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Differences between the Texas phoenix palm phytoplasma and the coconut lethal yellowing phytoplasma revealed by restriction fragement length polymorphism (RFLP) analysis of the *NUSA* and *HFLB* genes

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A nested polymerase chain reaction (PCR) assay was used to amplify the *nusA* gene from a DNA sample extracted from a cabbage or sabal (*Sabal palmetto*) palm showing symptoms of Texas Phoenix decline (TPD) and from three DNA samples from coconut (*Cocos nucifera*) palms showing symptoms of lethal yellowing (LY). TPD is caused by a 16SrIV-D phytoplasma and LY by a 16SrIV-A strain. From the sabal DNA sample and from one coconut DNA sample two copies of the *hfl*B gene were amplified by nested PCR. Restriction fragment length polymorphisms analysis of the PCR-amplified *nusA* and the *hfl*B gene copies showed that these genes vary in the phytoplasma strains examined. Four restriction enzymes were used for the *nusA* gene and 16 were used for the *hfl*B genes.

Key words: *nusA*, *hfl*B, phytoplasma, Texas Phoenix decline.

INTRODUCTION

Until the 1980s, differentiation and classification of phytoplasmas relied solely on biological properties such as plant host range specificity, vector, geographic distribution and symptom differences on affected plants. Nucleic acid-based methods introduced in the late 1980s for studies of phytoplasmas have been less time-consuming and more reliable (Lee et al., 2000; Bertaccini, 2007). These nucleic acid-based methods rely mainly on PCR-amplification and sequence analysis of amplified genomic regions/genes that are useful indicators of taxonomic classification. Of the genomic regions/genes used for classifying phytoplasmas, the 16S rRNA gene remains the primary reference. Although the 16S rRNA gene is a useful classifier for phytoplasmas, a plethora of other genes or genomic regions may be used to provide the much needed alternative evaluation and diagnostic tools. Among the genes useful in phytoplasma classification and which can be used in alternative diagnostic methods are the *nusA* and *hfl*B genes The *nusA* gene, a transcription termination factor gene, is ubiquitous and conserved among bacteria (Borukhov et al., 2005). In a previous study, the *nusA* gene was found useful in classifying phytoplasmas, with the branching order of a phylogenetic tree inferred from the *nusA* gene sequence similar to the branching order inferred from the 16S rRNA gene sequence for the same phytoplasma isolates (Shao et al., 2006). The consistency of the *nusA* gene in resolving phytoplasma strains was also demon-

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Table 1. Phytoplasma samples included in the study, listed with palm species, location and strain identity.

Strain	Gene amplified	Source (host plant)	Classification	Location
Sabal1	*nusA*, *hfl*B	*Sabal palmetto*	16SrIV-D	Hillsborough, Florida USA
LYFL	*nusA*	*Cocos nucifera*	16SrIV-A	Broward, Florida USA
LYJAM	*nusA*	*C. nucifera*	16SrIV-A	Jamaica
LYMEX5	*nusA*, *hfl*B	*C. nucifera*	16SrIV-A	Mexico

strated by correlations between *nusA* phylogenetic trees with trees inferred from the sequences of ribosomal protein genes (Lee et al., 2004), *tuf* gene sequences (Marcone et al., 2000; Schneider et al., 1997), as well as *gcp* gene sequences (Davis et al., 2003). This demonstration of *nusA* as a pertinent taxonomic tool argued for using *nusA* to genetically compare the TPD phytoplasma from *S. palmetto* and the LY phytoplasma from *C. nucifera*.

The *hfl*B gene is possibly associated with strain virulence (Beier et al., 1997; Lithgow et al., 2004). Its inclusion in this study was based on its success in resolving strains of '*Candidatus* Phytoplasma mali' (Seemüller and Schneider, 2007).

In the present study, we used sequence analysis (restriction fragment length profiles) of these two genes to compare the Texas Phoenix palm decline phytoplasma (TPD) isolated from cabbage [*Sabal palmetto* (Walter) Lodd. ex Schult. & Schult. f.] palm, a dominant native palm of the state of Florida with the lethal yellowing (LY) phytoplasma isolated from coconut (*Cocos nucifera* L.) palm. Based on the fact that there were differences in the restriction profiles of these genes between the TPD and LY strains, phytoplasma diagnosis of *S. palmetto*, *C. nucifera* and other palms can be based on, among other techniques, restriction profiles of *nusA* and *hfl*B, especially for mixed and rare infections. Moreover, our study serves to confirm the difference between the TPD phytoplasma and the LY phytoplasma which were found by 16S rDNA analysis (Harrison et al., 2009).

