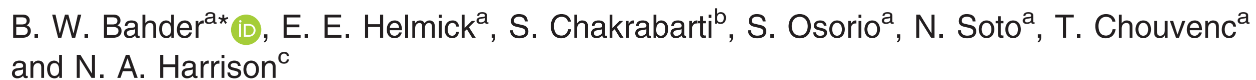

Disease progression of a lethal decline caused by the 16SrIV-D phytoplasma in Florida palms

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Recently, a new phytoplasma was discovered in Hillsborough County in the state of Florida, USA. This phytoplasma belongs to the 16SrIV taxonomic group and is classified as subgroup D. It is the causal agent of lethal bronzing disease (LBD) of palm. Since the discovery of LBD in 2006, the disease has spread throughout much of the state. In 2014 and 2015, stands of cabbage palm and queen palms that had been present at the University of Florida's Fort Lauderdale Research and Education Center in Davie, FL began showing symptoms of LBD. After confirming the presence of the LBD phytoplasma in initially infected palms by nested PCR and RFLP analysis, all palms were systematically sampled over the period of 1 year to monitor and quantify disease spread. A total of 30 cabbage palms were tested monthly by qPCR, with five testing positive on the first sample date. By the end of the study period, 16 cabbage palms had died from the infection. A total of 16 queen palms were surveyed, with three palms initially testing positive. By the end of the study, four queen palms had tested positive and died from the infection. To the authors' knowledge, this study is the first to document and quantify spread of palm-infecting phytoplasmas. This data provides important insights into the ecology of palm-infecting phytoplasmas and highlights the impact that the movement of infective insects can pose to established stands of palms.

Keywords: disease, lethal bronzing, palm, phytoplasma, vector

Introduction

In 2006, a new phytoplasma disease was discovered in Hillsborough County, Florida, USA and was found to be infecting palms in the genus *Phoenix* as well as the queen palm (*Syagrus romanzoffiana*) (Harrison *et al.*, 2008). The disease was determined to be the same 16SrIV-D phytoplasma that also caused lethal decline of *Phoenix* spp. in Texas and was called Texas phoenix palm decline (TPPD). In 2008, this phytoplasma was also isolated from declining cabbage palms (*Sabal palmetto*) in Florida (Harrison *et al.*, 2009). Due to an expansion of the known host range, as well as its presence in Florida and in Mexico (Lara *et al.*, 2017), the name TPPD has been dropped and the disease is currently being referred to as lethal bronzing disease (LBD; Bahder *et al.*, 2018). This name was selected due to the bronze colouration present in plants with symptoms that are susceptible to the phytoplasma. However, the bronze colour on affected leaves is not the only symptom, and can sometimes be misleading due to similar colouration from ganoderma butt rot (Elliott & Broschat, 2001). Diagnosis of the disease based on symptoms typically requires a combination of symptoms that are observed over time. When fruit is present, the first symptom is premature fruit drop and inflorescence necrosis, followed by bronzing of the oldest layer of leaves that progresses upwards, impacting younger leaves. At approximately 50% canopy discolouration, the spear leaf typically collapses, resulting in death of the palm (Harrison & Elliott, 2016).

Since its discovery, LBD has spread across the state and is confirmed from 22 counties (Harrison & Elliott, 2016) and recently confirmed from two more counties, Okeechobee and Martin (B. W. Bahder, unpublished data). Currently, little is known about the biology of this phytoplasma. Due to the rapid spread across the state of Florida and the impact that the disease has on ornamental and naturally occurring palms, recent advances have focused on optimizing detection of the phytoplasma to increase the speed and sensitivity of diagnostic assays (Bahder *et al.*, 2017). While these tools can help to implement management strategies more rapidly due to a faster turnaround of samples sent for diagnosis, they will also facilitate detailed studies on the biology and epidemiology of the disease. Important aspects of the disease that need to be addressed are the identification of the vector, alternative host plants for both the phytoplasma and vector, the rate of spread of the disease, and the impact this disease may have on natural habitats.

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The primary means of widespread movement of plant pathogens is human-mediated and occurs when infected plant material is moved and planted in new locations (Krenz *et al.*, 2014; Sudarshana *et al.*, 2015). Once established in a given location, secondary spread can then occur by movement of the pathogen via an insect vector already present in the region where the pathogen was introduced. Alternatively, the introduction of insects that become established and classified as invasive, causing economic damage to various crops, is well documented (Pimentel *et al.*, 2005) and can also contribute to the spread of a pathogen in an adventive region. Currently, it is unclear how LBD was introduced into the state of Florida. Possible means of introduction include: (i) human-mediated movement of infected palms into the region; (ii) introduction of infective individuals of the vector species; (iii) movement of infected alternative phytoplasma plant hosts; or (iv) movement of phytoplasma via the vector through natural causes such as hurricanes (Anderson *et al.*, 2010; Andraca-Gómez *et al.*, 2015). It is also unclear how much of the disease spread is occurring as a result of palm-to-palm transmission, via the vector, rather than transmission from a reservoir host plant. In the case of lethal yellowing (LY), another lethal decline of palms caused by phytoplasmas that primarily impacts coconut palms (*Cocos nucifera*), various weed species including *Emilia fosbergii*, *Synedrella nodiflora* and *Vernonia cinerea* have been shown to harbour the LY phytoplasma (Brown *et al.*, 2008a,b), and could be contributing to disease epidemiology. Because of this, the transmission of a phytoplasma implicated with palm lethal declines from a weed into a susceptible palm host could be contributing to the current spread of LBD in Florida.

