MOLECULAR PROPERTIES OF TEXAS PHOENIX DECLINE PHYTOPLASMA, A SUBGROUP 16SrIV-D STRAIN ASSOCIATED WITH LETHAL DISEASES OF SABAL PALMETTO AND OTHER PALMS IN FLORIDA

By

KHAYALETHU NTUSHELO

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To my family for their unconditional love and support
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TABLE OF CONTENTS

ACKNOWLEDGMENTS ........................................................................................................ 4
LIST OF TABLES .................................................................................................................. 8
LIST OF FIGURES ................................................................................................................ 9
ABSTRACT .......................................................................................................................... 11

CHAPTER

1 REVIEW OF DECLINE DISEASES OF PALMS WITH SPECIFIC REFERENCE TO THE MOLECULAR PROPERTIES OF SUBGROUP 16SrIV-D PHYTOPLASMA CAUSING TEXAS PHOENIX DECLINE OF SABAL PALMETTO ................................................................. 13

Introduction .................................................................................................................. 13
General Characteristics of Phytoplasmas ........................................................................ 13
Taxonomy of Phytoplasmas .......................................................................................... 14
Group 16SrIV Phytoplasmas ....................................................................................... 17
The Sabal Genus ........................................................................................................... 20
  Taxonomy .................................................................................................................. 20
  Distribution and Ecology ......................................................................................... 20
  Morphology ............................................................................................................... 21
  Sabal palmetto ......................................................................................................... 21
Symptoms of Texas Phoenix Decline on Sabal palmetto ........................................... 22
Molecular Characterization of Phytoplasmas with Reference to Texas Phoenix Decline on Sabal palmetto ................................................................................................................. 23
  Ribosomal RNA Genes .......................................................................................... 23
  The 16S-23S Intergenic Spacer Region ................................................................... 24
  nusA Gene ............................................................................................................... 25
  hflB Gene ............................................................................................................... 25
  O-sialoglycoprotein Endopeptidase Gene, the Glycoprotease (gcp) Gene .......... 26
Problem Statement and Purpose of the Study ............................................................. 27

2 MOLECULAR SURVEY OF THE TEXAS PHOENIX DECLINE PHYTOPLASMA POPULATION .................................................................................................................. 30

Introduction .................................................................................................................. 30
Materials and Methods ............................................................................................... 31
  Plant Material and DNA Extraction ........................................................................ 31
  Polymerase Chain Reaction .................................................................................... 32
  Cloning ..................................................................................................................... 33
3 DIFFERENTIATION OF PHYTOPLASMA STRAINS CAUSING DECLINE OF SABAL PALMETTO AND COCONUT LETHAL YELLOWING BASED ON SEQUENCES OF THE RIBOSOMAL RNA OPERON ................................................................. 42

   Introduction ........................................................................................................ 42
   Materials and Methods ..................................................................................... 42
   Plant Material, DNA Extraction Polymerase Chain Reaction and Cloning ....... 42
   Sequence Analysis .......................................................................................... 43
   Results ............................................................................................................... 43
   Discussion ........................................................................................................... 45

4 GENETIC CHARACTERIZATION OF SUBGROUP 16SrIV-D PHYTOPLASMA INFECTING SABAL PALMETTO USING HFLB, NUSA AND GLYCOPROTEASE GENE SEQUENCES ....................................................................................... 59

   Introduction ........................................................................................................ 59
   Materials and Methods ..................................................................................... 60
   Plant Material and Polymerase Chain Reaction .............................................. 60
   Cloning, Sequencing and Restriction Fragment Length Polymorphisms .......... 60
   Sequence Analysis .......................................................................................... 61
   Results and Discussion .................................................................................... 61
   \textit{nusA} Gene ................................................................................................. 61
   Polymerase chain reaction and analysis by restriction fragment length polymorphism ................................................................. 61
   Molecular comparisons ................................................................................... 62
   \textit{hflB} Gene ................................................................................................. 62
   Polymerase chain reaction .............................................................................. 62
   Analysis by restriction fragment length polymorphism ................................. 63
   Molecular comparisons ................................................................................... 63
   \textit{gcp} Gene ................................................................................................. 64
   Polymerase chain reaction and analysis by restriction fragment length polymorphism ................................................................. 64
   Molecular comparisons by phylogenetic analysis ........................................... 64

5 RESULTS OBTAINED AND CONCLUDING REMARKS ................................... 76

APPENDIX: SOME PHYTOPLASMAS REFEREED TO IN THIS MANUSCRIPT .... 78

LIST OF REFERENCES .......................................................................................... 79

BIOGRAPHICAL SKETCH ...................................................................................... 85
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Phytoplasma samples included in the study, listed with palm host species,</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>location and strain identity.</td>
<td></td>
</tr>
<tr>
<td>3-1</td>
<td>Primers and product size of polymerase chain reaction amplification of</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>ribosomal RNA operon genes.</td>
<td></td>
</tr>
<tr>
<td>4-1</td>
<td>Phytoplasma samples included in this study listed with host palm species</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>and location.</td>
<td></td>
</tr>
<tr>
<td>4-2</td>
<td>Primers used to amplify phytoplasma gene products from total DNA</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>extracted from symptomatic plants.</td>
<td></td>
</tr>
<tr>
<td>4-3</td>
<td>Sequence comparisons of the <em>nusA</em> gene.</td>
<td>67</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>Orientation of genes of the ribosomal RNA operon of five phytoplasma species.</td>
<td>28</td>
</tr>
<tr>
<td>1-2</td>
<td>The syndrome of Texas Phoenix decline in <em>Sabal palmetto</em> caused by a 16SrV-D phytoplasma in Hillsborough county in west central Florida.</td>
<td>29</td>
</tr>
<tr>
<td>2-1</td>
<td>Agarose gel showing polymerase chain reaction amplification of the 16S-23S intergenic spacer region.</td>
<td>39</td>
</tr>
<tr>
<td>2-2</td>
<td>Restriction fragment length profiles of phytoplasma DNA (ca. 800 bp) amplified from symptomatic palms.</td>
<td>40</td>
</tr>
<tr>
<td>2-3</td>
<td>Inferred molecular relationships of phytoplasma strains based on the 16S-23S intergenic spacer sequence.</td>
<td>41</td>
</tr>
<tr>
<td>3-1</td>
<td>Sequence comparison of the ribosomal RNA operon.</td>
<td>49</td>
</tr>
<tr>
<td>3-2</td>
<td>Schematic representation of the ribosomal operon of the Texas Phoenix decline phytoplasma strain isolated from <em>Sabal palmetto</em>.</td>
<td>50</td>
</tr>
<tr>
<td>3-3</td>
<td>Inferred molecular relationship of phytoplasma strains based on ribosomal RNA operon.</td>
<td>51</td>
</tr>
<tr>
<td>3-4</td>
<td>Inferred molecular relationship of phytoplasma strains based on 16S ribosomal RNA (rrns) gene using the neighbor-joining method.</td>
<td>53</td>
</tr>
<tr>
<td>3-5</td>
<td>Inferred molecular relationship of phytoplasma strains based on 16S-23S intergenic spacer using the neighbor-joining method.</td>
<td>55</td>
</tr>
<tr>
<td>3-6</td>
<td>Inferred molecular relationship of phytoplasma strains based on 23S ribosomal RNA (rrns) gene using the neighbor-joining method.</td>
<td>57</td>
</tr>
<tr>
<td>4-1</td>
<td>Nested PCR products amplified using primer pair nusA-F1 and nusA-R1 followed by nusA-F2 and nusA-R2.</td>
<td>68</td>
</tr>
<tr>
<td>4-2</td>
<td>Restriction fragment length polymorphism of nusA-F2 and nusA-R2 PCR product.</td>
<td>68</td>
</tr>
<tr>
<td>4-3</td>
<td>Restriction fragment length polymorphism of phytoplasma <em>hfB</em> gene copies.</td>
<td>69</td>
</tr>
<tr>
<td>4-4</td>
<td>Agarose gel electrophoresis showing amplification of the glycoprotease (<em>gcp</em>) gene in different DNA samples collected from symptomatic palms.</td>
<td>72</td>
</tr>
<tr>
<td>4-5</td>
<td>Restriction fragment length polymorphisms of a polymerase chain reaction (PCR) fragment amplified with primer pair GCPFI/GCPR1.</td>
<td>73</td>
</tr>
</tbody>
</table>
Molecular tree of the glycoprotease (gcp) gene sequences of palm lethal disease strains inferred by neighbor-joining method.
A decline of sabal or cabbage palm (Sabal palmetto) with symptoms similar to those of coconut (Cocos nucifera) lethal yellowing (LY) was first observed in west central Florida, USA in 2008. Characterization of the causal agent implicated a phytoplasma, which closely resembled a subgroup 16SrIV-D phytoplasma previously associated with declining Canary Island date palms (Phoenix canariensis), edible date (P. dactylifera), silver date (P. sylvestris) and Queen (Syagrus romanzoffiana) palms. The phytoplasma strain associated with the Florida native S. palmetto was therefore also referred to as Texas Phoenix decline (TPD) phytoplasma, and classified as a subgroup 16SrIV-D strain. Although this phytoplasma had been classified based on the 16S rRNA sequence, further characterization of its genome remained crucial. Twenty five S. palmetto plants in west central Florida showing symptoms of decline were selected for sampling of the phytoplasma associated with the disease. DNA was extracted from tissue samples and the intergenic spacer region between 16S rRNA and 23S rRNA genes was amplified by a nested polymerase chain reaction assay. The sequence of the 16S-23S intergenic spacer (IGS) of TPD phytoplasma infecting S.
*palmetto* was compared with IGS sequences derived from six 16SrIV-D phytoplasma strains infecting *Phoenix* spp., a 16SrIV-D strain infecting *Syagrus romanzoffiana*, a 16SrIV-F strain infecting *Washingtonia robusta*, two strains each comprising a mixture of 16SrIV-A and 16SrIV-F phytoplasmas, one LY phytoplasma strain from *P. canariensis* and finally those of three 16SrIV-A strains associated with LY disease of *C. nucifera*. The TPD phytoplasma population associated with *S. palmetto* appears homogenous. On the basis of the ribosomal operon, the TPD phytoplasma in *S. palmetto* is similar to other TPD strains found in *Phoenix* spp. and *S. romanzoffiana*, but different from the subgroup 16SrIV-A phytoplasma associated with *C. nucifera* and *Phoenix* spp. For further analysis, three genes were selected: the *nusA* gene, the *hflB* gene, and the *gcp* gene. All genes (*nusA*, *hflB* and *gcp*) differentiated the strains in this study similarly, TPD phytoplasma strain infecting *S. palmetto* is similar to the TPD strain infecting *Cocos* and *Phoenix* spp. but different from the LY strains associated with *C. nucifera*. 
CHAPTER 1
REVIEW OF DECLINE DISEASES OF PALMS WITH SPECIFIC REFERENCE TO THE
MOLECULAR PROPERTIES OF SUBGROUP 16SrlV-D PHYTOPLASMA CAUSING
TEXAS PHOENIX DECLINE OF SABAL PALMETTO

Introduction

A widespread yellows disease on aster was first reported in 1902 (Kunkel 1926 cited by Lee et al. 2000). Yellows diseases were originally considered to be caused by viruses until a group of Japanese scientists using electron microscopy discovered mycoplasma-like organisms (MLOs) in ultrathin sections of the phloem of plants showing typical yellows symptoms (Doi et al. 1967 cited by Lee et al. 2000). However, an inability to culture MLOs in cell-free media limited further efforts to study and understand them. Later adoption of molecular-based techniques, especially sequencing of the ribosomal operon, facilitated their classification. Based upon the sequence of the 16S rRNA gene, MLOs share between 88% and 99% similarity among themselves and between 87% and 88.5% similarity with acholeplasmas, which proved to be their closest relatives (Gundersen et al. 1994; Seemüller et al. 1998). Subsequently the name phytoplasma was adopted by the Phytoplasma Working Team of the 10th Congress of International Organization of Mycoplasmology to refer to MLOs (Int. Comm. Syst. Bacteriol., Subcomm. Taxon. Mollicutes. 1993; Int. Comm. Syst. Bacteriol. Subcomm. Taxon. Mollicutes. 1997).