MATERIALS AND METHODS

Plant material and DNA extraction

Apical bud tissues were obtained from a TPD symptomatic *S. palmetto* palm in west central Florida (Sabal1), a LY symptomatic *C. nucifera* palm from Broward county in southeastern Florida (LYFL), a LY symptomatic *C. nucifera* palm in Jamaica (LYJAM) and an LY sample from a symptomatic palm in Mexico (LYMEX5) (Table 1). These apical bud tissue samples were collected by felling the palm and excising immature leaf bases of the stem apex.

Total nucleic acids were extracted from 100 g of the bud tissues. DNA from bud tissues was extracted following the phytoplasma enrichment method of Harrison et al. (1994). Nucleic acid was precipitated with 95% ethanol and pellets were recovered by centrifugation at 12000 x *g* for 15 min. The pellets were resuspended in 200 µL TE buffer (10 mM Tris, 1 mM ethylene diamine tetra-acetic acid [EDTA, pH 8]). Presence of DNA in the the pellets was confirmed by agarose gel electrophoresis.

Polymerase chain reaction

DNA preparations from the symptomatic plants were evaluated by PCR assay, together with a negative control, which consisted of DNA from a healthy plant, and a water control (no DNA template). The PCR reaction was conducted using primer pair nusA-F1 5'-ATTTTGTTATATTTTGAAGGAGTGTT-3'/nusA-R1 5'-CAAAAAGCTTCATGACCCGGAGTATCTA-3' followed by a nested primer pair nusA-F2 5'-ACATCTAAAGCTGAATTAGGACA-3'/nusA-R2 5'-GCACCAATATGTTGAGTAATTCCA-3'. Primers hflB2-f1 5'-CCAGAAAATTATGATCCAGATGTTATA-3'/hflB2-r1 5'-CTACAGGAAAACTCTCAATAAG-3' and hflB1-f2 5'-TGTTTTGGAACCAGAAGATCCTTATT-3'/hflB1-r2 5'-TTGTTGTCCGTGTTGAGAAAATTG-3' were used to amplify the *hfl*B gene copies. All primers used in this study were designed using sequences of the LY phytoplasma obtained by 454 sequencing. Positions of these primers on the LY genome cannot yet be ascertained as the sequencing of this genome is not complete.

Each PCR reaction contained 33.8 µL H_2O; 5 µL buffer (1.675 µL H_2O; 1.25 µL 1 M KCl; 1 µL 1 M Tris; 0.5 µL 5% Tween 20; 0.5 µL 1% gelatin; 0.075 µL 1 M $MgCl_2$); 0.1 µg of each of the two primers; 0.04 mM of each of the dNTPs and 0.2 µL Taq DNA polymerase (New England BioLabs, Waverley, MA, USA). The reagent made a total volume of 50 µL and the PCR reaction consisted of 35 cycles, each consisting of 94°C for 1 min, a temperature between 50°C and 60°C (depending on the primer pair) for 2 min, and 72°C for 3 min. The 35 thermal cycles were preceded by 1 min initial denaturation at 94°C and succeeded by 7 min final elongation at 72°C. Ten microliter (10 µL) of the PCR end mixture was mixed with 7 µL of gel loading dye, run by electrophoresis through a 1% agarose gel using TAE buffer (40 mM Tris acetate, 1 mM EDTA) and visualized by UV transillumination following staining with ethidium bromide.

Cloning

Purified PCR products were quantified by visualizing on agarose gel with a serial dilution of uncut lambda DNA. The PCR fragments were ligated (mixed with and incubated at 4°C overnight) with pGEM-T vector (Promega Corp, Madison, WI). The ligated PCR product was transformed into Top 10 chemically competent *Escherichia coli* cells (Invitrogen Life Technologies, Carlsbad, CA, USA). The transformed bacterial cultures were grown overnight at 37°C on Luria-Bertani (LB) media amended with isopropyl ß-D-1-thiogalactopyranoside and X-gal for blue/white colony screening. After the 37°C incubation white colonies, which were regarded as carrying the cloned PCR fragment, were selected, inoculated into LB broth and incubated at 37°C with gentle shaking for 24 h. Cells were lysed, using lysis buffer, recovered, purified, resuspended in TE buffer and submitted for sequencing. Sequencing was necessary so that the *nusA* and *hfl*B gene sequences could be run