In 2014, LBD was detected for the first time at the entrance of the University of Florida's Fort Lauderdale Research and Education Center (FLREC) in Davie, FL. At the entrance of FLREC was a stand of 30 *S. palmetto* that had been present since at least the mid-1970s and had been healthy for the entire period until 2014 (N. A. Harrison, personal observation). In 2014, construction work on a water treatment facility onsite was completed and new sod (composed of St Augustine grass, *Stenotaphrum secundatum*) was laid in the disturbed area. Approximately 5 months after the sod had been laid, October/November of 2014, the first *S. palmetto* trees began showing symptoms. Several of the *S. palmetto* showing declining symptoms were tested for phytoplasma by nested PCR and RFLP in November of 2014 and tested positive for the 16SrIV-D phytoplasma. In February 2015, several *S. romanzoffiana* also began exhibiting symptoms associated with phytoplasma infection. After confirming the presence of the 16SrIV-D phytoplasma in *S. palmetto* palms showing symptoms, a systematic sampling strategy to monitor the progression of the disease in both the *S. palmetto* and *S. romanzoffiana* stands was established.

The primary objective of this study was to monitor and quantify the spread and titre increase of the LBD phytoplasma in palms where an infective vector was introduced, resulting in a localized outbreak. This study will provide valuable data on the pattern and behaviour of this disease in an urban landscape, how quickly it can spread, and the impact it can have on local palm populations, that can contribute to developing management programmes.

Materials and methods

Study site and palm species

Disease progression was monitored in two different palm species at FLREC in Davie, FL, USA (26°05′02.42″N, 80°14′14.75″W). The two species included in this study were *S. palmetto* and *S. romanzoffiana*. The stand of *S. palmetto* contained 30 individuals (labelled RECSP-1 to RECSP-30) and was located on both the north and south sides of the east entrance gates to FLREC (Fig. 1). The *S. palmetto* stand was split into two different clusters, one north of the entrance gate and one south of the entrance gate (Fig. 1). The stand of *S. romanzoffiana* contained a total of 16 individuals (labelled QP1 to QP16), where seven of the queen palms (QP10–QP16) were located in a cluster just north of the northern *S. palmetto* cluster and the remaining nine (QP1–QP9) palms were located further north of the cluster along the east fence line (Fig. 1). *Syagrus romanzoffiana* samples QP1–QP9 were separated by 6 m in a single row and had been transplanted to the current location from another plot on the FLREC property in 2001 (Elliott & Broschat, 2001). At the time of relocation, the age of the *S. romanzoffiana* palms was estimated to be 20–25 years old (Elliott & Broschat, 2001). *Syagrus romanzoffiana* palms QP10–QP16 were planted in a double row with approximately 2–3 m between palms and between rows. For both species, all individuals were approximately the same age and height. The *S. palmetto* palms are not original to the front entrance of the FLREC; however, they have been onsite and established in their current location since the early 1970s (N. A. Harrison, personal observation).

Sample collection

After the first appearance of symptoms and confirmation by RFLP analysis, each palm in the *S. palmetto* stand was sampled on a monthly basis for 1 year, from December 2014 to November 2015, regardless of the presence or absence of visible symptoms. Initial sampling data for December 2014 included two sample sets, one at the beginning and one at the end of the month. In total, 360 tissue samples were taken from 30 palms over the 12-month period.

For the *S. romanzoffiana* stand, four palms (QP1, QP2, QP4 and QP9) were tested on the first date due to visual presence of symptoms. Palms QP1 and QP4 were cut down and the palm heart tissues stored for future analyses. The remaining 14 palms were tested each month from February to November 2015 (10 months), with the exception of QP2 and QP9, for which data was not collected for the second sampling date, but for all dates thereafter. In total, there were 121 tissue samples taken from 14 palms over the 10-month sample period.

For each sample, 3–6 g of trunk tissue was extracted from each palm at each sample date as per the protocol of Harrison *et al.* (2013). Samples were taken at various heights on the trunk, ranging from 0.3 to 1.0 m above the root base of the tree, and were also taken at different areas around the