General Characteristics of Phytoplasmas

Phytoplasmas are mollicutes that inhabit phloem sieve tube elements of their respective plant hosts. Ultrastructural observation of phytoplasmas frequently reveal cells with a rounded pleiomorphism with a diameter between 200 and 800 nm. Other observations indicated that phytoplasmas are at times filamentous in form [(Haggis and
For dissemination, they depend upon phloem-feeding insect vectors of the order Hemiptera (Kirkpatrick 1992 cited by Liefting et al. 2004). These insects, primarily leafhoppers, planthoppers and psyllids, transmit phytoplasmas to different plants in a plant-insect-phytoplasma relationship known as the matrimonial triangle. Phytoplasmas are associated with diseases of hundreds of plant species worldwide (Lee at al. 2000; Seemüller et al. 1998). Typical symptoms on infected plants include virescence (development of green flowers and the loss of flower pigmentation), phyllody (development of leafy appearance on flowers), sterility of flowers, witches’-brooms, abnormal internode elongation, stunting, discoloration of the foliage, leaf distortion, malformation of stem tips, and eventual plant decline. However, some plant species are tolerant of phytoplasma infection and consequently display mild or no symptoms. Phytoplasmas possess small genomes ranging between 530 and 1200 kb, a G+C content between 23 and 29%, two rRNA operons (Figure 1-1) (except for Western-X disease phytoplasma which has only one (Kirkpatrick et al. 1987)), few tRNAs and limited metabolic activity (Bai et al. 2006; Bové 1997; Kube et al. 2008; Marcone et al. 1999; Oshima et al. 2004; Razin 1985; Tran-Nguyen et al. 2008). Because phytoplasmas are unculturable, thus far, their taxonomic characterization has been limited primarily to molecular-based methods.

**Taxonomy of Phytoplasmas**

Before a comprehensive classification was devised, phytoplasmas were named according to prominent biological properties such as symptoms or types of the diseases they caused. The shortfall of this system was that molecularly distinct phytoplasmas can cause very similar or the same symptoms on shared plant hosts and, as such, might be
assigned the same name. Molecular-based methods that had gained prominence in the study of prokaryotes were then adopted to design a new classification scheme. Initial molecular methods to identify and classify unknown phytoplasmas were based on phylogenetic analysis of the 16Sr RNA gene (Kuske and Kirkpatrick 1992; Namba et al. 1993). The difficulty with this method of classification was that many phytoplasma laboratories were ill-equipped to obtain reliable sequences of the 16S rRNA gene. A classification scheme based on universal amplification and restriction fragment length polymorphism (RFLP) of the 16S rRNA gene was then adopted and originally reported by Lee et al. (1993) and used to differentiate a collection of 40 phytoplasmas from three continents into nine major groups and 14 subgroups. This classification scheme was later revised by further differentiation of the 14 major groups into 32 strain subgroups using RFLP data derived from PCR amplified ribosomal protein genes (Lee et al. 1998, 2000). With the wide acceptance of 16S rRNA analysis as a reliable tool for identification and classification, an in silico RFLP analytical method was developed (Wei et al. 2008) and automated (Zhao et al. 2009). Used to examine 16S rRNA sequences of phytoplasmas available in the public nucleotide databases, this method delineated 28 major groups and at least 100 subgroups of strains (Wei et al. 2007).

Phylogenetic analysis of 16S rRNA gene sequences (Gundersen et al. 1994; Namba et al. 1993; Seemüler et al. 1998) were consistent with a molecular classification of phytoplasmas based upon RFLP analysis. Based upon a global analysis of the 16S rRNA gene and ribosomal protein gene operon sequences, phytoplasmas form a distinct monophyletic clade within the class Mollicutes (Gundersen et al. 1994; Lim and Sears 1992). Within the clade, numerous subclades (i. e. major groups) of
phytoplasmas were delineated and presumed to represent separate species. With the advent of phylogenetic classification of prokaryotes, based upon the evolutionary conserved 16SrRNA gene, to accommodate assignment of binominal names to unculturable taxa of prokaryotes defined by very limited data, such as nucleotide sequence, derived from a very small portion of the genome (e.g. 16Sr RNA gene), Murray and Schleifer (1994) proposed the ‘Candidatus Phytoplasma’ genus. Subsequently, it was proposed to accommodate phytoplasmas within the novel genus ‘Candidatus Phytoplasma’ (IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma taxonomy group 2004).

Within the genus ‘Ca. Phytoplasma’, species designations have been separately assigned to reference strains representative of 27 phytoplasma groups. Current guidelines for ‘Ca. Phytoplasma’ species description are based upon identification of a significantly unique 16S rRNA gene sequence >1,200 bp in length. The strain from which the sequence is obtained should be designated as the reference strain. Strains with minimal differences in the 16S rRNA sequence, relative to the reference strain, should be referred to as related strains. In general, two phytoplasma strains are the same species if they share more than 97.5% of their 16S rRNA gene (IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma taxonomy group 2004). However, if two such strains that share more than 97.5% of their 16S rRNA are vectored by different insects, have different hosts, behave differently in the same host, or are molecularly distinct in hybridization tests with cloned DNA, or in serotyping or in PCR assays, then two separate species may be proposed (IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma taxonomy group 2004).
Group 16SrIV Phytoplasmas

Yellowing syndrome of coconut palm has been known for more than one hundred years. This syndrome was rife in the Cayman Islands in the 1830s, but it was not until the mid-1900s that the first reliable reports of mortality of coconut palm were made (Nutman and Roberts 1955 cited by Eden-Green 1997). In these first credible reports of coconut palm mortality which were made in Jamaica, Nutman and Roberts (1955) cited by Eden-Green (1997), first used the term lethal yellowing (LY) to refer to this disease.

Symptoms observed on the highly susceptible Jamaica tall variety (i.e. Atlantic tall ecotypes) begin with premature falling of most or all coconuts (fallen coconuts have a brownish discoloration on the part immediately under the calyx of the stem). Closely following the nut fall is the blackening of new inflorescences. Then yellowing of leaves begins at the base of the crown, gradually advancing to reach mid-crown and eventually discoloring the entire crown. The leaves ultimately turn brown, droop and fall. Foliage discoloration is accompanied by death of the spear leaf. Finally, the tree canopy falls, leaving a bare trunk.

More reports of LY on coconut palm came from Cuba, the Dominican Republic, Haiti, the Bahamas, and the disease became established in Florida in the USA in the early 1970s (McCoy et al. 1983). Other outbreaks were also reported in the Yucatán Peninsula of Mexico and Belize (Eden-Green 1997). By 1997 LY of palms was present on the Pacific coast of Mexico (Harrison et al. 2002a). Most lethal yellows disease reports were made on Cocos nucifera L, which has been devastated in various epidemics. In Florida and Jamaica, LY epidemics were responsible for the death of most coconut palms of the “Jamaican Tall” variety from the 1970s until the 1990s. This occurred concurrently with spread of this disease to neighboring regions including the
Pacific coast of the Americas (Harrison et al. 2002a). In the late 1990s, most disease activity was along the Atlantic coasts of Belize and Honduras (Ashburner et al. 1996; Harrison and Oropeza 1997). Diseases symptomatically resembling LY have been reported from East and West Africa since the beginning of the 1900s [(Bull 1955; Ekpo and Ojomo 1990; Osagie and Asemota 1995; Schuiling et al. 1992) cited by Eden-Green 1997]. In some communities these epidemics caused severe economic losses. In subtropical southern Florida, where a wide selection of palm species are cultivated for use in landscape and amenity plantings, at least 35 species in addition to coconut are known to be affected by LY.

The initial application of DNA-based molecular diagnostics to studies on phytoplasma diseases have identified and classified phytoplasmas that are consistently associated with LY disease of palms in the Caribbean region, Mexico and Central America as members of the 16S RFLP group 16SrIV (Lee et al. 1998) or as the representative of group VII according to a classification system based on phylogeny of 16S rRNA gene (Gundersen et al. 1994). Within group 16SrIV several subgroups have been resolved (Harrison et al. 1992; Harrison and Richardson 1994; Harrison et al. 1994; Harrison et al. 2002a; Harrison et al. 2008). Although LY is most associated with coconut, LY-type diseases have a significant impact in other palm species.

In Canary Island date palms (Phoenix canariensis Chab.), a LY-type phytoplasma was detected in Brownsville and Rio Grande Valley in southern Texas during the late 1970s (McCoy et al. 1980). The same symptoms were later observed on the Canary Island date palms in Corpus Christi, Texas in 2001 and classified as subgroup 16SrIV-D (Harrison et al. 2002b). Most recently, records of LY-type diseases
on palms were also made on silver date \( [P. \textit{sylvestris} \text{(L.) Roxb.}] \), Canary Island date, edible date \( (P. \textit{dactylifera} \text{L.}) \), Queen \( [\text{Syagrus romanzoffiana} \text{(Cham.) Glassman}] \), and Mexican fan \( (\text{Washingtonia robusta} \text{Wendl.}) \) palms in west central Florida (Harrison et al. 2008). A lethal decline of sabal or cabbage palms \( [\text{Sabal palmetto} \text{(Walter) Lodd. ex Schult. & Schult. f.}] \) was also recorded in west central Florida in 2008 (Harrison et al. 2009). For the lethal decline in \( S. \textit{palmetto} \), initial DNA-based characterization found a phytoplasma which was identical to the strain previously affecting \( \text{Phoenix} \text{spp.} \) in Texas and later on \( P. \textit{dactylifera}, P. \textit{sylvestris} \) and \( S. \textit{romanzoffiana} \) in west central Florida (Harrison et al. 2009; McCoy et al. 1980).

Work done on the project herein focused on the phytoplasma strain infecting \( S. \textit{palmetto} \). Infected cabbage palms develop a reddish-brown foliage followed by decline of the spear leaf, and eventual mortality. Infected palms die within a few months once symptoms become visible. Unlike the LY phytoplasma associated with \( C. \textit{nucifera} \), this phytoplasma strain appears to spread slowly and its intensity was still confined in the area of first disease records, namely Hillsborough and Manatee counties in west central Florida, two years after its discovery, with fewer disease records in Polk and Desoto counties. This introduction of the pathogen by shipment could warrant restrictions on movement of plants given the evidence that it can perpetuate this disease. The new strain is similar to the Texas Phoenix palm decline (TPD) phytoplasma, the strain originally associated with Phoenix palms in Corpus Christi, Texas. It was also referred to as TPD, a subgroup 16SrI\(\text{V-D} \) phytoplasma.
The Sabal Genus

Sabal has always been an important part of the new world. Ecologically, its fruit provides food to migratory birds, and historically, it was used for roof thatching and crafting because of its tough but pliant leaves. It is also an important ornamental plant.

