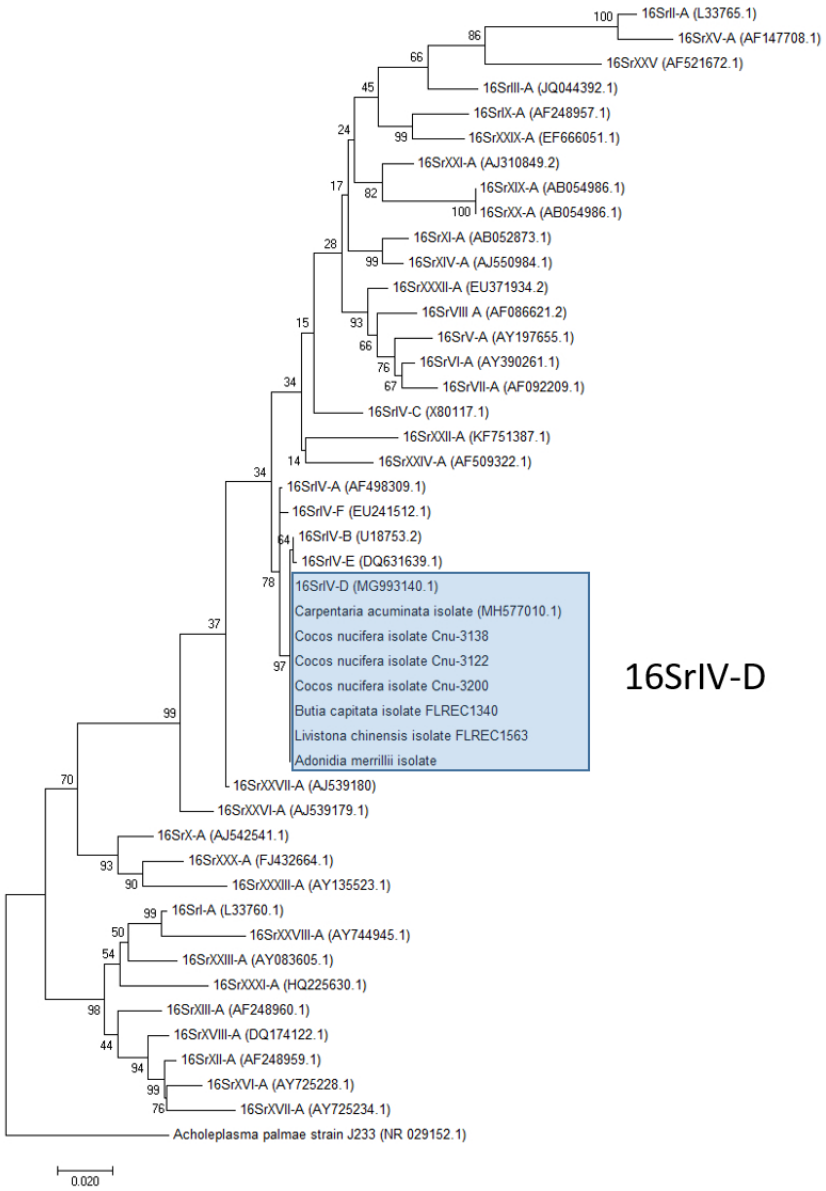

Figure 6. Maximum likelihood tree generated from the 16S gene sequences for all known subgroups of the 16SrIV phytoplasmas and the A subgroup from all known phytoplasma taxonomic 16Sr group with Acholeplasma palmae as an outgroup. Values on branches indicate bootstrap support based on 1000 samples. Sequences from new hosts Butia capitata, Cocos nucifera, Livistona chinensis and Carpentaria acuminata are identical to the 16SrIV-D reference sequence.

132x190mm (150 x 150 DPI)

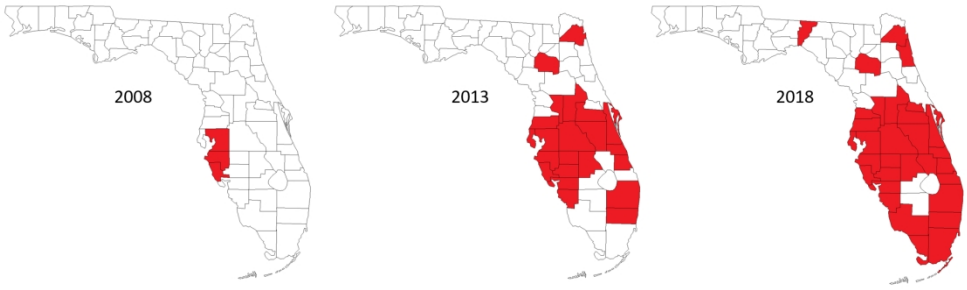

Figure 7. Distribution of the 16SrIV-D phytoplasma in Florida by county around the time of discovery in 2008, five years post discovery, around 2013, and a decade post discovery, around late 2018.

338x105mm (150 x 150 DPI)