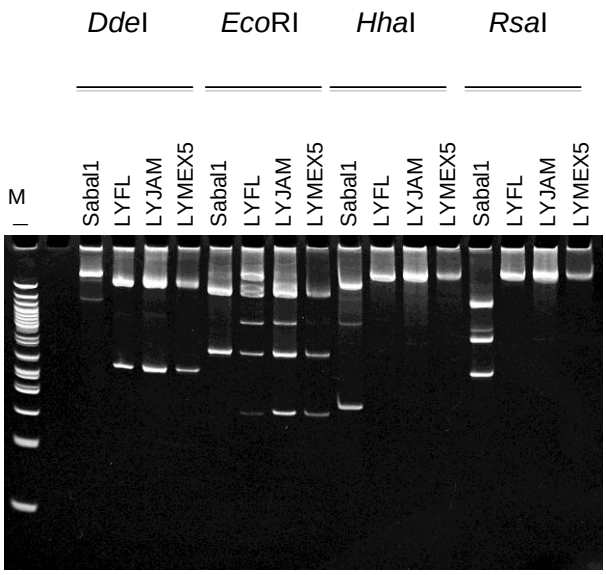

Figure 1. Restriction fragment length polymorphism of nusA-F2 and nusA-R2 PCR product. The *nusA* gene fragment was amplified from DNA fractions of symptomatic *Sabal palmetto* (Sabal1) and *Cocos nucifera* (LYFL, LYJAM, LYMEX5) and then digested with restriction enzymes *Dde*I, *Eco*RI, *Hha*I and *Rsa*I. M on the first lane is pGEM molecular (bp) size marker in descending order: 2465, 1605, 1198, 676, 517, 460, 396, 350, 222, 179, 126, 75, 65, 51 and 36. The second lane is empty.

on a virtual RFLP test using pDRAW32 (AcaClone, http://www.acaclone.com). Sequencing of cloned fragments was done using the M13 forward and M13 reverse primers by the University of Florida's Core DNA Sequencing Service Laboratory, Gainesville.

Sequence analysis

Sequences of the cloned fragments were assembled with SeqMan software (Lasergene™ 7.1; DNASTAR, Madison, WI, USA). To check the validity of the sequences database sequence similarity searching was performed using BLAST in NCBI (website: http://www.ncbi.nlm.nih.gov/BLAST). For the *nusA* gene the sequences were deposited in NCBI GenBank under accession numbers HQ613891, HQ613892, HQ613893. For the *hfl*B1 gene HQ613898, HQ613899. For the *hfl*B2 gene the sequences were deposited under HQ613896, HQ613897.

Restriction fragment length polymorphisms

Polymerase chain reaction products of the PCR amplified *nusA* gene were digested separately using restriction enzymes *Dde*I, *Eco*RI, *Hha*I and *Rsa*I (incubation at 37°C for a minimum of 16 h). For the *hfl*B gene, separate digestions of the obtained PCR products were performed using, for the primers hflB2-f1/hflB2-r1, restriction enzymes *Alu*I, *Apo*I, *Dra*I, *Eco*RI, *Hha*I, *Hind*III, *Mse*I, *Rsa*I, *Sau*3AI, *Ssp*I, and *Taq*I, and for the primers hflB1-f2/hflB1-r2, restriction enzymes *Alu*I, *Apo*I, *Ase*I, *Bst*UI, *Dde*I, *Dra*I, *Hae*III, *Hha*I, *Hind*III, *Mse*I, *Sau*3AI, *Ssp*I and *Tsp*I. These enzymes best differentiated between the phytoplasma strains as shown in a virtual test of sequence data using pDRAW32 (AcaClone, http://www.acaclone.com). Note that we could not use the virtual test alone to draw conclusions to our study because of possible sequencing defects. Real restrictions profiles were therefore necessary. Products of the restriction digests were separated by electrophoresis through 8% denaturing polyacrylamide gel in TBE buffer (90 mM Trisborate, 2 mM EDTA). Profiles were visualized using a UV transillumination following staining with ethidium bromide.