Taxonomy

Kingdom: Plantae
Order: Arecales
Family: Arecaceae
Subtribe: Sabalinae
Tribe: Corypheaee
Genus: Sabal

Distribution and Ecology

Fossil records of Sabal point to a range of distribution that includes the Soviet Union (Takhtajan 1958 cited by Zona 1990), Great Britain (Reid and Chandler 1933 cited by Zona 1990), Alaska (Wolfe 1972 cited by Zona 1990), Vancouver Island and Japan (Kryshtofovich 1918 cited by Zona 1990) and the states of New Jersey, Delaware, Maryland, South Carolina, Kentucky, Tennessee, Arkansas, Texas, Montana, Wyoming, Colorado, New Mexico and California in the USA [(Daghlian 1978; Noe 1936; Read and Hickey 1974) cited by Zona 1990]. Presently Sabal is only found in the new world. Primarily it is found in Mexico, southeastern USA, and the Caribbean (including Bermuda). Principal pollinators for the genus are Hymenoptera, especially solitary bees of the Megachilidae and Halictidae (Zona 1990). The fruits are dispersed by birds (Guppy 1917 cited by Zona 1990), and according to Brown (1973) cited by Zona (1990), water-dispersal is the primary mode for long-range dispersal for S. palmetto.
Morphology

In *Sabal*, stem formation begins underground where seedling growth first proceeds. Aerial stem growth is preceded by many years of this underground growth. For fully matured plants the aerial trunk ranges from 3 to 25 m tall. Most caulescent species reach heights between 5 and 15 m tall. Trunk diameter is between 15 cm and 60 cm, with most species ranging between 35 and 45 cm. *Sabal* has an extensive adventitious root system. Leaf petioles are between 30 and 250 cm long depending on conditions during growth. The leaves are alternate and spirally arranged, flabelliforus, with 15 to 120 segments with varying degrees of “costapalm”. The inflorescence may be between 0.4 to 3 m in length with varying degrees of branching density. The flowers protrude singly with a creamy-white appearance and a pungent sweet fragrance. They are ca. 3.5 to 7 mm in diameter. Generally, across species, there is uniformity in flower morphology. *Sabal* berries range in size from 6.5 to 27.5 mm in diameter (Zona 1990).

*Sabal palmetto*

*Sabal* or cabbage palm, *Sabal palmetto*, is a palm native in the central part of the Americas. Used as an indicator of poor soil, *S. palmetto* is important in the ecology of its habitat. Culturally, it is an important ornamental plant and also a very important symbol in the identity of the states of Florida and South Carolina (official state trees in both states). *S. palmetto* is most abundant in Cuba and the Bahamas, southeastern United States, specifically Florida, along the coast of the states of Georgia and South Carolina and its range also extends to Cape Fear, Smiths Island and North Carolina. Its natural habitat is mesic hammocks, pine forests, and along water bodies (rivers and the beach). It survives well in environments with salt spray and brackish water ([Brown 1973; Zona])
In the northern part of its geographic range, *S. palmetto* flowers in July and very sparingly during the remainder of the year. It flowers between June and August in central Florida, throughout the year in southern Florida and the Bahamas, and only in spring in Cuba (Zona 1990). *S. palmetto*'s horticultural characteristics include high salt tolerance, high drought tolerance, wide soil adaptation, high light requirement and low nutritional requirements. Morphologically, it has a solitary canopy, and the stem is gray, smooth and usually covered with split leaf bases. The leaves are costapalmate, induplicate with a dull green color. The plant is monoecious and has bisexual flowers which are white (Meerow 2006).

**Symptoms of Texas Phoenix Decline on *Sabal palmetto***

Phytoplasma infection of mature *S. palmetto* palms begins with inflorescence necrosis, which is closely followed by foliage discoloration beginning with the oldest leaves which turn varying shades of reddish-brown to dark brown to grey. The onset of foliage discoloration is accompanied by death of the spear leaf and shortly thereafter by the mortality of the apical meristem. Palms with advanced symptoms can be pushed-over easily indicating a loss of the structural integrity of the root system due to decay. Eventually the remaining canopy declines before toppling to the ground leaving just a bare stem, often referred to as a “telephone pole” (Harrison et al. 2009). Certain stages of disease development are depicted in Figure 1-2.
Molecular Characterization of Phytoplasmas with Reference to Texas Phoenix Decline on Sabal palmetto

Because phytoplasmas are unculturable, their classification is presently best achieved by the use of molecular techniques. Among the molecular procedures useful in characterizing phytoplasmas is the analysis of conserved genes (Gundersen et al. 1994; Schneider et al. 1997). For the purposes of the research reported herein, focus was on characterizing the sequences of ribosomal RNA genes and the intergenic spacer between the 16S rRNA and 23S rRNA genes. However, less conserved, non-ribosomal protein genes are required to differentiate closely related phytoplasmas (phytoplasmas within a given 16Sr group). Moreover, some of these non-ribosomal coding genes may have different evolutionary rates and, therefore, may add to the knowledge on evolution and adaptation. Attention was focused additionally on using the sequences of the transcription factor gene nusA, the hflB gene, which is a possible virulence factor, and an ATP-dependent membrane-associated Zn\(^{2+}\) protease and gcp gene, which is a metal dependent endopeptidase gene.

Ribosomal RNA Genes

Ribosomal RNA genes are an essential component of the protein synthesis apparatus and are therefore universally present in all organisms. They are conserved but have sufficient variation to allow distinction between taxa (Woose 1987). Signature sequences of the 16S rRNA gene have been used to distinguish phytoplasmas from other prokaryotes. Ribosomal RNA coding genes occur in multiple copies in eukaryotic genomes sometimes reaching several thousand copies. In prokaryotes, the rRNA gene copy number is far less, averaging three or four copies in a single genome (Fogel et al. 1999). Schneider and Seemüller (1994) analyzed 28 phytoplasmas and found two
copies of rRNA operons, and their work has been confirmed by the sequencing the entire genomes of ‘*Candidatus Phytoplasma asteris*’ (aster yellows witches’ broom phytoplasma), ‘*Ca. Phytoplasma australiense*’, ‘*Ca. Phytoplasma mali*’, and ‘*Ca. Phytoplasma asteris*’ (onion yellows phytoplasma OY-M) (Oshima et al. 2004; Bai et al. 2006; Kube et al. 2008; Tran-Nguyen et al. 2008), which also revealed different gene orientations (Figure 1-1). The exception is Western X-disease phytoplasma, which may have only one copy of the rRNA operon (Kirkpatrick et al. 1987). Phytoplasma ribosomal RNA operons also contain a tRNA\textsuperscript{Ile} in the intergenic spacer between the 16S and the 23S rRNA genes (Razin et al. 1998; Smart et al. 1996). Although analysis of the 16S rRNA gene is the primary parameter for classification of phytoplasmas, the 23S rRNA gene, which is almost twice the size of the 16S rRNA gene, has potential to provide additional information for differentiating strains (Guo et al. 2000). Successful differentiation of the 16SrIV (LY and TPD) strains using 23S rRNA gene, including the TPD strain infecting *S. palmetto*, remain to be explored.

**The 16S-23S Intergenic Spacer Region**

Length and nucleotide polymorphisms of the 16S-23S intergenic spacer (IGS) region between the 16S rRNA and the 23S rRNA genes offers an alternative for classification of phytoplasmas. The IGS region is less conserved than the 16S rRNA gene, has fewer evolutionary constraints, and has proved useful in classifying subspecies of the gram-positive bacterium *Clavibacter michiganensis* (Li and DeBoer 1995). This IGS region of phytoplasmas also has a highly conserved tRNA\textsuperscript{Ile} flanked by variable regions (Lim and Sears 1989; Kuske and Kirkpatrick 1992). These qualities make it useful for detection and differentiation of closely related phytoplasma strains. The classification scheme of Lee and associates (1998), which groups phytoplasmas
according to RFLP profiles of the 16S rRNA gene when combined with sequence analysis of ribosomal protein genes, categorizes phytoplasmas causing lethal diseases of palms as members of group 16SrIV, with the TPD (P. canariensis) phytoplasmas infecting palms in coastal southwestern Texas (Harrison et al. 2002b) and other palm species in west central Florida (Harrison et al. 2008, 2009) as subgroup 16SrIV-D strain. Although a preliminary analysis of the 16S rRNA gene identified the TPD phytoplasma infecting S. palmetto as subgroup 16SrIV-D strain, a population wide survey would allow determination of the diversity of the strain population in Florida and the qualities of the 16S-23S region spacer would be useful for accomplishing this task.

**nusA Gene**

The nusA gene, like the 16S rRNA gene, is ubiquitous and conserved among bacteria (Borukhov et al. 2005). The branching order of a phylogenetic tree inferred from the nusA gene sequence was similar to the branching order inferred from the 16S rRNA gene sequence for the same phytoplasma isolates (Shao et al. 2006) suggesting that this gene has a potential for strain typing. The consistency of the nusA gene in resolving phytoplasma strains was also demonstrated by correlations between nusA phylogenetic trees with trees inferred from the sequences of ribosomal protein genes (Lee et al. 2004), tuf gene sequences (Schneider et al. 1997; Marcone et al. 2000), as well as glycoprotease gene sequences (Davis et al. 2003). This demonstration of nusA as a pertinent taxonomic tool argued for using nusA to differentiate between the TPD and the LY phytoplasmas.

**hflB Gene**

Until a wider array of gene sequences became available, the scope of strain differentiation in phytoplasmas was restricted to the conserved 16S rRNA gene (Lorenz
et al. 1995; Martini et al. 2008; Seemüller and Schneider 2007; Danet et al. 2008). Information generated from completing the sequences of four phytoplasma genomes has made it easier to find genes that have a potential for strain differentiation. In most bacteria, the \textit{hflB} gene is present as a single copy, but up to 24 copies may be present in phytoplasmas (Bai et al. 2006; Arashida et al. 2008). Because the \textit{hflB} gene is present in many copies in phytoplasmas, it is reasonable to suspect that it is a critical biological component. This gene is possibly associated with strain virulence (Beier et al. 1997; Lithgow et al. 2004). Characterizing the \textit{hflB} gene, which possibly has adaptive traits, could give clues to the adaptability of group 16SrIV phytoplasmas. Its inclusion in the differentiation of TPD and LY phytoplasmas was based on its success in resolving strains of ‘\textit{Candidatus Phytoplasma mali}’ (Seemüller and Schneider 2007).

\textbf{O-sialoglycoprotein Endopeptidase Gene, the Glycoprotease (\textit{gcp}) Gene}

Phytoplasmas share a common \textit{gcp} gene that is derived from their bacterial ancestors. This is clearly shown by the homology of portions of the phytoplasmal \textit{gcp} gene with bacterial \textit{gcp} genes. Only one copy of the \textit{gcp} gene is present in phytoplasmas (Gundersen et al. 1994). The protein encoded by the \textit{gcp} gene, O-galactosidase endopeptidase, is possibly a host adaptation and virulence factor and is a member of the M22 peptidase family (Rawlings and Barret 1995 cited by Davis et al. 2003). Because branching of phytoplasmas from other bacteria shown by analysis of the \textit{gcp} gene sequence was similar to the pattern shown by the 16SrRNA gene (Gundersen et al. 1994), the \textit{gcp} gene may have taxonomic value, and its ability to differentiate phytoplasmas within a 16Sr group was an objective of this project. In the study reported herein, the sequence of the \textit{gcp} gene in the TPD phytoplasma was compared with sequences of the gene in other phytoplasma strains.
Problem Statement and Purpose of the Study

Since the first record of TPD in *S. palmetto* in west central Florida in 2008, initial work was directed at identifying this new phytoplasma strain using sequence analysis of 16S rRNA gene. In this initial DNA-based classification, a nested polymerase chain reaction (PCR) assay employing primer pairs P1m5'-TCCTGGGCTCAGGATTAAC-3'/LY16-23Sr5'-TTGAGAATTACGTTGGTTTATCTAC-3' and LY16Sf25'-AACGGGTGAGTAACACGTAAG-3'/LY16-23Sr25'-TTAGACTGGTGCCCTAAATG-3' followed by restriction fragment length polymorphism of the resulting PCR product revealed that this phytoplasma is similar to the strain previously affecting *Phoenix canariensis*, *P. dactylifera*, *P. sylvestris* and *Syagrus romanzoffiana* in Texas and Florida (Harrison et al. 2009). This preliminary work set the stage for complete elucidation of the TPD phytoplasma infecting *S. palmetto*. Although the phytoplasma strain has been classified based on the 16S rRNA sequence as subgroup 16SrIV-D, many questions still remained. For example, how different is the TPD phytoplasma associated with diseased *S. palmetto* from the LY phytoplasma that infects *C. nucifera* and from the TPD phytoplasma previously associated with *P. canariensis* in Texas and Florida and from other palm-associated phytoplasma strains distributed throughout Mexico, the Caribbean and Africa? This study was aimed at genetically characterizing, using informative genes, the TPD phytoplasma that infects *S. palmetto* in Florida and, where possible, compare this pathogen with the TPD strain infecting *P. canariensis*, the coconut LY phytoplasma, and other strains from symptomatic *C. nucifera* or other palms in different geographic locations.
Figure 1-1. Orientation of genes of the ribosomal RNA operon of five phytoplasma species. rrn stands for ribosomal RNA operon.
Figure 1.2. The syndrome of Texas Phoenix decline in *Sabal palmetto* caused by a 16SrIV-D phytoplasma in Hillsborough county in west central Florida. A) Initial stages of the disease beginning with the lower leaves. B) The disease has advanced to mid-crown. C) The spear leaf and inflorescences are dead. D) The entire palm is dead (see white arrow).
CHAPTER 2
MOLECULAR SURVEY OF THE TEXAS PHOENIX DECLINE PHYTOPLASMA POPULATION

Introduction

Until the 1980s, differentiation and classification of phytoplasmas relied solely on biological properties such as host plant range specificity, vector, geographic distribution and symptom differences on affected plants. Because determination of biological properties is time-consuming and sometimes unreliable, there was a need to develop more efficient and reliable methods of study. Nucleic acid-based methods introduced in the late 1980s for studies in phytoplasmas have been less time-consuming and more reliable (Lee et al. 2000; Bertaccini 2007). To date numerous phytoplasma universal primer pairs for use in polymerase chain reaction (PCR) assays have been designed, making detection and differentiation of phytoplasmas more practical. According to molecular analyses based on the PCR-amplified 16S rRNA gene, phytoplasmas form a distinct monophyletic clade within the class Mollicutes (Gundersen et al. 1994; Lim and Sears 1992).