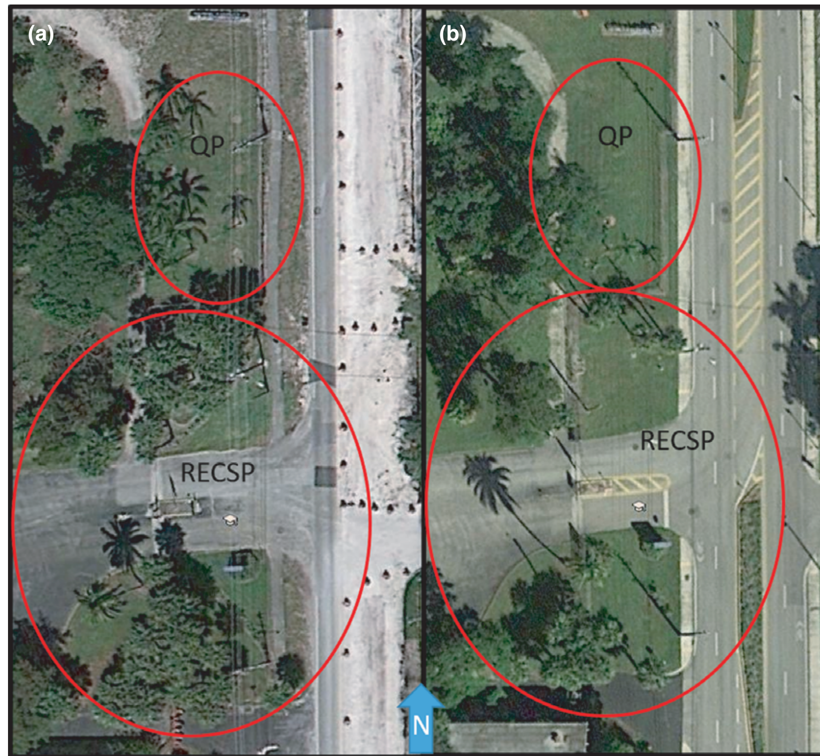

Figure 1 Map showing location of the outbreak of lethal bronzing disease at FLREC before its introduction (a) and current view of the study site (b). [Colour figure can be viewed at wileyonlinelibrary.com]

circumference of the trunk. All trunk tissues were stored at 4 °C until DNA extractions were completed.

Visual symptoms of each palm were documented for each sampling date, including the presence/absence of a spear leaf, fruit (premature drop), inflorescence and canopy condition. Canopy condition was included because of observed (N. A. Harrison & E. Helmick, personal observation) changes in fullness of the canopy ('drooping', separation), stunted growth of newest leaves and discolouration of lower leaves (not due to natural die-off of oldest leaves). Additionally, all palms that did not die during the sampling period were visually inspected for symptoms in April 2016. The surviving palms were tested twice more, once in November 2016 and again in February 2018, after the last sample date, to ensure that surviving symptomless palms were still negative for the phytoplasma.

DNA extraction, purification and quantification

Total genomic DNA (gDNA) was extracted from 3 g of trunk tissue following the protocol by Harrison *et al.* (2013). Depending on the size of the resulting gDNA pellet, 100–300 μL of sterile TE buffer, pH 8.0, was used for resuspension. A 50 μL aliquot of the resuspended gDNA was cleaned using the Wizard DNA Clean-Up system (Promega Corp.) as per the manufacturer's protocol, and eluted in 40 μL of sterile dH_2O heated to 65 °C. One microlitre each of the cleaned and crude gDNA samples was quantified with a Qubit 2.0 fluorometer (ThermoFisher Scientific), using the dsDNA BR Assay kit, as per the manufacturer's protocol. Samples were diluted using sterile dH_2O to 50 ng μL^{-1} for use in the primary and nested PCR assays, and to 25 ng μL^{-1} for use in the qPCR assay. All crude and cleaned gDNA were stored at 4 °C.

PCR assays and sequence identification

Amplification of phytoplasma from gDNA was conducted using a standard nested PCR assay. Primary amplification of gDNA was performed using forward primer P1m (Harrison *et al.*, 2008) and reverse primer LY16S-23R (Harrison *et al.*, 2002), followed by a nested PCR assay using forward primer LY16Sf2 and reverse primer LY16-23Sr2 (Harrison *et al.*, 2008). Each primary PCR contained 50 ng gDNA as DNA template, 1 U *Taq* DNA polymerase (New England Biolabs), 0.125 mM of each dNTP, 50 ng each of forward and reverse primer, 1.5 mM $MgCl_2$ in a standard PCR buffer (Innis & Gelfand, 1990) and sterile dH_2O to a final volume of 50 μL. The primary PCR cycling conditions were as follows: initial denaturation 95 °C for 60 s; 34 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 60 s, extension at 72 °C for 120 s; and a final extension of 72 °C for 8 min.

For the nested PCR assay, using primers LY16Sf2/LY16-23Sr2, the primary P1m/LY16S-23R products were diluted 1:10 and 2 μL of this dilution was used as the nested PCR template using the same PCR components and ratios as for the primary PCR. The nested PCR cycling conditions were as follows: initial denaturation 94 °C for 120 s; 34 cycles of denaturation at 94 °C for 60 s, annealing at 60 °C for 120 s, extension at 72 °C for 3 min; and a final extension of 72 °C for 7 min.

For each PCR assay, 10 μL of the resulting PCR amplifications were electrophoresed on a 1% agarose gel, stained with

ethidium bromide and visualized using UV transillumination. Known positive lethal yellowing (LYDA) and Texas phoenix palm decline (Sab1) samples were used as positive controls, and healthy *S. palmetto* DNA was used as a negative control, along with sterile dH_2O as a nontemplate negative control for the PCR assays.

Nine *S. palmetto* and two *S. romanzoffiana* palm samples were amplified in a nested PCR assay (as described above) in triplicate, combined and cleaned using ExoSAP-IT PCR Product Clean-up Reagent (ThermoFisher Scientific) as per the manufacturer's protocol. The cleaned PCR products were sent to the University of California, Davis, DNA Sequencing Laboratory for Sanger sequencing. Resulting sequences were assembled, visually inspected and corrected for sequencing errors using DNA BASER v. 4.36 (Heracle Biosoft), sequences were aligned using MEGA 7 (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).

