

# Detection and identification of lethal yellowing phytoplasma 16SrIV-A and D associated with *Adonidia merrillii* palms in Mexico

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**Abstract** *Adonidia merrillii* is a palm species native to the Philippines and commonly known as the manila palm. Lethal yellowing-type symptoms were first observed for the manila palm in Florida, USA. Manila palms in Mexico have not been reported to carry the lethal yellowing-type phytoplasma and moreover we have documented other symptoms that are not commonly associated to lethal yellowing (LY) disease. Therefore, our objective was to use molecular techniques to identify and characterize the presence of phytoplasmas in manila palms from southeastern Mexico. Thirty-three manila palm plants were analyzed. In general, three syndromes were observed: mature leaf browning, spear leaf necrosis and spear leaf opening. The presence of phytoplasma DNA was detected by nested-PCR using universal and LY-specific primers. Of the 33 palms analyzed, 24 (73%) were positive for the presence of phytoplasmas using universal primers, and 18 (55%) were positive for phytoplasma DNA using the LY-specific primers. By a TaqMan/real-time PCR assay for LY detection, 82% of palms tested were positive for LY-specific phytoplasma DNA. The restriction enzyme profiles of the DNA tested showed two clear patterns. One pattern was similar to that of the phytoplasma 16SrIV-A strain associated with coconut LY, and the other corresponded to the *Pritchardia pacifica* 16SrIV-D strain. The 16SrIV-A strain is associated with mature leaf-browning syndrome. The palms

infected with the 16Sr-IV-D phytoplasma exhibited spear leaf necrosis and opening. This is the first report of two phytoplasma strains belonging to the subgroups 16SrIV-A and 16SrIV-D in manila palms.

**Keywords** Manila palm · Lethal yellowing disease

## Introduction

Phytoplasmas are small bacteria that colonize plant phloem via insects and that affect a great variety of economically important plant species (Hogenhout et al. 2008). Phytoplasmas are transmitted by insects and are difficult to culture in vitro (Hogenhout et al. 2008). Therefore, phytoplasma characterization is based in molecular methods, such as polymerase chain reaction assays with primers from the 16S rRNA gene, restriction fragment length polymorphism (RFLP) analysis and sequencing of 16S rRNA (Lee et al. 2000). Known phytoplasmas have been grouped into one sole clade and more than 40 subgroups according to the 16S rRNA RFLP classification system devised by Lee et al. (1998).

In the southeast of Mexico, the lethal yellowing (LY) disease related to the 16SrIV-A subgroup, classified as ‘*Candidatus* Phytoplasma palmae’, is associated with many palm species. The other 16SrIV subgroups that are associated with LY symptoms in palms in this geographic region are B, C, D, E and F (Narvaez et al. 2006; Roca et al. 2006; Harrison et al. 2002; Vázquez-Euán et al. 2011; Harrison et al. 2008; Martínez et al. 2008; Narvaez et al. 2016).

*Adonidia merrillii* is a palm species native to the Philippines and is commonly known as the manila palm or Christmas palm (Dransfield et al. 2008). The manila palm

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grows in a tropical climate with an annual temperature range of 22 °C to 32 °C, an average annual relative humidity of 80–90% and a mean annual rainfall of 2000–2500 mm (Lim 2012). In Mexico, manila palms are cultivated along the coastal plain of the Gulf of Mexico, from the northern state of Tamaulipas to the Yucatan Peninsula. Manila palms are used mainly as ornaments in streets, parks and gardens. In Panama, the seeds are used for making necklaces, bracelets and earrings, which are sources of income for many families (Johnson 1996).

McCoy et al. (1983) first reported symptoms of LY-like disease in manila palms in Florida, USA. The presence of phytoplasmas had been previously confirmed by Thomas and Norris (1980) via electron microscopy. However, manila palms did not present yellowing of the leaves and instead showed brown leaves with water-soaked marks along the pinnae that eventually progressed to the rest of the frond. Eventually, Tsai and Harrison (2003) and Harrison and Jones (2004) reported that the 16SrIV-A phytoplasma was associated to the LY symptoms observed in these palms in Florida. Currently, these palms are being devastated by a LY-like disease in southeastern Mexico, and many palms show symptoms similar to those described by McCoy in Florida. However, no reports of the presence of phytoplasma among manila palms in Mexico exist, moreover, we have observed other symptoms in the manila palms that are not commonly associated with LY disease. Therefore, our objective is to use molecular techniques to identify and characterize the presence of phytoplasmas in manila palms from this area of Mexico.