Although the 16S rRNA gene is informative in phytoplasma classification, its usefulness in differentiating closely related phytoplasmas has been limited because of its relatively high level of conservation. Similarities in the 16S rRNA gene between two distinct phytoplasma 16Sr groups range from 88 to 94% and between two subgroups within a given 16Sr group from 95 to 98% (Gundersen et al. 1994). When classifying closely related phytoplasmas, the non-transcribed intergenic spacer (IGS) between the 16S rRNA and the 23S rRNA may offer more variation because of less evolutionary constraints on this region than on the 16S rRNA gene (Barry et al. 1991). The 16S-23S intergenic spacer has a highly conserved tRNA^{Ile} flanked by variable regions (Lim and
Sears 1989; Kuske and Kirkpatrick 1992). Its usefulness was demonstrated when it successfully reinforced clustering of phytoplasmas based on the well-characterized 16S rRNA gene (Kirkpatrick et al. 1994). Association of the Texas Phoenix decline (TPD) phytoplasma with the native sabal or cabbage palm (*Sabal palmetto*) has caused great concern as its biology, its genetic characteristics and the extent of devastation to be expected were not known. The purpose of this work was to survey the composition of the TPD phytoplasma population in west central Florida using the 16S-23S rRNA intergenic spacer region sequence. Reference strains of the lethal yellowing (LY) and other decline phytoplasmas from beyond the west central Florida region were included for comparative purposes.

**Materials and Methods**

**Plant Material and DNA Extraction**

Samples were taken from symptomatic plants. The first set of samples (a total of 24) consisting of interior tissue shavings was collected from the lower stems of symptomatic *S. palmetto* plants with foliar symptoms indicative of decline in west central Florida. Samples were harvested from symptomatic palms in the adjacent counties of Hillsborough and Manatee where diseased *S. palmetto* were most numerous. A second set of stem samples was obtained from nine symptomatic *Phoenix* palms [(Canary Island date (*P. canariensis*), edible date (*P. dactylifera*) and silver date (*P. sylvestris*)], one symptomatic Queen palm (*Syagrus romanzoffiana*) and one symptomatic Mexican fan palm (*Washingtonia robusta*). The latter samples also originated mostly from palms in Hillsborough and Manatee counties, however, three were from palms in Sarasota county. The second set of samples also formed part of a previous study reported by Harrison et al. (2008). A final set of samples were obtained from apical bud tissues from
a symptomatic *S. palmetto* palm in west central Florida (Sabal1), a symptomatic *Cocos nucifera* palm from Broward county in southeastern Florida (LYFL) a symptomatic *C. nucifera* palm in Jamaica (LYJAM) and a symptomatic palm in Mexico (LYMEX5). Information on the phytoplasma samples is given in Table 2-1.

Stem samples were removed from palms by drilling the stem using a portable electric drill fitted with a wood boring bit as previously described (Harrison et al. 2002b), and shavings were collected into clean sealable plastics bags. Apical bud tissues were collected by felling the palm and excising immature leaf bases of the stem apex. Total nucleic acids were extracted from 3 g quantities of stem tissue or from 100 g of bud tissues. DNA from bud tissues was extracted following the phytoplasma enrichment method of Harrison and associates (1994). From stem tissue, DNA was extracted using CTAB extraction buffer according to the procedure of Doyle and Doyle (1990). 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 ethylenediaminetetraacetic acid [EDTA, pH 8]). Presence of DNA in 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 16S1064F 5'-'TTGGAGGAAGGTGGGGATTAC-3'/23SRev 5'-TTCGCTTTCCCTCACGGTACT-3' for the primary reaction and primer pair TPD-16-23SF 5'-AGCTTAAACGCGAGTTTTTGCAAA-3'/TPD-16-23SR5'-GTTTCGCTCGCTGCTACTACCAGA-3' for the nested reaction. These primers were
designed specifically for this study to amplify the 16S-23S rRNA IGS region. Each PCR reaction contained 33.8 µL H₂O; 5 µL buffer (1.675 µL H₂O; 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 MgCl₂); 0.1 µg of each of the two primers; 0.04 mM of each of the dNTPs and 0.2 µL Taq DNA polymerase. The total volume per reaction was 50 µL and the reaction was run for 35 cycles. Each cycle was 94˚C for 1 min, 60˚C for 2 min, and 3 min at 72˚C. The 35 thermal cycles were preceded by 1 min initial denaturation at 94˚C and succeeded by a 7 min final elongation at 72˚C. At the end of the PCR run, 10 µL of the PCR mixture was mixed with 7 µL of gel loading dye, electrophoresed through 1% agarose gel using TAE buffer (40 mM Tris acetate, 1 mM EDTA) and visualized by UV transillumination following staining with ethidium bromide.

**Cloning**

PCR products were purified using Wizard PCR-preps purification kit (Promega Corp, Madison, WI) and then 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 at 37˚C on Luria-Bertani (LB) media amended with isopropyl β-D-1-thiogalactopyranoside and X-gal for blue/white colony screening. After 24 hour 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 hours. Cells were lysed, using lysis buffer, to recover the ligated plasmid vectors. The plasmids were purified, resuspended in TE buffer and sent for sequencing. 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). Database sequence similarity searching was performed using BLAST in NCBI (website: http://www.ncbi.nlm.nih.gov/BLAST). Sequences were compared pairwise using ClustalW (Larkin et al. 2007). Almost all sequences were submitted to NCBI. A phylogenetic tree was constructed from the alignment by the neighbor-joining method using MEGA 4.1 software (Tamura et al. 2007). Only sequences from a subset of the strains were used to infer the phylogenetic tree.

**Restriction Fragment Length Polymorphisms**

Polymerase chain reaction products of the PCR amplified 16S-23S intergenic spacer region were digested separately using restriction enzymes, Asel, Hhal and Rsal (New England BioLabs, Waverley, MA, USA) at 37°C for a minimum of 16 h. These enzymes best differentiated between the phytoplasma strains as shown in a virtual test of sequence data using pDRAW32 (AcaClone, http://www.acaclone.com). 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**

From DNA samples from 39 symptomatic palms, PCR fragments ca. 800 bp in length were amplified by nested PCR assay. No amplification of products was observed in reactions containing DNA from the healthy palm or the water control (Figure 2-1).
Assembled nucleotide sequences derived from PCR fragments were submitted for similarity analysis using BLAST to http://www.ncbi.nlm.nih.gov/BLAST. Significant BLAST matches of phytoplasma origin gave assurance that the PCR fragments were amplified from phytoplasma DNA. Based on analysis of RFLP profiles generated by digestion of each amplified PCR fragment with Asel restriction enzyme, 16SrIV-D strains all had the same profiles which were distinct from the profiles attributed to 16SrIV-A strains (Figure 2-2). However, SP6 and SP7, samples obtained from S. palmetto had each an additional band which distinguished them from all other TPD samples. This could be a result of heterogeneity between the ribosomal RNA operons possessed by phytoplasmas, assuming that 16SrIV-D phytoplasmas have more than one ribosomal operon copy. Two samples (BCT and VW), each containing a mixture of strains 16SrIV-A and 16SrIV-F, had different profiles, one was most similar to the 16SrIV-D subgroup of strains and the other similar to the 16SrIV-A group. The segregation of the mixed strains in this manner was probably due to selective PCR amplification of one strain in the mixture. The 16SrIV-D strain in S. romanzoffiana and the 16SrIV-F strain collected from W. robusta had Asel RFLP profiles most similar to those of the TPD strains.

Restriction enzyme Hhal differentiated the strains similarly except that two strains collected from S. palmetto (SP6 and SP7) showed unique profile (Figure 2-2). Restriction enzyme Rsal also differentiated the strains similarly with SP6 and SP7 again showing unique profiles (Figure 2-2). Restriction fragment digestion by Rsal of the S5-PS PCR-amplified fragment revealed a probable mixed infection. The secondary bands
in some samples in the RFLP profiles are common whenever DNA is amplified from palm samples, and probably indicating non-specific PCR amplification.

The phylogenetic tree that was inferred from the sequence comparisons of the 16S-23S intergenic spacer region showed that all 16SrlV-D strains tested were similar and clustered separately from the 16SrlV-A strains (Figure 2-3) as in the RFLP profiles, with strains VW and BCT that each represented a 16SrlV-A and 16SrlV-F mixture segregating (falling into different clusters - BCT was similar to 16SrlV-A strains and VW to 16SrlV-D strains) as in the RFLP profiles and SP6 and SP7 that showed unique RFLP profiles clustering with other TPD strains. Texas Phoenix decline phytoplasma was first reported in S. palmetto in west central Florida in 2008 (Harrison et al. 2009). Although a similar phytoplasma had been previously reported in P. canariensis in Corpus Christi, Texas and in west central Florida, the attack of S. palmetto by a phytoplasma caused grave concern because S. palmetto is a native species important in the natural landscape of the state of Florida and other states in the southern USA. Characterizing the pathogen population was important in order to understand the phytoplasma population diversity. The 16S-23S intergenic spacer region was chosen in this analysis because it has sufficient heterogeneity to differentiate phytoplasma strains that are closely related. Sequence analysis of 16S-23S rRNA intergenic spacer region from the strains representing the population of the TPD phytoplasma in west central Florida showed that in this region of Florida the phytoplasma population is homogenous. The homogeneity of the TPD phytoplasma is found across host palm species (S. palmetto, Phoenix spp. and S. ramonzoftiana). However, it should be understood that this conclusion was based only on sequence analysis of the 16S-23S intergenic spacer...
region and that other parts of the genome might reveal differences between the phytoplasma strains that were homogenous based on the ribosomal intergenic spacer region. Sequence homogeneity of the 800 bp 16S-23S intergenic spacer region could also mean that only one strain of the phytoplasma was introduced into west central Florida, and since its introduction this strain has multiplied and spread throughout this part of the state. Should this be the case, prediction of the TPD epidemic should be easier than if the phytoplasma population had been heterogeneous. Presently the question still remains about the apparent sudden extension of the host to include S. palmetto. Further characterization of the 16SrIV-D using other regions of the genome is necessary to determine the genetic basis of host specificity of 16SrIV-D phytoplasmas. The clustering of the phytoplasma strains included in this study was significantly correlated with 16SrIV subgroup designation (Table 2-1 and Figure 2-3). In some instances rRNA gene nucleotide polymorphisms may imply biological differences. For instance, in ash yellows phytoplasma, differences in rDNA RFLP patterns were correlated with differences in aggressiveness (Sinclair et al. 2000; Sinclair and Griffiths 2000).
Table 2-1. Phytoplasma samples included in the study, listed with palm host species, location and strain identity. The location is a Florida, United States of America county unless otherwise stated.