Restriction fragment length polymorphism (RFLP) verification

Restriction fragment length polymorphism verification of positive nested PCR products (LY16Sf2/LY16-23Sr2) for the *S. palmetto* and *S. romanzoffiana* palms to be tested for phytoplasma via nested PCR assay was conducted using the restriction enzyme *Hha*I (New England Biolabs) which had previously been shown to unambiguously distinguish between 16SrIV-A and 16SrIV-D phytoplasma (Harrison *et al.*, 2008).

Plasmid standard preparation

Standard PCR, using primers LY16S-LSF/LY16-LSR (Córdova *et al.*, 2014), was used for amplification of gDNA from sample RECSP-12, which had tested positive for LBD. Each PCR contained 5× GoTaq Flexi Buffer, 25 mM $MgCl_2$, 10 mM dNTPs, 10 mM each primer, 10% PVP-40 and 2.5 U GoTaq Flexi DNA polymerase, and sterile dH_2O to a final volume of 25 μL. Thermocycling parameters were as follows: 94 °C for 1 min initial denaturation; 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s; and a final extension of 72 °C for 5 min. Three microlitres of each product was electrophoresed on a 1.5% agarose gel stained with GelRed nucleic acid gel stain (Biotium) and visualized using UV transillumination.

PCR products were cloned using a TOPO TA Cloning kit into vector pCR2.1-TOPO (Invitrogen) as per the manufacturer's protocol. The cloning constructs were transformed into TOPO One Shot Chemically Competent *Escherichia coli* cells and plated on LB plates containing 50 μg mL^{-1} kanamycin. Plates were incubated overnight at 37 °C and transformed colonies were chosen for colony PCR using primers LY16S-LSF/LY16-LSR to verify that they contained the correct insert. Clones with the insert of the correct size were incubated on a shaker overnight in 20 mL LB broth with 50 μg mL^{-1} kanamycin. Plasmids were extracted using a QIAprep Spin Miniprep kit (QIAGEN) as per the manufacturer's protocol. Plasmid concentrations were quantified using either a NanoDrop spectrophotometer (ThermoFisher Scientific) or a Qubit 2.0 fluorometer using the Qubit dsDNA BR Assay kit (Invitrogen). Ten-fold serial dilutions were created, 10^8–10^3, for use as standards in the qPCR assay.

qPCR assay parameters

All qPCRs were performed on a StepOnePlus Real-Time PCR system (Applied Biosystems) and consisted of 10 μL of TaqMan Universal Master Mix II, with UNG, 10 μM of each LY16S-LSF and LY16-LSR primer, 10 μM of LY16S probe, 10% PVP, 25 ng gDNA (1 μL), and sterile dH_2O to a final volume of 20 μL. Córdova *et al.* (2014) designed both primers and probe used in this assay. Reactions were subject to the following cycling parameters: 95 °C for 10 min; then 35 cycles of 95 °C for 30 s, 60 °C for 60 s, and 72 °C for 30 s. Data was collected and analysed using the STEPONE v. 2.3 software (Life Technologies) supplied with the StepOnePlus Real-Time PCR system.

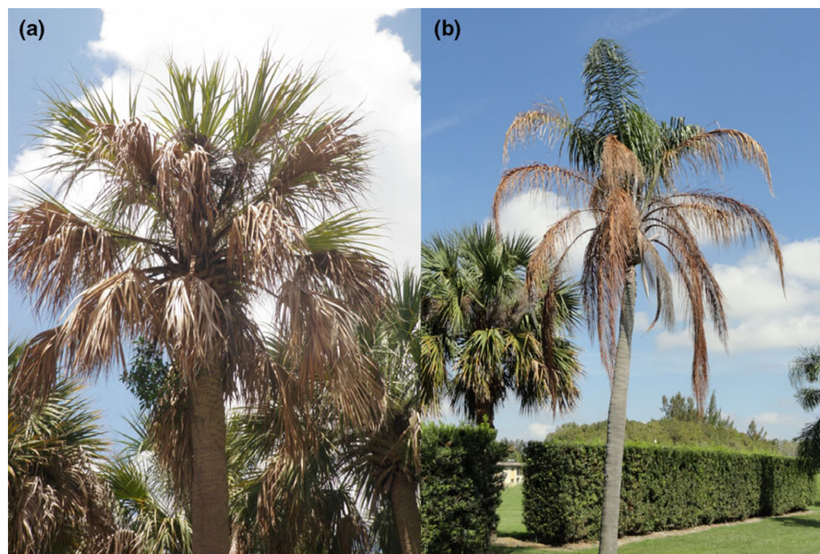

Figure 2 *Sabal palmetto* with 75% bronzing of canopy, necrotic inflorescences, and lacking spear leaf (a) and *Syagrus romanzoffiana* with 75% bronzing of canopy, necrotic inflorescences and lacking spear leaf (b). [Colour figure can be viewed at wileyonlinelibrary.com]