RESULTS AND DISCUSSION

Restriction fragment length polymorphisms

The nested primers nusA-F1/nusA-R1 and nusA-F2/nusA-R2 targeting the *nusA* gene amplified a fragment ca. 1.2 kb from DNA samples collected from four symptomatic plants (Sabal1, LYFL, LYJAM, and LYMEX5). No amplification was observed from the healthy palm or the water controls. PCR products resulting from amplification of the *nusA* gene fragment from diseased *S. palmetto* (Sabal1) and *C. nucifera* (LYFL, LYJAM and LYMEX5) were separately digested with *Dde*I, *Eco*RI, *Hha*I and *Rsa*I restriction enzymes. Based on the profiles obtained by using these enzymes, the 16SrIV-D phytoplasma from *S. palmetto* was clearly distinguished from the 16SrIV-A phytoplasma from *C. nucifera* (LYFL, LYJAM and LYMEX5) (Figure 1), similar to the prediction by the pDRAW32 software (AcaClone, http://www.acaclone.com). These RFLP results suggest that the phytoplasma strain causing decline of *S.*

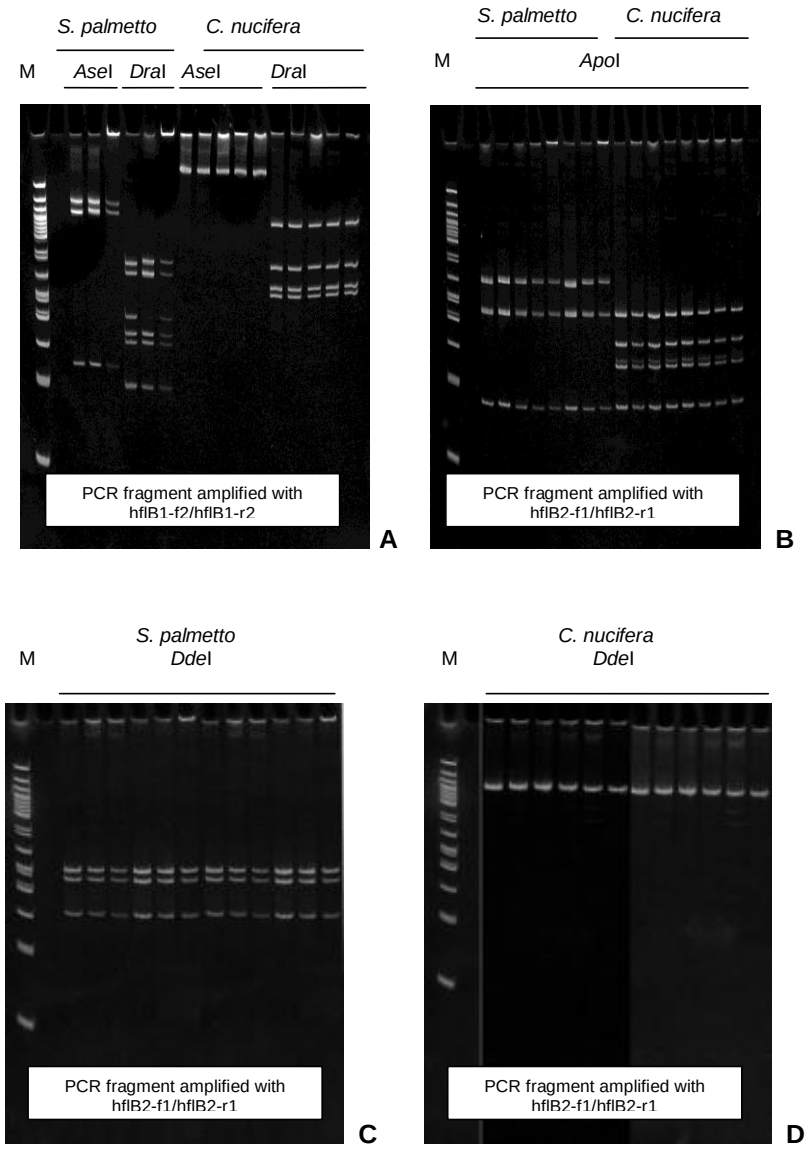

Figure 2. Restriction fragment length polymorphism of phytoplasma *hfl*B gene copies. Polymerase chain reaction (PCR) amplicons were amplified with different primer pairs from DNA of declining *Sabal palmetto* (Sabal1) and *Cocos nucifera* (LYMEX5) palms. **A)** PCR fragment amplified with hflB1 f2/hflB1-r2 was digested with *Ase*I and *Dra*I. **B)** PCR fragment amplified with hflB2-f1/hflB2-r1 was digested with *Apo*I. **C and D)** PCR fragment amplified with hflB2-f1/hflB2-r1 was digested with *Dde*I.