<table>
<thead>
<tr>
<th>Phytoplasma sample identity</th>
<th>Palm species</th>
<th>Location</th>
<th>Phytoplasma strain identity</th>
</tr>
</thead>
<tbody>
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<td><em>Sabal palmetto</em></td>
<td>Hillsborough</td>
<td>16SrIV-D</td>
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<td><em>S. palmetto</em></td>
<td>Hillsborough</td>
<td>16SrIV-D</td>
</tr>
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</tr>
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<td>Manatee</td>
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<td><em>P. dactylifera</em></td>
<td>Sarasota</td>
<td>16SrIV-A and 16SIV-F</td>
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<td>16SrIV-F</td>
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<td><em>Cocos nucifera</em></td>
<td>Broward</td>
<td>16SrIV-A</td>
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<td><em>C. nucifera</em></td>
<td>Jamaica</td>
<td>16SrIV-A</td>
</tr>
<tr>
<td>LYMEX5</td>
<td><em>C. nucifera</em></td>
<td>Mexico</td>
<td>16SrIV-A</td>
</tr>
</tbody>
</table>
Figure 2-1. Agarose gel showing polymerase chain reaction amplification of the 16S-23S intergenic spacer region. The fragment (ca. 800 bp) was amplified from DNA extracted from symptomatic palm samples included in this study. The first lane (marked by M) represents uncut lambda DNA which was used as a size marker and the last two lanes show no amplification and represent a healthy plant control and a water control (no DNA template) respectively.
Figure 2-2. Restriction fragment length profiles of phytoplasma DNA (ca. 800 bp) amplified from symptomatic palms. See Table 2-1 for sample identity. The PCR amplification was done by primer pair 16S1064F/23SRev followed by primer pair TPD-16-23SF/TPD-16S23SR. A-C) Digestion was with AseI, D-F) Digestion was with HhaI and G-I) Digestion was with Rsal. M stands for the pGEM molecular size (bp) markers in descending order: 2465, 1605, 1198, 676, 517, 460, 396, 350, 222, 179, 126, 75, 65, 51 and 36.
Figure 2-3. Inferred molecular relationships of phytoplasma strains based on the 16S-23S intergenic spacer sequence. The tree was constructed by the neighbor-joining method and bootstrap values are shown on branches. rRNA means ribosomal RNA operon. The National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) GenBank accession numbers are written in brackets.
CHAPTER 3
DIFFERENTIATION OF PHYTOPLASMA STRAINS CAUSING DECLINE OF SABAL PALMETTO AND COCONUT LETHAL YELLOWING BASED ON SEQUENCES OF THE RIBOSOMAL RNA OPERON

Introduction

According to nested polymerase chain reaction (PCR) assays using primers P1m/LY16-23Sr followed by LY16Sf2/LY16-23Sr2 and subsequent sequence analysis of the amplified fragment, as well as restriction fragment length polymorphisms (RFLPs) of the PCR product, the lethal decline of sabal or cabbage palm (Sabal palmetto) observed in west central Florida in 2008 appears similar to the Texas Phoenix decline (TPD) phytoplasma originally causing decline of the Canary Island date palm (Phoenix canariensis) in Texas, the same strain which was later recorded in edible date (P. dactylifera), silver date (P. sylvestris), Queen (Syagrus romanzoffiana) palms and also in P. canariensis in west central Florida. The phytoplasma infecting S. palmetto belongs to subgroup 16SrIV-D (Harrison et al. 2009).

The ribosomal operon of the TPD phytoplasma infecting S. palmetto was sequenced and annotated, and molecular comparisons with the operons of other palm phytoplasma strains were performed. Other strains belonging to other 16Sr groups were also included in these analyses. Furthermore, the usefulness of the different portions of the ribosomal operon in differentiating between palm phytoplasma strains was also investigated.

Materials and Methods

Plant Material, DNA Extraction Polymerase Chain Reaction and Cloning

Some of the DNA preparations from chapter 2 (Sabal1, PCT 3, LYJAM, S5-PS, S1-QP, SA1) (see Table 2-1) were included in this study. The sequence of the
ribosomal operon of LY from Florida (LYFL) had already been obtained by 454 sequencing (N. Harrison, unpublished data). Fragments of the ribosomal RNA operon were amplified with primer pairs P1/P7; 16S1064F/23SRev; 23S25F/23S1684C; 23S-791F/23S-2757C and C41-8640F/tRNA-phe-31C (Table 3-1) from the DNA preparations. Two controls, DNA from a healthy palm and a water control (no DNA template) were included in the analysis. PCR was conducted as described in chapter 2. For the PCR products that required cloning, the cloning was performed following the procedure outlined in chapter 2.

**Sequence Analysis**

Sequencing, similarity searches and inference of molecular trees were performed as described previously in chapter 2. Ribosomal operon from an entry of *Acholeplasma laidlawii* (GenBank accession number CP000896.1) was used to root the tree. The distance tree drawn using the neighbor-joining algorithm was based on alignment performed using ClustalW (Thompson et al. 1994) with the following settings: gap opening penalty – 5.0, gap extension penalty – 0.2, delay divergent cutoff 30% and DNA transition weight – 0.5.

**Results**

Polymerase chain reaction products of different sizes representing overlapping portions of the ribosomal operon were amplified in the PCR runs. Only fractions from the diseased palms had bands showing amplification by the five primer pairs used. No amplification was observed on the healthy palm or the water control. The PCR fragments were sequenced, and failed sequencing reactions resulted in certain portions of the ribosomal RNA missing in some of the strains included in the study. From each of the two samples whose entire operon was sequenced [*S. palmetto* from west central
Florida (Sabal1) and Cocos nucifera from Jamaica (LYJAM)], the assembled sequence was approximately 5 kb, with a GC content of ca. 44%. Further comparative analysis of the sequence of ribosomal RNA amplified from the TPD phytoplasma showed high similarity between the S. palmetto TPD and other strains included in this study. The ribosomal operon of TPD from S. palmetto was 98% similar to each of the operons of the LY (16SrIV-A) phytoplasmas from C. nucifera or P. canariensis (Figure 3-1). Any of the ribosomal RNA portions amplified from the S. palmetto TPD phytoplasma were 100% similar to corresponding portions from any of the subgroup 16SrIV-D phytoplasmas except for the near full-length 16S rRNA gene of P. sylvestris (S5-PS) which was 99% similar to the S. palmetto TPD 16S rRNA gene (Figure 3-1). The gene orientation from the assembled sequence for each of the strains sequenced in this study was 5′16SrRNAgene-tRNAIlegene-23SrRNAgene-5SrRNAgene-tRNATValgene3′ as represented by the ribosomal RNA operon with defined boundaries for the TPD S. palmetto phytoplasma strain (Figure 3-2).

Phylogenetic trees were inferred using the complete sequences of the ribosomal RNA operon obtained in this study and NCBI BLAST entries of the ribosomal RNA operon of phytoplasmas belonging to other 16Sr groups, Loofah witches’ broom (GenBank accession number AF086621.2), ‘Candidatus Phytoplasma mali’ (GenBank accession number CU469464.1), ‘Ca. Phytoplasma asteris’ OY-M strain (GenBank accession number AP006628.2), ‘Ca. Phytoplasma asteris’ aster yellows witches’ broom strain (GenBank accession number CP000061.1) and ‘Ca. Phytoplasma’ australiense (GenBank accession number AM422018.1). Both ribosomal operons from each of the phytoplasma strains retrieved from NCBI and for the LY strain from C.
nucifera in Florida were included in this molecular analysis except for Loofah witches’–broom for which only one ribosomal operon was analysed.

Phylogenetic analysis based on the entire ribosomal operon showed that although the TPD phytoplasma infecting S. palmetto is different from the LY strains, all the 16SrIV phytoplasmas are more similar to each other than to phytoplasmas belonging to other 16Sr groups (Figure 3-3). Grouping of the phytoplasmas included for molecular analysis agreed with the widely accepted phytoplasma classification of Lee and associates (1998) that employs RFLP profiles of the 16S rRNA gene combined with sequence analysis of ribosomal protein genes, in that group 16SrIV strains, namely LY and TPD, formed a distinct clade from other phytoplasma strains representing other 16Sr groups. All four phylogenetic trees inferred using either 16S rRNA, 16S-23S intergenic spacer, 23S rRNA, or the entire ribosomal RNA operon differentiated the group 16SrIV strains similarly (Figures 3-3, 3-4, 3-5 and 3-6).

**Discussion**

The organization of the ribosomal RNA operon found in the palm yellows phytoplasma strains, whether subgroup 16SrIV-A or 16SrIV-D (i.e. 5’16S-tRNA^Ile^-23S-5S3’) is similar to that of ‘Candidatus Phytoplasma asteris’ OY-M rRNA (Oshima et al. 2004), ‘Ca. Phytoplasma asteris’ aster yellows witches’ broom strain rRNA (Bai et al. 2006), ‘Ca. Phytoplasma mali’ rRNA and rRNB (Tran-Nguyen et al. 2008) and the rRNs of Loofah witches’ broom (Ho et al. 2001). This similarity in gene composition and orientation can be understood by studying Figure 1-1. To amplify the 16S rRNA the universal primer pair P1/P7, which has shown consistency in detecting phytoplasmas (Deng and Hiruki 1991; Smart et al. 1996), was used. Other primers used in this study
were designed, specifically for this study using conserved stretches of the ribosomal operons and on the tRNA\textsuperscript{Phe} gene downstream of the 5S rRNA gene.

The assembled ca. 5 kb sequence covered all the genes of the ribosomal operon. Alignment of the 16SrIV-D phytoplasma infecting \textit{S. palmetto} with LY phytoplasmas (16SrIV-A) showed that there are numerous polymorphisms between the \textit{S. palmetto} TPD and the LY phytoplasmas. Because those polymorphisms occur on a non-coding region, they are unlikely to have biological significance. Biologically, TPD and LY have been recorded in different host plants. Texas Phoenix palm decline is prevalent in \textit{Phoenix} spp. and since 2008 has been observed in \textit{S. palmetto}, whereas LY is associated mainly with \textit{C. nucifera}. Presently no link between the differences in the ribosomal operons of TPD and LY to the differences in the biology of these palm yellows phytoplasma strains can be made. The 2\% dissimilarities in the 16S rRNA gene between TPD and any of the LY strains is insufficient to explain the difference between these phytoplasma strains and, therefore, more work is needed to explore other portions of the genome.