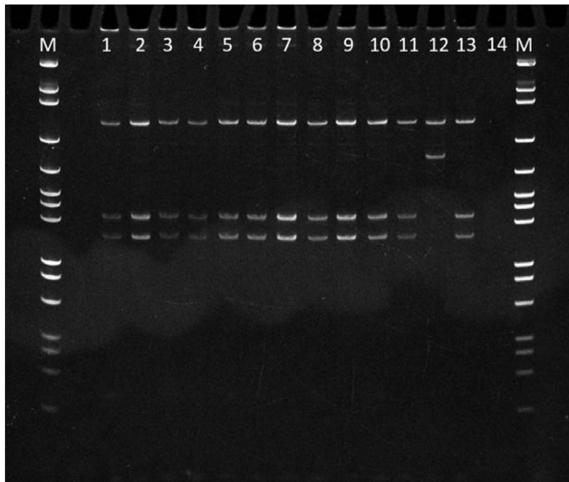

Figure 3 RFLP analysis using *Hha*I for verification of phytoplasma identity in cabbage palms (RECSP) and queen palms (QP) at study location: M, molecular weight marker pGEM; 1, RECSP-3; 2, RECSP-7; 3, RECSP-13; 4, RECSP-14; 5, RECSP-19; 6, RECSP-20; 7, RECSP-22; 8, RECSP-30; 9, QP3; 10, QP11; 11, QP16; 12, positive control (LY-16SrIV-A); 13, positive control (LBD-16SrIV-D); 14, water control.

Results

Disease introduction and verification

The first symptoms that appeared were on *S. palmetto* and were noticeable in the autumn of 2014, and the first symptoms in *S. romanzoffiana* appeared in early 2015 (Fig. 2). Three *S. palmetto* palms, RECSP-7, RECSP-19 and RECSP-30, were the first to display symptoms and were initially tested for phytoplasma using the standard nested PCR assay, resulting in a 1.6 kb rDNA product for all three palms. The PCR products were then subjected to RFLP analysis using enzyme *Hha*I to distinguish between LY and LBD phytoplasmas. The *Hha*I digest indicated that all three palms were infected with the phytoplasma associated with 16SrIV-D (Fig. 3).

Four *S. romanzoffiana* palms (QP1, QP2, QP4 and QP9) initially displaying symptoms were tested by nested PCR assay and all with the exception of QP9 yielded a 1.6 kb rDNA product, indicating that they were positive for phytoplasma, and following RFLP analysis identified as 16SrIV-D (data not shown).

Sanger sequencing of the 16S rDNA nested PCR products from 11 palms identifying as 16SrIV-D from RFLP analysis (Fig. 2), nine *S. palmetto* and two *S. romanzoffiana* palms, produced an average sequence length of 1540 bp. For initial disease verification, all sequences were aligned in MEGA 7 to a known positive 16SrIV-D *S. palmetto* sequence (GenBank accession no. FJ217386; Harrison *et al.*, 2009). All sequences were compared to sequences within GenBank and all were a 100% nucleotide match to the isolates from Florida (Sabal1, HQ613895.1), Texas (AF434989.1), Louisiana (MF491872.1) and Mexico (KY508691.2). The sequence data for the 11 samples was submitted to GenBank (accession numbers MG993138–MG993148).

Disease progression in *S. palmetto*

On the first sample date, all 30 *S. palmetto* plants were tested by qPCR, with five testing positive and the remaining palms testing negative (Table 1). The five palms that were initially infected subsequently tested positive throughout the sampling period and demonstrated a steady increase in phytoplasma titre over the course of the study (Table 1). On the final sample date, 14 palms tested positive for the phytoplasma, with samples RECSP-4, RECSP-9, RECSP-11 and RECSP-12 testing positive for the first time, with a low titre being detected (Table 1). These new infections occurred almost 1 year after the first palms tested positive. In May 2017, a total of 16 palms had died as a result of the infection. All palms that had tested positive for phytoplasma were dead as of May 2017 with the exception of one palm. The palm RECSP-1 was dead as of May 2017 but never tested positive for phytoplasma or exhibited symptoms during the sampling period (Table 1). Other than RECSP-1, all palms that tested negative and appeared healthy on the last sample date were symptomless and appeared healthy as of May 2017 (Table 1). In February 2018, the remaining *S. palmetto* palms were sampled and tested, with two of the remaining 14 palms testing positive (Table 2).

Disease progression in *S. romanzoffiana*

Of the four palms initially tested for phytoplasma, palms QP1, QP2 and QP4 were positive for phytoplasma infection (Table 3). Queen palms QP1 and QP4 were cut down and removed after the first sampling date. *Syagrus romanzoffiana* palm QP2 continued to test positive for phytoplasma throughout the 10 month sampling period (Table 3), even though the palm had completely died and the top had fallen off by June 2015. In subsequent sampling dates, QP11, QP3 and QP16 tested positive in April, June and October 2015, respectively (Table 3). Palm QP9, although displaying general disease symptoms, did not test positive for phytoplasma throughout the sampling period; the discolouration and poor health of the palm was probably due to nutritional deficiency and/or fungal disease. In total, six out of 16 *S. romanzoffiana* palms died from the phytoplasma disease within the 10-month sampling period. Subsequently, QP15 died from confirmed ganoderma butt rot (conch visible at base of tree) and QP7 also died of symptoms that were consistent with ganoderma.