palmetto is different from LY strains in *C. nucifera*, reinforcing subgroup differentiation based on the 16S rRNA gene (Harrison et al., 2009). We used only two DNA samples for the *hfl*B gene analysis. From both symptomatic samples (Sabal1 and LYMEX5), bands of approximately 1.3 kb were amplified in the PCR assays using primer pair hflB1-f2/hflB1-r2. The 1.3 kb fragment represented one *hfl*B copy. Smaller fragments about 0.6 kb (second *hfl*B copy) were amplified from the same samples when primer pair hflB2-f1 and hflB2-r1 were used. No amplification was observed in the healthy palm or the water controls. The two sets of PCR products from each of the successfully amplified palm DNA samples were digested separately using restriction enzymes. Each PCR product represented a different paralog of the *hfl*B gene. Patterns representing these digestions are illustrated in Figure 2. Sabal1 and LYMEX5 were differentiated clearly when the PCR fragment obtained by amplification using hflB1-f2/hflB1-r2 were digested with restriction enzymes *Apo*I, *Ase*I, *Dde*I, *Dra*I, *Hha*I, *Hind*III,

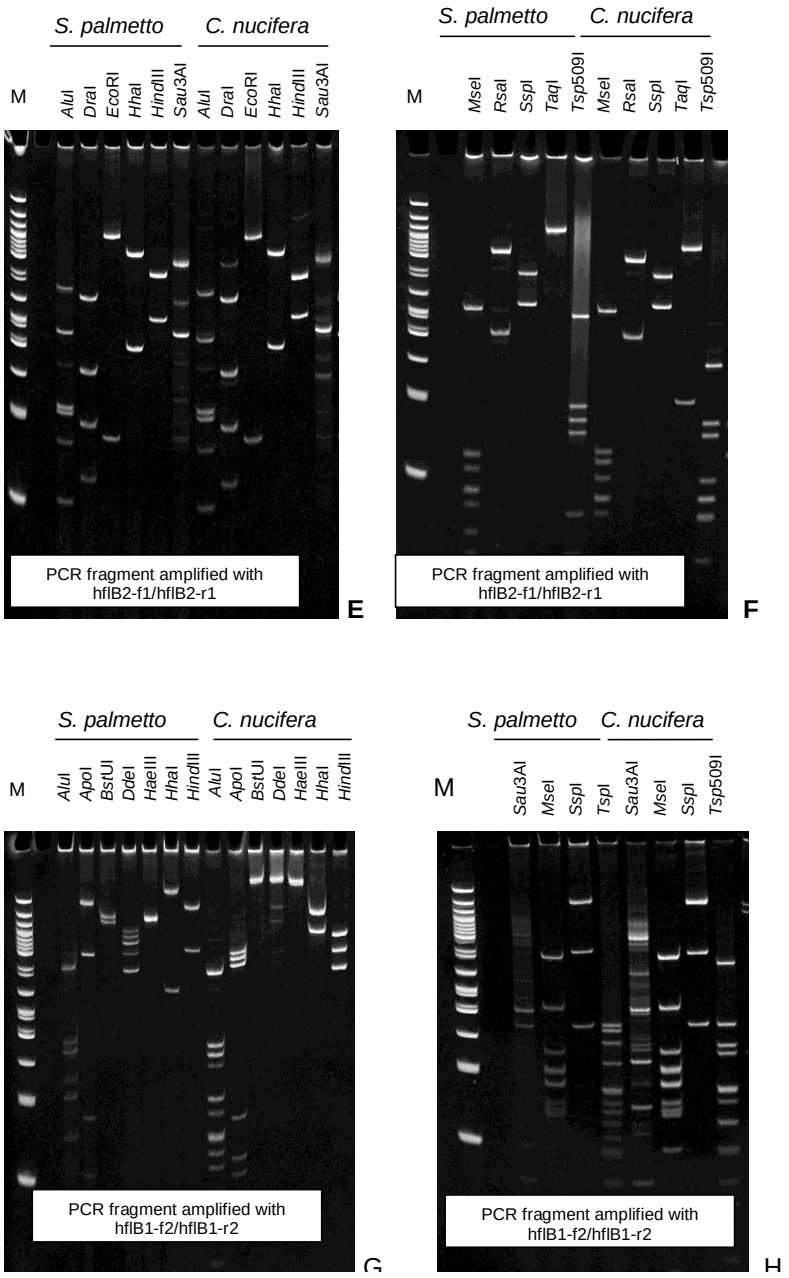

Figure 2 Contd. Restriction fragment length polymorphism of phytoplasma *hfl*B gene copies. Polymerase chain reaction (PCR) amplicons were amplified with different primer pairs from DNA of declining *Sabal palmetto* (Sabal1) and *Cocos nucifera* (LYMEX5) palms. **E)** PCR fragment amplified with hflB2-f1/hflB2-r1 was digested with *Alu*I, *Dra*I, *Eco*RI, *Hha*I, *Hind*III, *Sau*3AI. **F)** PCR fragment amplified with hflB2-f1/hflB2-r1 was digested with *Mse*I, *Rsa*I, *Ssp*I, *Taq*I and *Tsp*509I. **G)** PCR fragment amplified with hflB1-f2/hflB1-r2 was digested with *Alu*I, *Apo*I, *Bst*UI, *Dae*I, *Hae*III, *Hha*I, *Hind*III. **H)** PCR fragment amplified with hflB1-f2/hflB1-r2 was digested with *Sau*3AI, *Mse*I, *Ssp*I and *Tsp*I. M on the first lane is for pGEM molecular size (bp) marker in descending order: 2465, 1605, 1198, 676, 517, 460, 396, 350, 222, 179, 126, 75, 65, 51 and 36. Lane 2 is empty.

*Sau*3AI and *Tsp*509I (Figure 2 A, G and H). Differences between Sabal1 and LYMEX5 were also demonstrated when the PCR fragment primed with the hflB2-f1 and hflB2-r1 primer pair was digested with enzymes *Apo*I,