All of the molecular trees — i.e. the trees based on the entire ribosomal operon, 16S rRNA gene, 16S-23S and 23S rRNA gene — were similar, which suggested that ribosomal operon genes evolve uniformly. This similarity in strain clustering, shown with the analysis using the different ribosomal portions, is in agreement with analysis of the ‘\textit{Ca. Phytoplasma}’ genus by Hodgetts and associates (2008) who showed that trees inferred with 16S rRNA, 16S-23S intergenic spacer region and 23S rRNA are similar except for differences in branch lengths. Because the 16S rRNA gene is highly conserved and offers little use in differentiating closely related phytoplasma strains, the
16S-23S intergenic spacer and 23S rRNA were also used to differentiate between phytoplasmas belonging to 16SrIV group. The 23S rRNA gene which is almost twice the size of the 16S rRNA gene was expected to provide additional information for differentiating the strains because previous work conducted on the 23S rRNA gene showed that this gene is useful in differentiating phytoplasma groups with the potential for finer differentiation among the groups (Guo et al. 2000). The 100% sequence similarity between the TPD strain from *S. palmetto* and other decline strains from *Phoenix* spp. and *S. romanoffiana* may mean that a common 16SrIV-D strain infects all these palm species. Although the first record of TPD in the southern USA was made in *P. canariensis* in Texas, it cannot be concluded that Texas is the original source of TPD. The phytoplasma could have long been in west central Florida. Because the vector for the phytoplasma is not known, it is difficult to trace the movement of the phytoplasma. The difference between 16SrIV-D and 16SrIV-A phytoplasmas had already been demonstrated in previous studies (Harrison et al. 2008: Harrison et al. 2009). Studies on palm phytoplasmas have so far shown that the hosts for 16SrIV-D phytoplasmas are *Phoenix* spp., *S. romanoffiana* and *S. palmetto*, and those of 16SrIV-A phytoplasmas are *C. nucifera* and *Phoenix* spp. (Harrison et al. 2008). Presently no host determinants have been discovered in these phytoplasmas and, therefore, the potential host range for each is not known. Because of this, it is important to monitor the landscape and sample tissue from symptomatic palms to identify new palm-phytoplasma associations.
Table 3-1. Primers and product size of polymerase chain reaction amplification of ribosomal RNA operon genes. The genes were amplified from coconut lethal yellowing phytoplasma from Jamaica (LYJAM) and from the Texas Phoenix decline (Sabal1) phytoplasma infecting *Sabal palmetto* in Florida.

<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>Size of the amplified fragment (base pairs)</th>
<th>References for the primers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LYJAM</td>
<td>Sabal1</td>
</tr>
<tr>
<td>P15'AAGAGTTTGATCTGGCTTCTAGATT3'</td>
<td>1847</td>
<td>1849</td>
</tr>
<tr>
<td>P75'CGTCCCTTCATCGGCTT3'</td>
<td>1117</td>
<td>1106</td>
</tr>
<tr>
<td>16S1064F5'TTGGAGGAAGGTGGGGATTAC3'</td>
<td>1626</td>
<td>1626</td>
</tr>
<tr>
<td>23SRev5'TTCGCCCTTCCCTACCGGTAC T3'</td>
<td>1969</td>
<td>1969</td>
</tr>
<tr>
<td>23S25F5'GATGAAAGGACGCAATTAACG3'</td>
<td>807</td>
<td>867</td>
</tr>
<tr>
<td>23S1684C5'TGCCAGTTCCCTTAACAAGA3'</td>
<td>807</td>
<td>867</td>
</tr>
<tr>
<td>23S-791F5'TCTCCCGAAATAGCTTTAGG3'</td>
<td>807</td>
<td>867</td>
</tr>
<tr>
<td>23S-2757C 5'GTCTTTCAGGGATCTCATAGG3'</td>
<td>807</td>
<td>867</td>
</tr>
<tr>
<td>C41-8640F5'GCATGGAAGGGGCCAGTACTC3'</td>
<td>807</td>
<td>867</td>
</tr>
<tr>
<td>tRNA-phe-31C5'GCCACTGCTCCTACCGACTGA3'</td>
<td>807</td>
<td>867</td>
</tr>
</tbody>
</table>

The phytoplasma samples listed are the ones whose entire ribosomal operon was sequenced in this study.
Figure 3-1. Sequence comparison of the ribosomal RNA operon. Texas Phoenix decline (TPD) phytoplasma (isolate Sabal1) was compared with other phytoplasma strains sampled from palms showing symptoms of lethal yellowing (LY) and decline. The numbers indicate base positions on the Sabal1 operon. Red lines indicate 100% sequence similarity, green 99%, and brown 98%. Strains compared are LY from Cocos nucifera in Jamaica (LYJAM), LY phytoplasma from C. nucifera in Florida (LYFL), TPD strain from Phoenix canariensis in Texas (PCT4), TPD strain from P. sylvestris in Florida (S5-PS), TPD strain from Syagrus romanzoffiana in Florida (S1-QP) and an LY strain from P. canariensis in Florida (SA1). rRNA stands for ribosomal RNA operon. The diagram is not drawn according to scale. The sequences are represented in the 5’ to 3’ direction.
Figure 3-2. Schematic representation of the ribosomal operon of the Texas Phoenix decline phytoplasma strain isolated from *Sabal palmetto*. Numbers represent base positions of the boundaries of the four genes that make up the operon as well as the tRNA\textsuperscript{Val} downstream of the 5S rRNA gene. Gene orientation is in the 5' to 3' direction.
Figure 3-3. Inferred molecular relationship of phytoplasma strains based on ribosomal RNA operon (rrns). Genes 16S rRNA: tRNAile. 23S rRNA: 5S rRNA were analyzed using the neighbor-joining method. For lethal yellowing strain from Florida (LYFL), ‘Candidatus Phytoplasma mali’, ‘Ca. Phytoplasma australiense’, ‘Ca. Phytoplasma asteris’ (OY-M strain), and ‘Ca. Phytoplasma asteris’ Aster yellows witches’ – broom (AYWB), both ribosomal RNA copies were included, whereas one copy was included for Texas Phoenix decline (Sabal1) phytoplasma and the lethal yellowing strain from Jamaica (LYJAM). Phytoplasma strains that had their ribosomal RNA operon sequenced in this study are in bold type. Numerical labels at branch nodes are for bootstrap values. National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) GenBank accession numbers are written in brackets.
Figure 3-4. Inferred molecular relationship of phytoplasma strains based on 16S ribosomal RNA (rrns) gene using the neighbor-joining method. For lethal yellowing strain from Florida (LYFL), ‘Candidatus Phytoplasma mali’, ‘Ca. Phytoplasma australiense’, ‘Ca. Phytoplasma asteris’ (OY-M strain), and ‘Ca. Phytoplasma asteris’ Aster yellows witches - broom (AYWB), both ribosomal RNA copies were included, whereas one copy was included for Texas Phoenix decline (Sabal1) phytoplasma and the lethal yellowing strain from Jamaica (LYJAM). Phytoplasma strains that had their 16S rRNA gene sequenced in this study are in bold type. Numerical labels at branch nodes are for bootstrap values. National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) GenBank accession numbers are written in brackets.
Figure 3-5. Inferred molecular relationship of phytoplasma strains based on 16S-23S intergenic spacer using the neighbor-joining method. For lethal yellowing strain from Florida (LYFL), ‘Candidatus Phytoplasma mali’, ‘Ca. Phytoplasma australiense’, ‘Ca. Phytoplasma asteris’ (OY-M strain), and ‘Ca. Phytoplasma asteris’ Aster yellows witches - broom (AYWB), both intergenic spacer copies were included, whereas one copy was included for each of Texas Phoenix decline (Sabal1) phytoplasma and lethal yellowing strain from Jamaica (LYJAM). Phytoplasma strains that had their 16S-23S intergenic spacer sequenced in this study are in bold type. Numerical labels at branch nodes are for bootstrap values. National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) GenBank accession numbers are written in brackets.
Loofah witches-broom phytoplasma (AF086621)

Acholeplasma laidlawii rrna (CP000896)

Sabal1 rrn (HQ613895)

LYJAM rrn (HQ613873)

LYFL rrna (HQ613874)

LYFL rrnb (HQ613875)

0.02
Figure 3-6. Inferred molecular relationship of phytoplasma strains based on 23S ribosomal RNA (rrns) gene using the neighbor-joining method. For lethal yellowing strain from Florida (LYFL), ‘Candidatus Phytoplasma mali’, ‘Ca. Phytoplasma australiense’, ‘Ca. Phytoplasma asteris’ (OY-M strain), and ‘Ca. Phytoplasma asteris’ Aster yellows witches - broom (AYWB), copies from both ribosomal RNA operons were included, whereas one copy was included for Texas Phoenix decline (Sabal1) phytoplasma and lethal yellowing strain from Jamaica (LYJAM). Phytoplasma strains sequenced in this study are in bold type. Numerical labels at branch nodes are for bootstrap values. National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) GenBank accession numbers are written in brackets.
LYFL rRNA (HQ613874)
LYFL rRNB (HQ613875)
LYJAM rRN (HQ613873)
Sabal1 rRN (HQ613895)
Loofah witches-broom phytoplasma (AF086621)
‘Ca. P. mali’ rRNA (CU469464.1)
‘Ca. P. mali’ rRNB (CU469464.1)
‘Ca. P. australiense’ rRNA (inverted) (AM422018.1)
‘Ca. P. australiense’ rRNB (AM422018.1)
‘Ca. P. asteris’ OY-M rRNA operon (AP006628.2)
‘Ca. P. asteris’ OY-M rRNB operon (AP006628.2)
‘Ca. P. asteris’ AYWB rRNA operon (CP000061.1)
‘Ca. P. asteris’ AYWB rRNB operon (CP000061.1)
Acholeplasma laidlawii rRNA (CP000896)
CHAPTER 4
GENETIC CHARACTERIZATION OF SUBGROUP 16SrIV-D PHYTOPLASMA INFECTING SABAL PALMETTO USING HFLB, NUSA AND GLYCOPROTEASE GENE SEQUENCES

Introduction

Based on consistent observations of phytoplasmas in symptomatic tissues of palms growing in Florida, lethal diseases of palms are caused by phytoplasmas in that part of the world (Thomas 1979). In addition, remission of symptoms on palms in response to tetracycline treatment further implicates phytoplasmas as the causal agents of palm lethal diseases in Florida (McCoy et al. 1983). The Texas Phoenix decline (TPD) phytoplasma identified in sabal or cabbage palm (Sabal palmetto) in west central Florida in 2008 had to be characterized to elucidate its identity, and based on the sequence of portions of the ribosomal RNA operon, this phytoplasma is similar to the TPD strains found in Canary Island date (Phoenix canariensis), edible date (P. dactylifera), silver date (P. sylvestris) and Queen (Syagrus romanzoffiana) palms and different from all known subgroups 16SrIV-A strains associated with Cocos nucifera and P. canariensis (Harrison et al. 2009). By characterization of the ribosomal RNA operon of the phytoplasma strain from S. palmetto, it was found that this strain was similar to an already known strain - i.e. the strain that infects Phoenix spp. and S. romanzoffiana, and different from all 16SrIV-A strains. However, since S. palmetto was a new host and also an endemic palm species, more characterization of the phytoplasma strain was necessary. The purpose of this study was to amplify, sequence and characterize the nusA, hflB and gcp genes from the subgroup 16SrIV-D phytoplasma causing TPD in S. palmetto and use the obtained sequences to compare S. palmetto TPD with other TPD
strains found in *Phoenix* spp. and *S. romanzoffiana* as well as with palm lethal yellowing (LY) and LY-like phytoplasma strains collected from different geographic localities.

**Materials and Methods**

**Plant Material and Polymerase Chain Reaction**

Details of samples used in the experiment are illustrated in Table 4-1. For the *nusA* gene, nested polymerase chain reactions (PCR) were conducted using the primer pair nusA-F1/nusA-R1 followed by nusA-F2/nusA-R2. For the two copies of the *hflB* gene PCR amplification was done using primers hflB2-f1/hflB2-r1 for one copy and primers hflB1-f2/hflB1-r2 for the other copy. The nested PCR assay for the *gcp* gene required GCPF3/GCPR2 for the primary and GCPF1/GCPR1 for the nested run. Primer sequences for the PCR amplifications are listed in Table 4-2. PCR and PCR product purification were conducted as previously described in chapter 2 except that the annealing temperature was 50°C in the *nusA* gene PCR runs and 56°C in the *hflB* gene PCR runs.