## Materials and methods

### Tissue sampling for PCR analysis.

A total of 33 samples were collected from manila palms showing LY-like symptoms in different locations of Merida, Mexico. Trunk and young leaf tissues from three manila palms without any symptoms were collected as well. The trunk tissue samples were obtained using methods described by Oropeza et al. (2011) with slight modifications as outlined by Córdova et al. (2014). A portable electric drill was used, and the sawdust was collected into a sealable plastic bag. To prevent cross-contamination of the samples, the drill bit was washed first with 70% alcohol followed by a 0.6% of NaClO solution and finally rinsed with sterile distilled water prior to sampling the next palm. After collection, harvested tissue samples were stored on ice for transport to the laboratory and stored at –80 °C until further use.

### DNA extraction

A protocol described by Harrison et al. (1994) was used with minor modifications as described by Córdova et al. (2014) to extract manila palm DNA. Briefly, 1 g of the tissue was incubated in cetyltrimethylammonium bromide (CTAB) buffer (20 mM EDTA pH 8.0, 100 mM Tris-HCl pH 8.0, 2% CTAB, 1.4 M NaCl, and 2-mercaptoethanol in sterile distilled water) for 20–30 min at 65 °C and then vigorously mixed with a solution of phenol-chloroform isoamyl alcohol. The fine sawdust tissue obtained was mixed directly with CTAB buffer for DNA extraction. After centrifugation, the supernatant was precipitated using cold isopropanol and sodium acetate and then incubated at –20 °C for 1 h. The DNA was pelleted by centrifugation and dried at room temperature. Finally, the DNA was resuspended in 100 µL of pure water.

### Detection of LY phytoplasma by nested-PCR.

The presence of LY-phytoplasma was confirmed in diseased plants by PCR according to methods described by Oropeza et al. (2011). The PCR was run for 35 cycles in a programmable PCR Express (Thermo Hybaid, Ashford, UK) using the universal phytoplasma rRNA primer pair P1/P7 (Deng and Hiruki 1991; Smart et al. 1996) with the following parameters: denaturation for 60 s at 94 °C; annealing at 54 °C for 50 s; and extension at 72 °C for 1 min 20 s (10 min for final cycle). The products of the P1/P7-primed PCR were diluted 1:40 with sterile ultrapure water and re-amplified for 35 cycles using the universal phytoplasma 16S rRNA gene primer pair R16F2n/R16R2 (Gundersen and Lee 1996) or the LY-group 503f/LY16Sr specific primer pair as described by Harrison et al. (1999). The amplification with universal primers was performed as follows: denaturation at 94 °C for 90 s followed by 35 cycles at 94 °C for 39 s, 56 °C for 50 s and 72 °C for 90 s with a final extension step at 72 °C 10 min. For specific primers, the thermal cycling parameters were as follows: denaturation for 30 s at 94 °C; annealing at 60 °C for 50 s; and extension at 72 °C for 1 min 20 s (10 min for the final cycle). Aliquots (5 µL) of each final reaction were electrophoresed on 1% agarose gels, stained with ethidium bromide, viewed under UV transillumination and photographed with the GelDoc (Bio-Rad; Hercules, CA, USA) device.

### Detection of LY phytoplasma by real-time PCR.

The detection of LY-phytoplasma by real-time PCR using a TaqMan probe and a set of primers was

completed as described by Córdova et al. (2014). Amplification was performed with a CFX96 real-time PCR System (Bio-Rad, Hercules, CA, USA). The PCR was carried out as follows: 2 min at 50 °C to activate the AmpErase UNG, 10 min at 95 °C to activate the AmpliTaq Gold DNA polymerase and 40 cycles at 95 °C for 15 s and 61 °C for 1 min. All DNA samples including controls were assessed in duplicate. The threshold cycle (Ct) values of each PCR reaction were manually set to intersect the exponential phase of the amplification curves, but the baseline was automatically set using CFX manager software IQ (Bio-Rad). The eukaryotic 18S rRNA TaqMan probe kit (Applied Biosystems, cat. No. 4333760 T) was used as endogenous control. The amplification conditions used were the same as those employed in the TaqMan assay.