*Dde*I, *Taq*I and *Tsp*509I (Figure 2 B-F). In Figure 2 A-D multiple fragments from different clones of each of the strains were tested whereas one fragment from a single clone was tested in the rest of the figures. This was believed to be sufficient to demonstrate differences between the two phytoplasma strains. Findings obtained by RFLP analysis of the *hfl*B gene also show that the TPD phytoplasma (16SrIV-D) from *S. palmetto* is different from the LY phytoplasma (16SrIV-A) from *C. nucifera*.

The open reading frame (ORF) of the PCR fragment amplified with the hflB1-f2/hflB1-r2 primer pair from the LYMEX5 sample was ca. 120 bp longer than the ORF of the fragment amplified by the same primers from the phytoplasma infecting *S. palmetto*. The differences between these phytoplasma strains were also shown by a few base substitutions between the two PCR fragments amplified with hflB1-f2/hflB1-r2. The ORF of the PCR fragment amplified from the *S. palmetto* phytoplasma with the hflB2-f1 and hflB2-r1 primers was almost twice as long as the fragment amplified from the DNA sampled from *C. nucifera* showing symptoms of LY. A few base substitutions also showed the differences between the TPD and the LY phytoplasmas in the fragment amplified by the hflB2-f1 and hflB2-r1 primers. In this work, differences between the TPD and the LY phytoplasma were demonstrated. The variation of both the *nusA* and *hfl*B genes between the TPD and the LY phytoplasmas can be exploited to diagnose palms showing symptoms of either TPD or LY.

Texas Phoenix decline phytoplasma, 16SrIV subgroup D, was first reported in *S. palmetto* in west central Florida in 2008 (Harrison et al., 2009). Although this phytoplasma subgroup had been previously reported in *P. canariensis* in Corpus Christi, Texas (Harrison et al., 2002), and in west central Florida (Harrison et al., 2008), the attack of *S. palmetto* by a phytoplasma was a surprise as no indigenous palms had been documented as being affected by phytoplasmas in Florida or Texas prior to this time. *S. palmetto* is a native species that is important in the natural landscape of the state of Florida and other states in the southern USA. We recommend further studies to explore the usefulness of other genes in differentiating between TPD and LY. This becomes useful in the face of phytoplasmas spreading and phytoplasma diseases of palms becoming significant as recently found in Mexico (Vázquez-Euán et al., 2011).

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