**Cloning, Sequencing and Restriction Fragment Length Polymorphisms**

Cloning and sequencing PCR fragments was conducted according to the procedure explained in chapter 2. Analysis of restriction fragment length polymorphism was performed on the PCR fragments of the *nusA*, *hflB* and *gcp* genes. For the *nusA* gene, the restriction enzymes used were *Ddel*, *EcoRI*, *Hhal* and *Rsal* (incubation at 37°C for a minimum of 16 h). For the *hflB* gene, separate digestions of the obtained PCR products were performed using, for the primers hflB2-f1/hflB2-r1, restriction enzymes *AluI*, *Apol*, *Dral*, *EcoRI*, *Hhal*, *HindIII*, *Msel*, *Rsal*, *Sau3AI*, *Sspl*, and *TaqI* and for the hflB1-f2/hflB1-r2 PCR product *AluI*, *Apol*, *Asel*, *BstUI*, *Ddel*, *Dral*, *HaellIII*, *Hhal*, *HindIII*, *Msel*, *Sau3AI*, *Sspl* and *Tspl*. For the *gcp* gene *Rsal* was selected for the RFLP
analysis. These restriction enzymes were purchased from New England BioLabs, Waverley, MA, USA. Virtual tests performed on pDRAW32 (AcaClone, http://www.acaclone.com) preceded the actual RFLP experiment. Products of the restriction digests were separated by electrophoresis through 8% denaturing polyacrylamide gel in TBE (90 mM Trisborate, 2 mM EDTA) buffer. Profiles were visualized using a UV transillumination following staining with ethidium bromide.

**Sequence Analysis**

Sequences of the amplified DNA fragments of the *nusA*, *hflB* and *gcp* genes from tissue of the symptomatic plants examined were assembled as described in chapter 2. Database similarity searches and inference of the phylogenetic tree were also done following the procedure previously described in chapter 2. Pairwise similarity comparison was performed by ClustalW (Larkin et al. 2007). For the *gcp* gene a phylogenetic tree was constructed from the alignment by the neighbor-joining method using MEGA 4.1 software (Tamura et al. 2007). The tree was based on sequences of the *gcp* gene obtained in this study and sequences of organisms retrieved through the NCBI (www.ncbi.nlm.nih.gov) BLAST.

**Results and Discussion**

*nusA* Gene

**Polymerase chain reaction and analysis by restriction fragment length polymorphism**

The nested primers nusA-F2/nusA-R2 targeting the *nusA* gene amplified a fragment ca. 1.2 kb from DNA samples collected from symptomatic plants (Sabal1, LYFL and LYJAM) (Figure 4-1). No amplification was observed from the healthy palm or the water controls. Polymerase chain reaction products resulting from amplification of
the nusA gene fragment from *S. palmetto* (Sabal1), *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* (Sabal1) was clearly distinguished from the *C. nucifera* LY phytoplasmas (LYFL, LYJAM and LYMEX5) similar to the prediction by the pDRAW32 software (AcaClone, http://www.acaclone.com) (Figure 4-2). These RFLP results suggest that the phytoplasma causing decline of *S. palmetto* is different from LY strains in *C. nucifera* reinforcing the results obtained by analysis of the ribosomal RNA operon in chapters 2 and 3.

**Molecular comparisons**

Nested primers nusA-F2/nusA-R2 targeting the nusA gene amplified PCR fragment from DNA extracted from tissue of palms showing symptoms of decline and yellowing. Based on pairwise similarity comparison of approximately 600 bases of the nusA gene, the two LY strains were more similar to each other (99%) than to the *S. palmetto* (Sabal1) strain which was 92% or 93% similar to LY (Table 4-3). From the results of the nusA gene, it could be concluded that the phytoplasma infecting *S. palmetto* is different from the phytoplasma infecting *C. nucifera*. These results were positively correlated with those based on analysis of the ribosomal operon (Chapter 2 and Chapter 3).

**hflB Gene**

**Polymerase chain reaction**

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. Smaller fragments about 0.6 kb bases were amplified from the same samples when primers
hflB2-f1 and hflB2-r1 were used. No amplification was observed in the healthy palm or the water controls.

**Analysis by restriction fragment length polymorphism**

Two sets of products of PCRs, the first set primed by hflB2-f1 and hflB2-r1 from *S. palmetto* (Sabal1) and *C. nucifera* (LYMEX5), and the second primed by hflB1-f2/hflB1-r2 from the same plant samples, were digested separately using restriction enzymes. Patterns representing these digestions are illustrated in Figure 4-3. Sabal1 and LYMEX5 were differentiated clearly when the PCR fragment obtained by amplification using hflB1-f2/hflB1-r2 were digested with restriction enzymes *Apol, Asel, Ddel, Dral, Hhal, HindIII, Sau3AI* and *Tsp509I* (Figure 4-3 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 *Apol, Ddel, TaqI* and *Tsp509I* (Figure 4-3 B-F). In Figures 4-3 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 *hflB* gene also show that the TPD phytoplasma from *S. palmetto* is different from the LY phytoplasma from *C. nucifera*.

**Molecular comparisons**

The 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* (Sabal1). 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.

**gcp Gene**

Polymerase chain reaction and analysis by restriction fragment length polymorphism

Seventeen PCR products corresponding to approximately 1.5 kb nucleotides (with about 1 kb representing *gcp* gene) were amplified using the *gcp* gene primers for all the phytoplasmas strains listed in Table 4-1. No amplification was observed for the healthy plant or the water controls (Figure 4-4). The RFLP analysis of the *gcp* gene fragment shows that 16SrIV-D strains are different from 16SrIV-A strains as shown by the digestions of the *gcp* PCR fragment by *RsaI* restriction enzyme (Figure 4-5). The RFLP was performed on samples fewer than the samples on which the PCR testing was performed, due to the insufficiency of diseased plant tissue.

Molecular comparisons by phylogenetic analysis

Sequence analysis of the *gcp* gene showed that the strains belonging to subgroup 16SrIV-A, namely, the Mexican strains LYMEX3 and LYMEX5, the Florida strain LYFL, the Jamaican strain LYJAM, SA1 strain from *P. canariensis*, the subgroup 16SrIV-C Tanzanian strain LDT and the group 16SrXXII Nigerian strain Awka, are all similar (Figure 4-6). Another cluster grouped together the TPD phytoplasma strains (16SrIV-D), Sabal1 from *S. palmetto*, CID3, PCT3, PCT4, JLL, RPA and SEG obtained from
Phoenix spp., and the coconut lethal decline strain CLDO from C. nucifera in Honduras, and the COYOL strain from Acrocomia aculeata (Jacq.) Lodd. ex Mart. in Honduras (Figure 4-6). The TPD phytoplasma is the only phytoplasma known to be associated with S. palmetto. Analysis of the sequence of the gcp gene demonstrated that the TPD phytoplasma strain infecting S. palmetto is not different from the TPD strains infecting Phoenix spp., A. aculeata, C. nucifera and S. romanztsoffiana (Figure 4-6). When analyzing TPD phytoplasma strains, the gcp gene has not proved more variable relative to the 16S rRNA gene where the TPD strains are 98.2% to 100% similar (Harrison et al. 2008). It was also shown that the gcp gene is not variable among subgroup 16SrIV-A and group 16SrXXII strains. Based on evidence gathered in this study it can be concluded that the TPD strain infecting S. palmetto is similar to TPD strains infecting Phoenix spp. However, it is possible that strain variation may be revealed by studying other genes.
Table 4-1. Phytoplasma samples included in this study listed with host palm species and location. The origin of the samples is the United States of America unless otherwise mentioned.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Disease</th>
<th>Palm species</th>
<th>Strain</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Awka</td>
<td>Awka wilt</td>
<td><em>Cocos nucifera</em></td>
<td>16SrXXII</td>
<td>Nigeria</td>
</tr>
<tr>
<td>CID3</td>
<td>Texas Phoenix decline</td>
<td><em>Phoenix canariensis</em></td>
<td>16SrIV-D</td>
<td>Sarasota county, Florida</td>
</tr>
<tr>
<td>CLDO</td>
<td>Coconut lethal decline</td>
<td><em>C. nucifera</em></td>
<td>16SrIV-D</td>
<td>Olancho, Honduras</td>
</tr>
<tr>
<td>COYOL</td>
<td>Coyol palm decline</td>
<td><em>Acrocomia aculeata</em></td>
<td>16SrIV-D</td>
<td>Olancho, Honduras</td>
</tr>
<tr>
<td>JLL</td>
<td>Texas Phoenix decline</td>
<td><em>Phoenix spp.</em></td>
<td>16SrIV-D</td>
<td>Corpus Christi, Texas</td>
</tr>
<tr>
<td>LDT</td>
<td>Lethal disease</td>
<td><em>C. nucifera</em></td>
<td>16SrIV-C</td>
<td>Tanzania</td>
</tr>
<tr>
<td>LYFL</td>
<td>Lethal yellowing</td>
<td><em>C. nucifera</em></td>
<td>16SrIV-A</td>
<td>Broward county, Florida</td>
</tr>
<tr>
<td>LYJAM</td>
<td>Lethal yellowing</td>
<td><em>C. nucifera</em></td>
<td>16SrIV-A</td>
<td>Jamaica</td>
</tr>
<tr>
<td>LYMEX3</td>
<td>Lethal yellowing</td>
<td><em>C. nucifera</em></td>
<td>16SrIV-A</td>
<td>Yucatán Peninsula, Mexico</td>
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<tr>
<td>LYMEX5</td>
<td>Lethal yellowing</td>
<td><em>C. nucifera</em></td>
<td>16SrIV-A</td>
<td>Yucatán Peninsula, Mexico</td>
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<tr>
<td>PCT3</td>
<td>Texas Phoenix decline</td>
<td><em>P. canariensis</em></td>
<td>16SrIV-D</td>
<td>McClellan, Texas</td>
</tr>
<tr>
<td>PCT4</td>
<td>Texas Phoenix decline</td>
<td><em>P. canariensis</em></td>
<td>16SrIV-D</td>
<td>McClellan, Texas</td>
</tr>
<tr>
<td>RPA</td>
<td>Texas Phoenix decline</td>
<td><em>P. dactylifera</em></td>
<td>16SrIV-D</td>
<td>Hillsborough county, Florida</td>
</tr>
<tr>
<td>S1-QP</td>
<td>Texas Phoenix decline</td>
<td><em>Syagrus romanzoffiana</em></td>
<td>16SrIV-D</td>
<td>Manatee county, Florida</td>
</tr>
<tr>
<td>SA1</td>
<td>Lethal yellowing</td>
<td><em>P. canariensis</em></td>
<td>16SrIV-A</td>
<td>Manatee county, Florida</td>
</tr>
<tr>
<td>Sabal1</td>
<td>Texas Phoenix decline</td>
<td><em>Sabal palmetto</em></td>
<td>16SrIV-D</td>
<td>Hillsborough county, Florida</td>
</tr>
<tr>
<td>SEG</td>
<td>Texas Phoenix decline</td>
<td><em>P. canariensis</em></td>
<td>16SrIV-D</td>
<td>Hillsborough county, Florida</td>
</tr>
</tbody>
</table>

*a* Positive amplification with *nusA*-F2/nusA-R2.

*b* Positive amplification with *hflB2*-f1/hflB2-r1 and *hflB1*-f2/hflB1-r2.

*c* Positive amplification with GCPF1/GCPR1.