### RFLP analysis

Products (10 µL) amplified by nested PCRs primed with R16F2n/R16R2n of some manila palms were digested separately with restriction endonuclease *AluI* (10 U) (New England BioLabs, Waverley, MA, USA) at 37 °C for a minimum of 12 h. Digestion products were separated by electrophoresis with 8% non-denaturing polyacrylamide gels with TBE (90 mM Tris-borate, 2 mM EDTA) used as a running buffer at a current of 2 V/cm during two hours. The DNA fragment size standard was a 1 kb plus ladder (Invitrogen, Carlsbad, CA, USA). DNA fragment profiles in the gels were visualized and recorded as described above.

### Cloning and nucleotide sequences

Cloning and sequencing were completed using the protocols described by Narvaez et al. (2016). The products of nested-PCR amplification from *A. merrillii* DNA extracts with primers P1-P7/ R16F2n/R16R2 were purified with the QIAquick Gel Extraction kit (QIAGEN, Hilden, Germany) and cloned using the vector pGEM-T Easy kit (Promega, Fitchburg, WI, USA). For recombinant plasmid purification, the Plasmid Midiprep kit (Qiagen, Hilden, Germany) was used. Cloned inserts were sequenced in full on automated equipment (Davis Sequencing, Inc., CA).

### Phylogenetic analysis

Phylogenetic analysis of the 16S rRNA gene sequences was carried out with maximum-parsimony analysis performed by utilizing the close neighbor interchange algorithm (CNI) with a search level of 1 in the MEGA 5.05 software as described by Tamura et al. 2011. The initial

tree for the CNI search was obtained with the random addition of sequences (10 replicates). The reliability of the analysis was subjected to a bootstrap test with 1000 replicates. The phylogenetic tree and data used to generate it was submitted to TreeBASE (<http://purl.org/phylo/treebase/phyloids/study/TB2:S21088>).

## Results

### Syndromes observed in manila palms

Thirty-three manila palms with LY disease symptoms were observed at several locations in Merida City (Mexico) between February 2014 and November 2015. In general, three syndromes were recorded and classified according to the early symptoms presented: (1) browning of mature leaves (Fig. 1a), (2) spear leaf necrosis (Fig. 1b and c) and (3) spear leaf opening (Fig. 1d). Leaf browning began in the mature leaves and progressively affected all the leaves and eventually resulted in palm death. The spear leaf remained on the top of the dead palms. In the case of spear leaf necrosis and spear leaf opening, the rest of the leaves underwent necrosis and death. The adult palms in all of the cases presented necrotic inflorescences (Fig. 1e). A representative healthy manila palm and its inflorescence is presented in Figs. 1f and g, respectively. The duration from the appearance of the first symptom to palm death was 3–6 months. Of the 33 palms studied, 20 manila palms showed mature leaf browning, 9 showed spear leaf necrosis and 4 showed spear leaf opening.

### Detection of LY-phytoplasma in manila palms

The presence of phytoplasma DNA was detected by nested-PCR using the universal and specific LY-group primer pairs (Harrison et al. 1994) in extracts obtained from tissue samples of manila palms collected from Merida, Yucatán, a region affected by the LY disease. The results show that of the 33 analyzed palms, 24 (73%) were positive for phytoplasma DNA using the universal primers and 18 (55%) for the specific primer pair (Fig. 2). Using the 16S TaqMan/real-time PCR assay, which is more sensitive than nested-PCR and is specific for LY-phytoplasma (Córdova et al. 2014), 27 palms (82%) were found positive for LY-phytoplasma (Table 1). Details of the localization of the sampled palms are presented in Table 2. Three asymptomatic palms were also analyzed and showed no positive signals for phytoplasma DNA using any of the techniques described in this study (Fig. 2a). Additionally, the 18S ribosomal palm DNA was amplified to ensure that no