Discussion

The use of qPCR to study phytoplasmas in palms (16SrIV) is a relatively new area of research. Currently the utility of qPCR has been demonstrated for the detection of subgroups A, D and E (Córdova *et al.*, 2014), the detection and differentiation of subgroups A and D (Bahder *et al.*,

Table 1 qPCR results for 30 *Sabal palmetto* palms over a 1-year period following the introduction of the 16SrIV-D phytoplasma, overlaid with symptom data.

	10 December 2014		16 February 2015		18 April 2015		15 June 2015		20 November 2015	
Sample	C_t	Qty[a]	C_t	Qty	C_t	Qty	C_t	Qty	C_t	Qty
RECSP-1	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
RECSP-2	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
RECSP-3	No C_t	0	No C_t	0	No C_t	0	16.1	1 008 964	13.8	2 860 653
RECSP-4	No C_t	0	No C_t	0	No C_t	0	No C_t	0	30.4	1754
RECSP-5	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
RECSP-6	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
RECSP-7	19.2	238 096	15.3	1 932 803	16.4	5 381 685	16.1	1 047 116	13.0	3 962 905
RECSP-8	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
RECSP-9	No C_t	0	No C_t	0	No C_t	0	No C_t	0	33.0	565
RECSP-10	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
RECSP-11	No C_t	0	No C_t	0	No C_t	0	No C_t	0	31.5	1108
RECSP-12	No C_t	0	No C_t	0	No C_t	0	No C_t	0	31.9	919
RECSP-13	No C_t	0	No C_t	0	23.3	224 690	17.7	492 145	15.1	1 600 750
RECSP-14	No C_t	0	No C_t	0	29.3	5065	20.0	179 459	14.6	1 962 172
RECSP-15	No C_t	0	30.7	1436	28.0	11 492	20.1	168 494	16.3	946 097
RECSP-16	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
RECSP-17	15.0	2 145 460	16.1	1 333 947	14.7	2 549 827	16.1	1 024 797	12.7	4 619 835
RECSP-18	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
RECSP-19	15.0	2 203 865	13.3	4 979 990	13.2	3 685 186	17.2	636 906	13.2	3 685 186
RECSP-20	28.5	1193	23.2	47 330	18.9	3 460 169	17.9	447 203	13.6	3 125 119
RECSP-21	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
RECSP-22	No C_t	0	34.1	289	31.5	1291	21.0	112 749	16.4	880 754
RECSP-23	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
RECSP-24	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
RECSP-25	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
RECSP-26	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
RECSP-27	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
RECSP-28	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
RECSP-29	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
RECSP-30	18.3	345 397	14.9	2 257 982	17.1	2 549 726	19.0	274 406	16.9	718 253
Control (+)	16.2	956 788	17.1	677 999	16.0	1 001 237	16.5	871 222	16.9	745 555
Healthy (−)	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
Water (−)	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0

Unshaded, symptomless; shaded, symptoms present.
[a]Qty, copies μL^{-1}.

2017), and the detection of the phytoplasma in embryos of infected palms (Oropeza *et al.*, 2017). This study is the first to systematically monitor and quantify infection and disease progression of palm-infecting phytoplasmas by both nested PCR and qPCR in palms that were probably infected by the introduction of an infective insect vector, or due to the introduction of a reservoir host that was infected, allowing the pathogen to subsequently jump into volunteer palms.

The palms impacted by the disease at FLREC were present and healthy since the 1970s (N. A. Harrison, personal observation). Additionally, no other palms in adjacent properties to FLREC showed symptoms prior to the study period. Based on the timeline and events observed, it seems reasonable that the sod brought to the research centre contained an insect vector that was harbouring the phytoplasma, or the grass itself was infected with phytoplasma. The initial transmission appears to have occurred on five palms in the *S. palmetto* stand and three palms in the *S. romanzoffiana* stand. At least 2 months passed between the first detection of phytoplasmas in *S. palmetto* palms and the emergence of newly infected palms. It is likely that the initially infected palms were infected prior to the first sampling date, making the actual time between the first infections and secondary infections at least 3 months and possibly more. Additionally, four *S. palmetto* palms began testing positive approximately 1 year after first infection, with one palm testing negative throughout the study period but was dead approximately 1 year after the study period. Based on this timeline, it is likely that secondary transmission occurred from the first five *S. palmetto* infected to adjacent palms. Interestingly, the stand of *S. palmetto* was impacted more severely than the stand of *S. romanzoffiana*, with just over half of the *S. palmetto* palms dying, while 25% of the *S. romanzoffiana* palms were infected. This difference in infection could be due to either a higher level of resistance to the phytoplasma in *S. romanzoffiana*

Table 2 qPCR results from surviving cabbage palms after termination of monthly sampling during study period.