These primer pairs were tested on all the samples.
Table 4-2. Primers used to amplify phytoplasma gene products from total DNA extracted from symptomatic plants

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Primer sequence</th>
<th>Reference for the primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>nusA</td>
<td>nusA-F1 5'-ATTTTGTTATTTTTGAAGGAGTGTT-3'</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>nusA-R1 5'-AAAAAGCT TCATGACCCGGAGATCTA-3'</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>nusA-F2 5'-ACATCTAAAGCTGAATTAGGACA-3'</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>nusA-R2 5'-GCACCAATATGTTGAGTAATTCAA-3'</td>
<td>This study</td>
</tr>
<tr>
<td>hflB</td>
<td>hflB2-f1 5'-CCAGAAAATTATGATCCAGATGTTATA-3'</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>hflB2-r1 5'-CTACAGAAAAACTCTCAATAAG-3'</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>hflB1-f2 5'-TGGTTGGAACCAGAAGATCCTTATT-3'</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>hflB1-r2 5'-TTGTTGTCGGTGGAGAAAATTTG-3'</td>
<td>This study</td>
</tr>
<tr>
<td>gcp</td>
<td>GCPF3 5'GATAGGCCAGGTTCTTA3'</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>GCPR2 5' TCCGGAGGAAAACGAGTTA 3'</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>GCPF1 5' GGTACACGTCTAGCTGTTGTA 3'</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>GCPR1 5'CCGGAGGAAAACGAG TTATT3'</td>
<td>This study</td>
</tr>
</tbody>
</table>

Table 4-3. Sequence comparisons of the nusA gene. Comparison was between sequence of a fragment of the nusA gene of the Texas Phoenix decline (Sabal1) phytoplasma infecting Sabal palmetto with phytoplasma strains from Cocos nucifera [Lethal yellowing (LY) from Florida (LYFL) and LY from Jamaica (LYJAM)]

<table>
<thead>
<tr>
<th>Name</th>
<th>Length (base pairs)</th>
<th>Name</th>
<th>Length (base pairs)</th>
<th>Score (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LYFL</td>
<td>603</td>
<td>LYJAM</td>
<td>603</td>
<td>99</td>
</tr>
<tr>
<td>LYFL</td>
<td>603</td>
<td>Sabal1</td>
<td>602</td>
<td>92</td>
</tr>
<tr>
<td>LYJAM</td>
<td>603</td>
<td>Sabal1</td>
<td>602</td>
<td>93</td>
</tr>
</tbody>
</table>

The alignment and calculation of sequence similarity comparison was done by ClustalW (Larkin et al. 2007).
Figure 4-1. Nested PCR products amplified using primer pair nusA-F1 and nusA-R1 followed by nusA-F2 and nusA-R2. Lane 1. Lambda DNA-HindIII digest; Lane 2. Empty lane. Lane 3. Sabal palmetto (Sabal1); Lane 4. Lethal yellowing on coconut, Florida (LYFL); Lane 5. Lethal yellowing on coconut, Jamaica (LYJAM); Lane 6. Healthy control; Lane 7. Water control.

Figure 4-2. Restriction fragment length polymorphism of nusA-F2 and nusA-R2 PCR product. The nusA gene fragment was amplified from DNA fractions of Sabal palmetto (Sabal1), Cocos nucifera (LYFL, LYJAM, LYMEX5) and was digested with restriction enzymes Ddel, EcoRI, Hhal and Rsal. M on the first lane is for 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.
Figure 4-3. Restriction fragment length polymorphism of phytoplasma hflB gene copies. Polymerase chain reaction (PCR) amplicon was amplified with different primer pairs from DNA of declining *Sabal palmetto* and *Cocos nucifera* palms showing yellowing symptoms. A) PCR fragment amplified with hflB1-f2/hflB1-r2 from *C. nucifera* and *S. palmetto* was digested with Asel and DraI. B) PCR fragment amplified with hflB2-f1/hflB2-r1 from *C. nucifera* and *S. palmetto* was digested with *Apol*. C) PCR fragment amplified with hflB2-f1/hflB2-r1 from *S. palmetto* was digested with *Ddel*. D) PCR fragment amplified with hflB2-f1/hflB2-r1 from *C. nucifera* was digested with *Ddel*. E) PCR fragment amplified with hflB2-f1/hflB2-r1 from *S. palmetto* and *C. nucifera* was digested with *AluI, DraI, EcoRI, Hhal, HindIII, Sau3AI*. F) PCR fragment amplified with hflB2-f1/hflB2-r1 from *S. palmetto* and *C. nucifera* was digested with *Msel, Rsal, SspI, TaqI* and *Tsp509I*. G) PCR fragment amplified with hflB1-f2/hflB1-r2 from *S. palmetto* and *C. nucifera*. H) PCR fragment amplified with hflB1-f2/hflB1-r2 from *S. palmetto* and *C. nucifera* was digested with *Sau3AI, Msel, SspI* and *TspI*. 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.
<table>
<thead>
<tr>
<th></th>
<th>S. palmetto</th>
<th>C. nucifera</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>Ase I</td>
<td>Ase I</td>
</tr>
<tr>
<td></td>
<td>Dral I</td>
<td>Dral I</td>
</tr>
</tbody>
</table>

**PCR fragment amplified with**

- hflB1-f2/hflB1-r2
- hflB2-f1/hflB2-r1

**A**

**B**

<table>
<thead>
<tr>
<th></th>
<th>S. palmetto</th>
<th>C. nucifera</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ddel</td>
<td>Ddel</td>
</tr>
</tbody>
</table>

**PCR fragment amplified with**

- hflB2-f1/hflB2-r1

**C**

**D**
Figure 4-3. Continued
Figure 4-4. Agarose gel electrophoresis showing amplification of the glycoprotease (gcp) gene in different DNA samples collected from symptomatic palms. The first lane is HindIII digested lambda DNA serving as a size marker, and the last two lanes are healthy palm and water controls (no phytoplasma DNA) respectively.
Figure 4-5. Restriction fragment length polymorphisms of a polymerase chain reaction (PCR) fragment amplified with primer pair GCPFI/GCPR1. DNA preparations were from symptomatic tissue of palms from various localities. PCR fragment was digested with enzyme *RsaI*. 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.
Figure 4-6. Molecular tree of the glycoprotease (gcp) gene sequences of palm lethal disease strains inferred by neighbor-joining method. Approximately 470 bases of the gcp gene were used to infer the tree. Phytoplasma strains (as listed in Table 4-1) sequenced in this study are in bold type, and the rest were retrieved from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) GenBank. GenBank accession number are written in brackets.
PCT3 *P. canariensis* 16SrIV-D (HQ613877)
- JLL *Phoenix* spp. 16SrlV-D (HQ613888)
- COYOL *Acrocomia aculeata* 16SrlV-D (HQ613876)
PCT4 *P. canariensis* 16SrlV-D (HQ613884)
RPA *P. dactylifera* 16SrlV-D (HQ613878)
CLDO *Cocos nucifera* 16SrlV-D (HQ613887)
SEG *Phoenix canariensis* 16SrlV-D (HQ613879)
CID3 *P. canariensis* 16SrlV-D (HQ613886)
Sabal1 *Sabal palmetto* 16SrlV-D (HQ613883)

---

S1-QP *Syagrus romanzoffiana*
LYMex3 *C. nucifera* 16SrlV-A (HQ613881)
LDT *C. nucifera* 16SrlV-C (HQ613889)
Awka *C. nucifera* 16SrlXXII (HQ613885)
LYMex5 *C. nucifera* 16SrlV-A (HQ613882)
SA1 *P. canariensis* 16SrlV-A (HQ613880)
LYFL *C. nucifera* 16SrlV-A (HQ613890)
LYJAM *C. nucifera* 16SrlV-A (Identical to LYFL)

---

PnWB (AY327624)
CX (AY327623)
P-ulmi (AY327625)
P-mali (AY327626)
BBS (AY327620)
PaWB (AY327621)
AY-Md (AY327618)
IOWB (AY327622)
CPh (AY348874)
CPh (AY327627)
TBB (AY327619)
AYWB (CP000061)

*Acholeplasma laidlawii* (CP000896)

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0.0
CHAPTER 5
RESULTS OBTAINED AND CONCLUDING REMARKS

The population of TPD phytoplasma (subgroup 16SrIV-D) infecting *Sabal palmetto* in west central Florida was surveyed, and it was found that the phytoplasma strain population in that region is genetically homogenous. This was determined through molecular analysis of the 16S-23S intergenic spacer (IGS) region and verified by analysis of restriction fragment length polymorphisms. It was hypothesized that a single strain was introduced into west central Florida. Because this *S. palmetto* strain is similar to the strain infecting *Phoenix canariensis*, *P. dactylifera*, *P. sylvestris* and *Syagrus ramonzoffiana*, it was also hypothesized that the same phytoplasma strain affects these other palm species. Sequence analysis of the entire ribosomal RNA operon and glycoprotease (*gcp*) gene reinforced these conclusions. Furthermore, the TPD phytoplasma (subgroup 16SrIV-D) was shown to be different from the lethal yellowing (LY) phytoplasma (subgroup 16SrIV-A) common in *Cocos nucifera* as shown by sequence analysis of the ribosomal RNA operon, and the *nusA*, *hflB*, and *gcp* genes.

Although the development of TPD epiphytotics in west central Florida could not be completely elucidated, speculations about the most recent events could be made. TPD in *Phoenix* spp. was first observed in 2006, coinciding with massive property development, which resulted in increased *Phoenix* plantings. Presumably, the onset of mild winters in west central Florida could have favored disease development. In 2008, observations of *S. palmetto* with similar foliar discoloration in west central Florida as the *Phoenix* spp. were reported. The most plausible explanation for the infection of *S. palmetto* was that the same phytoplasma infecting *Phoenix* spp. was spread by a vector or vectors to the *S. palmetto* in the vicinity. This explanation can be hypothesized from
this work, which found similarities between the TPD phytoplasma in *Phoenix* spp. and the strain in *S. palmetto*. However, further comparative studies are required. Sequence analysis of more genes and a search for the insect vector for the TPD phytoplasma will help further understand the epidemiology of TPD.
## Appendix

### Some Phytoplasmas Referred to in This Manuscript

<table>
<thead>
<tr>
<th>Phytoplasma Identity</th>
<th>Host plant</th>
<th>16Sr group/subgroup</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Candidatus Phytoplasma asteris' OY-M</td>
<td>Onion</td>
<td>16SrI</td>
</tr>
<tr>
<td>'Ca. Phytoplasma asteris' aster yellows witches'-broom</td>
<td>Numerous</td>
<td>16SrI</td>
</tr>
<tr>
<td>'Ca. Phytoplasma australiense'</td>
<td>Numerous</td>
<td>16SrXII</td>
</tr>
<tr>
<td>'Ca. Phytoplasma mali'</td>
<td>Numerous</td>
<td>16SrIV</td>
</tr>
<tr>
<td>'Ca. Phytoplasma palmae' lethal yellowing (LY)</td>
<td>Cocos nucifera</td>
<td>16SrIV-A</td>
</tr>
<tr>
<td></td>
<td>Phoenix spp.</td>
<td></td>
</tr>
<tr>
<td>Lethal disease Tanzania</td>
<td>C. nucifera</td>
<td>16SrIV-C</td>
</tr>
<tr>
<td>Texas Phoenix decline</td>
<td>Sabal palmetto</td>
<td>16SrIV-D</td>
</tr>
<tr>
<td></td>
<td>Phoenix spp.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Syagrus romanzoffiana</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Washingtonia robusta</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phoenix spp.</td>
<td>16SrIV-F</td>
</tr>
</tbody>
</table>

Source: Harrison et al. 2008; Lee et al. 1998.
List of References


BIIOGRAPHICAL SKETCH

Khayalethu Ntushelo was born in South Africa in 1973. As a child, he developed a passion for learning. His interest in science grew in high school and after graduating with matric, he studied towards a Bachelor of Science (botany and microbiology) degree at the University of the Western Cape, South Africa. Obtaining his B.S. degree in 1995, he proceeded to study towards a Master of Science degree majoring in plant pathology at the University of Stellenbosch, South Africa. A short spell after earning his Master of Science degree found him with employment as a plant pathologist at the Agricultural Research Council (ARC) of South Africa. His career experiences at the ARC helped him to make informed choices on the field of study in which to undertake his Ph.D and also the university where he could enroll for his doctorate.

With the help of, among other people Drs. Raghavan Charudattan and Monica Elliott of the Department of Plant Pathology, University of Florida, Khayalethu would then pursue his ambition. He enrolled for a Ph.D degree in plant pathology at the Department of Plant Pathology, University of Florida, in the fall of 2007, and his research focused on molecular characterization of a phytoplasma associated with Sabal palmetto. He conducted his research under the guidance of Dr. Nigel Harrison in the Department of Plant Pathology, Fort Lauderdale Research and Education Center, University of Florida. Khayalethu received his Ph.D degree in December 2010.