**Fig. 1** Lethal yellowing-like symptoms in manila palms in Yucatan, Mexico. Manila palms with browning leaves (a). Manila palm with spear leaf necrosis (b) and a magnified view of a necrotic spear leaf (c). Manila palm with opened spear leaf (d). Manila palm with necrotic inflorescences (e). Uninfected manila palm (f) and close up of inflorescences (g)



amplifications were due to the absence of LY-phytoplasma DNA, Ct values were 18.6, 17.7 and 17.1 respectively, indicating that there were no inhibitors in the DNA samples.

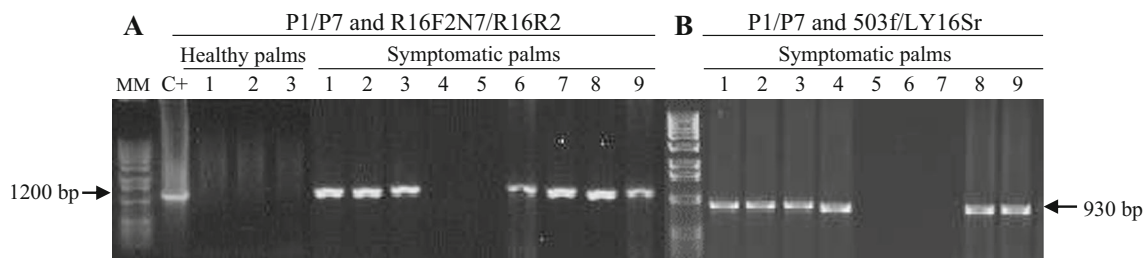
### RFLP analysis

To determine the possibility that two or more subgroups of the 16SrIV phytoplasma group are associated with different syndromes observed in manila palms, an RFLP analysis from the PCR product amplified with the primers R16F2n/R16R2 and using the restriction endonuclease *A*lulI were performed in palms showing the symptoms described above. Two different palms per syndrome were studied. The restriction profiles showed two clear patterns, one of which was similar to the pattern of the 16SrIV-A phytoplasma strain associated with coconut LY and the

other corresponded to the *Pritchardia pacifica* 16SrIV-D strain (Fig. 3). These results suggest the presence of two phytoplasma subgroups (16SrIV-A and 16SrIV-D) in manila palms with different syndromes.

### Sequence analysis

To confirm the presence of DNA phytoplasma in the manila palms and the subgroups to which these phytoplasma belonged, the products amplified with the PCR primers R16F2n/R16R2 of the DNA from the six palms analyzed by RFLP were cloned and sequenced. Four full high-quality sequences with replicates from four palms were obtained. The results revealed the presence of two types of phytoplasma isolates, 16SrIV-A and 16SrIV-D, from the manila palms. The blast analysis presented the highest similarities with a 99.86% identity



**Fig. 2** Nested-PCR amplifications from the DNA of tissues of manila palms using P1 and P7 and universal primers R16F2n/R16R2 (a). The 1.2-kb band indicates the presence of phytoplasma DNA in the sample. Lanes 1–3 represent DNA samples from healthy manila palms. Lanes 1–9 represent DNA samples from symptomatic manila palms. Nested-PCR

amplifications from the DNA of tissues of manila palms using P1 and P7 and specific primers 503f/LY16Sr (b). The 0.93-kb band indicates the presence of phytoplasma DNA in the sample. Lanes 1–9 represent DNA samples from symptomatic manila palms. . MM = 1 kb ladder. C+ positive control

**Table 1** Detection of LY phytoplasma in manila palms by real-time PCR

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Ct*	ND	24.8	28.0	26.6	ND	23.1	28.6	23.4	24.2	28.9	25.2	27.7	27.3	ND	21.5	21.1	18.8	27.2
Diagnostic	-	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+
Sample	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	+	-	
Ct*	25.4	27.0	28.6	ND	ND	ND	26.1	25.1	26.4	26.6	27.8	22.6	24.8	27.4	21.7	19.3	ND	
Diagnostic	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+			

\*Tested in duplicate. ND, no amplification detected. + positive control, - negative control

with Coconut LY phytoplasma for group 16SrIV-A (Acc. Num HQ613874.1) and a 99.93–100% identity with ‘*Sabal palmetto*’ decline phytoplasma (Acc. Num HQ613895.1) for group 16SrIV-D (Table 3).