Sample	May 2017 C_t	May 2017 Qty[a]	February 2018 C_t	February 2018 Qty
RECSP-2	No C_t	0	No C_t	0
RECSP-5	No C_t	0	No C_t	0
RECSP-6	No C_t	0	22.9	11 802
RECSP-8	No C_t	0	No C_t	0
RECSP-10	No C_t	0	No C_t	0
RECSP-16	No C_t	0	No C_t	0
RECSP-18	No C_t	0	No C_t	0
RECSP-21	No C_t	0	No C_t	0
RECSP-23	No C_t	0	No C_t	0
RECSP-25	No C_t	0	No C_t	0
RECSP-26	No C_t	0	19.5	112 647
RECSP-27	No C_t	0	No C_t	0
RECSP-28	No C_t	0	No C_t	0
RECSP-29	No C_t	0	No C_t	0
Control (+)	19.2	275 666	21.0	39 001
Healthy (−)	No C_t	0	No C_t	0
Water (−)	No C_t	0	No C_t	0

Unshaded, symptomless; shaded, symptoms present.
[a]Qty, copies μL^{-1}.

or due to feeding preferences of the vector, where *S. palmetto* is a preferred food source. Finally, the appearance of two new infections 2 years after the initial outbreak ended and occurring on opposite ends of the study site are interesting. These new infections could be the result of transmission from a reservoir because it was approximately 18 months since the last leaves with symptoms were present. While it is possible there could have been an 18 month latent period in these plants, it seems likely that the pathogen could be present in a grass or herbaceous weed that has served as a reservoir for infection. The presence of LY phytoplasmas in weeds is well documented in the Caribbean (Brown *et al.*, 2008a,b; Brown & McLaughlin, 2011) so it is not unreasonable to suspect this closely related strain can also persist in symptomless hosts. This aspect is crucial for management of the disease and needs to be investigated in the future.

This study is also, to the authors' knowledge, the first to demonstrate detection of 16SrIV phytoplasmas in *S. palmetto* palms during the latent stage by qPCR and monitor the increase of titre as symptoms begin to appear. These findings are consistent with those by Narváez *et al.* (2017) where phytoplasma was detectable in *Pritchardia pacifica* 1 month prior to symptom development. This is important because it demonstrates that qPCR is sensitive enough to detect phytoplasmas prior to symptom development in *S. palmetto* and *S. romanzoffiana*, and may also do so in other species of palms. Because qPCR is a more sensitive technique compared to traditional PCR, it was initially expected that it may be possible to detect infections in palms early, possibly before symptoms developed. With the exception of palms that demonstrated symptoms from the beginning of the study and samples RECSP-11, RECSP-13 and RECSP-14, all samples that developed symptoms tested positive by qPCR before the symptom onset. However, it was unexpected to find some palms with high titres of phytoplasma, equivalent to those with symptoms, that had not

Table 3 qPCR results for 16 *Syagrus romanzoffiana* palms over a 1-year period following the introduction of the 16SrIV-D phytoplasma, overlaid with symptom data.

Sample	16 February 2015 C_t	16 February 2015 Qty[a]	18 April 2015 C_t	18 April 2015 Qty	18 May 2015 C_t	18 May 2015 Qty	15 June 2015 C_t	15 June 2015 Qty	22 October 2015 C_t	22 October 2015 Qty
QP1[b]	22.7	38 902	—	—	—	—	—	—	—	—
QP2	n/a	n/a	n/a	n/a	17.9	560 113	18.0	530 505	24.1	9340
QP3	No C_t	0	No C_t	0	No C_t	0	30.8	411	16.7	621 557
QP4[b]	18.7	371 409	—	—	—	—	—	—	—	—
QP5	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
QP6	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
QP7	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
QP8	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
QP9	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
QP10	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
QP11	n/a	n/a	20.5	135 621	23.2	29 594	20.3	145 754	18.4	232 865
QP12	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
QP13	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
QP14	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
QP15	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
QP16	No C_t	0	No C_t	0	No C_t	0	No C_t	0	20.7	63 193
Control (+)	18.2	654 888	18.1	677 999	16.0	1 701 237	16.5	1 111 222	16.9	945 255
Healthy (−)	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0
Water (−)	No C_t	0	No C_t	0	No C_t	0	No C_t	0	No C_t	0

Unshaded, symptomless; shaded, symptoms present; n/a, qPCR results not available.
[a]Qty, copies μL^{-1}.
[b]Palm was removed after 16 February 2015 sampling.

yet developed symptoms. The presence of symptoms in qPCR negative palms that tested positive on the next sample date is probably due to sampling error, where the region sampled did not have phytoplasma, a result of the uneven distribution of phytoplasma throughout the palm (Harrison & Elliott, 2016).

This provides a benefit to stakeholders interested in sampling palms adjacent to palms with symptoms that could be at risk, or are at the beginning of the disease cycle. Confirming the presence of phytoplasma in palms prior to symptom development justifies not only treatment of symptomless palms with oxytetracycline (OTC) but also a proactive sampling regime that does not focus solely on palms with symptoms, but also healthy-appearing palms that are in the immediate vicinity. Currently, the period of time that a palm is infective is unknown. If the vector can acquire the phytoplasma prior to symptom development, then being able to detect the phytoplasma in palms soon after infection where they have not yet developed symptoms will allow stakeholders to either treat symptomless palms with OTC or remove them. This could reduce the time that infective palms are present in the environment and potentially reduce disease spread by implementing management practices sooner. Typically, palms are removed after the spear leaf has collapsed and it is probable that new infections have already occurred, making early detection and management crucial. While a relatively small number of individuals was studied in a single location, this information is valuable to urban landscapers and nursery owners because it highlights the damage that can occur if outbreaks are not properly managed. Conducting this type of research in the urban setting or nursery is not possible due to rapid removal of infected palms by property owners, but it serves as a valuable data set to demonstrate that if rapid management decisions are not implemented, further spread and losses are possible.