**Phylogenetic analysis**

To confirm the subgroup to which the phytoplasma strains detected in manila palms belonged, a phylogenetic tree with 16S rRNA gene sequences of phytoplasma strains belonging to the other subgroups of the 16SrIV group (Subgroups A, B, C, D and F) was obtained. In the phylogenetic tree these subgroups were grouped according to the *A. merrillii* phytoplasma sequences (Acc KU714843 and KU714844) clustered together within a clade with the reported phytoplasmas 16SrIV-A strain, associated with LY found in different palm species such as the coconut palm (Acc 498,309), *Phoenix canariensis* (Acc EU241517) and 16SrIV-D strain (Acc KU714837, KU714838, KU714839, KU714840 and KU714841), associated with LY in *Carludovica palmata* and *Phoenix dactylifera* (Acc AF237615; EU241518, respectively) (Fig. 4). Phylogenetic analysis confirmed the presence of the 16SrIV-A and 16SrIV-D phytoplasma subgroups in the manila palms in the Merida Yucatan region of Mexico.

**Association between the type of phytoplasma and syndromes**

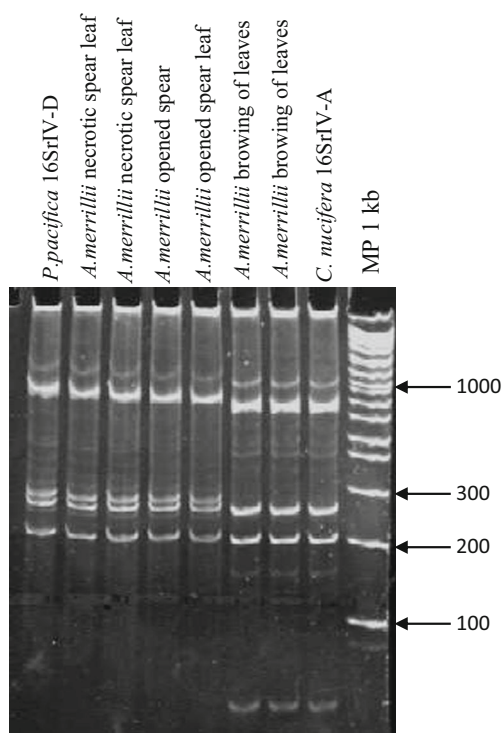
The syndromes were correlated with the phytoplasma strains associated with the manila palms. The 16SrIV-A strains were associated with the mature leaf browning syndrome whilst the palms with phytoplasmas from group 16Sr-IV-D presented the other syndromes, including spear leaf necrosis and opening (Table 4).

**Discussion**

The manila palm is an ornamental palm that is widely used in houses and gardens all over the world and particularly in the southeastern region of Mexico (Lim 2012). In recent years, many of these palms have died and exhibited different syndromes. McCoy et al. (1983) reported that manila palms in Florida, USA showed symptoms related to the same group of organisms that cause LY disease in the coconut palm. However, they did not identify the strain of phytoplasma responsible for this disease. Later, Harrison and Jones (2004) reported that the phytoplasma 16SrIV-A strain was associated with diseased manila palms in Florida. No studies have been reported in Mexico related to phytoplasma

**Table 2** Analysis of symptomatic manila palms at different sites in Merida, México for evidence of phytoplasma infection by nested PCR assay using phytoplasma rRNA operon primer pair P1/P7 followed by universal primers or LY-group primers

Sites (Localization)	Number of palms collected	Positive detection	
		Conventional nested-PCR: universal primers P1/P7-R16F2n/R16R2	Conventional nested-PCR: LY-group primers P1/P7-503f/LY16Sr
Las Américas (21°04'09.55"N; 89°39'10.45"W)	4	4	2
Francisco de Montejo (21°01'51.15"N; 89°38'46.70")	12	10	7
Gonzalo Guerrero (21°01'35.12"N; 89°37'26.43")	11	9	6
Kukulcan (20°56'21.93"N; 89°35'44.48"W)	2	0	0
Caucel (21°00'56.06"N; 89°42'16.26"W)	4	1	3



**Fig. 3** Restriction fragment length polymorphism (RFLP) of nested-PCR amplifications from the tissue DNA of manila palms using the P1 and P7 and specific primers R16F2n/R16R2 (1251 bp) following digestion with the restriction endonuclease *A**lu*I. The comparison was carried out with the profiles of phytoplasma 16SrIV-A and 16SrIV-D strains associated with LY

colonizing manila palms. In our study, we report an epidemic loss of manila palms in southeastern Mexico with symptoms similar to those of phytoplasma infection. Three different syndromes were recorded: leaf browning, spear leaf necrosis and spear leaf opening. The differences in the observed symptoms could be due to different strains of phytoplasma.

Our results show that the majority of manila palms with suspicious symptoms that were analyzed were positive for phytoplasma and 82% were positive for

phytoplasma associated with LY disease according to real-time PCR results. Negative results for phytoplasma presence in symptomatic plants may be due to low phytoplasma titers or related to low nutrient availability in the soil, thus affecting the palms physiological status.

The RFLP analysis showed that manila palms with different syndromes presented two patterns of digestion that could indicate that the palms were colonized with different strains of phytoplasma. The sequence and phylogenetic analysis corroborated that at least the following two strains of the LY group are associated with the manila palms sampled in México: subgroup 16SrIV-A and 16SrIV-D.

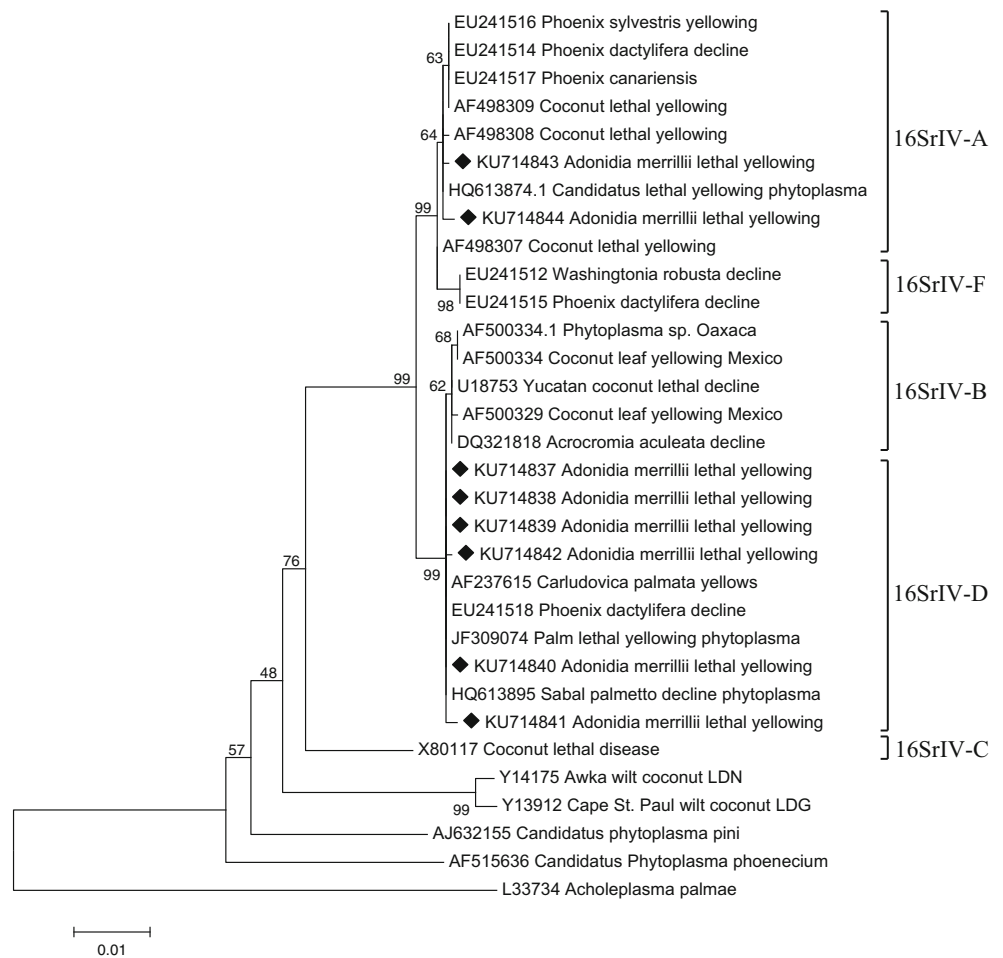
Coconut lethal yellowing (16SrIV) subgroup phytoplasmas were reported in native palms from the Americas with symptoms superficially resembling those of LY for *Trinax radiata* and *Cocotrinax readdi* (Subgroup A; Narvaez et al., 2006); *Acrocomia aculeata* in Honduras (subgroup B; Roca et al. 2006); *Cocos nucifera* in Tabasco, Mexico (subgroup C; Harrison et al. 2002); *Sabal mexicana* in Yucatán, Mexico (Subgroup A and D; Vázquez-Euán et al. 2011); *Pseudophoenix sargentii* in Yucatán, Mexico (Subgroup D; Vázquez-Euán et al. 2011), which also included *Washingtonia robusta* (Harrison et al. 2008); *Cocos nucifera* in the Dominican Republic (subgroup E; Martínez et al. 2008) and *Washingtonia robusta* (subgroup F; Harrison 2008). Recently, *Acrocomia mexicana* and *Roystonea regia* presented lethal yellow symptoms and were confirmed to contain phytoplasma associated with subgroup 16SrIV-A by Narvaez et al. (2016).