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References

Anderson KL, Deveson TE, Sallam N, Congdon BC, 2010. Wind-assisted migration potential of the island sugarcane planthopper *Eumetopina flavipes* (Hemiptera: Delphacidae): implications for managing incursions across an Australian quarantine frontline. *Journal of Applied Ecology* **47**, 1310–9.

Andraca-Gómez G, Ordano M, Boege K *et al.*, 2015. A potential invasion route of *Cactoblastis cactorum* within the Caribbean region matches historical hurricane trajectories. *Biological Invasions* **17**, 1397.

Bahder BW, Helmick EE, Harrison NA, 2017. Detecting and differentiating phytoplasmas belonging to subgroups 16SrIV-A and 16SrIV-D associated with lethal declines of palms in Florida using qPCR and high-resolution melt analysis (HRMA). *Plant Disease* **101**, 1449–54.

Bahder BW, Helmick EE, Mou D, Harrison NA, Davis R, 2018. Digital PCR technology for detection of palm infecting phytoplasmas belonging to group 16SrIV that occur in Florida. *Plant Disease* **102**, 1008–14.

Brown SE, McLaughlin WA, 2011. Identification of lethal yellowing group (16SrIV) of phytoplasmas in the weeds *Stachytarpheta jamaicensis*, *Macroptilium lathyroides* and *Cleome rutidosperma* in Jamaica. *Phytopathogenic Mollicutes* **1**, 27–34.

Brown SE, Been BO, McLaughlin WA, 2008a. First report of the presence of the lethal yellowing group (16SrIV) of phytoplasmas in the weeds *Emilia fosbergii* and *Synedrella nodiflora* in Jamaica. *Plant Pathology* **57**, 770.

Brown SE, Been BO, McLaughlin WA, 2008b. First report of lethal yellowing group (16SrIV) of phytoplasmas in *Vernonia cinerea* in Jamaica. *Plant Disease* **92**, 1132.

Córdova I, Oropeza C, Puch-Hau C *et al.*, 2014. A real-time PCR assay for detection of coconut lethal yellowing phytoplasmas of group 16SrIV subgroups A, D and E found in the Americas. *Journal of Plant Pathology* **96**, 343–52.

Elliott ML, Broschat TK, 2001. Ganoderma in Florida: observations and pathogenicity experiments on *Ganoderma zonatum* in Florida. *Palms* **45**, 62–72.

Harrison NA, Elliott ML, 2016. Texas Phoenix palm decline. Gainesville, FL, USA: University of Florida: UF/IFAS Extension Publication PP243. [http://edis.ifas.ufl.edu/pp163]. Accessed 10 May 2018.

Harrison NA, Narváez M, Almeyda H, Cordova I, Carpio ML, Oropeza C, 2002. First report of group 16SrIV phytoplasmas infecting coconut palms with leaf yellowing symptoms on the Pacific coast of Mexico. *Plant Pathology* **51**, 808.

Harrison NA, Helmick EE, Elliott ML, 2008. Lethal yellowing-type diseases of palms associated with phytoplasmas newly identified in Florida, USA. *Annals of Applied Biology* **153**, 85–94.

Harrison NA, Helmick EE, Elliott ML, 2009. First report of a phytoplasma-associated lethal decline of *Sabal palmetto* in Florida, USA. *Plant Pathology* **58**, 792.

Harrison NA, Davis RE, Helmick EE, 2013. DNA extraction from arborescent monocots and how to deal with other challenging hosts. In: Dickinson M, Hodgetts J, eds. *Phytoplasma. Methods in Molecular Biology (Methods and Protocols), vol. 938*. Totowa, NJ, USA: Humana Press, 147–58.

Innis MA, Gelfand DH, 1990. Optimization of PCRs. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR Protocols: A Guide to Methods and Applications*. San Diego, CA, USA: Academic Press, 3–12.

Krenz B, Thompson JR, McLane HL, Fuchs M, Perry KL, 2014. *Grapevine red blotch-associated virus* is widespread in the United States. *Phytopathology* **104**, 1232–40.

Kumar S, Stecher G, Tamura K, 2016. MEGA 7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* **33**, 1870–4.

Lara IC, Narváez LM, Hau CP, Oropeza C, Sáenz L, 2017. Detection and identification of lethal yellowing phytoplasma 16SrIV-A and D associated with *Adonidia merrillii* palms in Mexico. *Australasian Plant Pathology* **46**, 389–96.

Narváez M, Ortíz E, Silverio C, Santamaría JM, Espada F, Oropeza C, 2017. Changes observed in *Pritchardia pacifica* palms affected by a lethal yellowing-type disease in Mexico. *African Journal of Biotechnology* **16**, 2331–40.

Oropeza C, Cordova I, Puch-Hau C, Castillo R, Chan JL, Sáenz L, 2017. Detection of lethal yellowing phytoplasma in coconut plantlets obtained through *in vitro* germination of zygotic embryos from the seeds of infected palms. *Annals of Applied Biology* **171**, 28–36.

Pimentel D, Zuniga R, Morrison D, 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecological Economics* **52**, 273–88.

Sudarshana MR, Perry KL, Fuchs MF, 2015. Grapevine red blotch-associated virus, an emerging threat to the grapevine industry. *Phytopathology* **105**, 1026–32.