The subgroup 16SrIV-A phytoplasma that is associated with manila palms in our study presented the syndrome described by McCoy et al. (1983), which includes browning of mature leaves that eventually involves all leaves and is followed by necrosis of inflorescences and death. The subgroup 16SrIV-D correlates with the two other syndromes of spear leaf necrosis and opening. In the coconut palm, three strains with

**Table 3** Results of identity searches between the phytoplasma del LY amplified from Manila palms and GenBank accessions using nucleotide blast

Palm	Number accession	Highest identity with GenBank accessions	E value	% identity	Group and Subgroup
Clone 1.1	KU714837	' <i>Sabal palmetto</i> ' decline phytoplasma.	0.0	99.93	16SrIV-D
Clone 1.2	KU714838	' <i>Sabal palmetto</i> ' decline phytoplasma.	0.0	100	16SrIV-D
Clone 3.1	KU714839	' <i>Sabal palmetto</i> ' decline phytoplasma.	0.0	99.72	16SrIV-D
Clone 3.2	KU714840	' <i>Sabal palmetto</i> ' decline phytoplasma.	0.0	100	16SrIV-D
Clone 5.1	KU714841	' <i>Sabal palmetto</i> ' decline phytoplasma.	0.0	99.79	16SrIV-D
Clone 5.2	KU714842	' <i>Sabal palmetto</i> ' decline phytoplasma.	0.0	99.72	16SrIV-D
Clone 6.2	KU714843	Coconut lethal yellowing phytoplasma.	0.0	99.93	16SrIV-A
Clone 6.3	KU714844	Coconut lethal yellowing phytoplasma.	0.0	99.86	16SrIV-A

**Fig. 4** Phylogenetic tree derived from analysis of 16SrRNA gene sequences of representative palm phytoplasmas associated with 16SrIV subgroups A, B, C, D and F. Phytoplasma strains sequenced in this study are indicated with ♦. Other phytoplasma groups are presented, including those of the *Acholeplasma palmae* that served as an out-group during phylogenetic tree construction. The number of branches indicate the percentage of 1000 bootstrap replications supporting the particular nodes



different symptoms have been reported. Palms associated with the 16SrIV-A phytoplasma subgroup that causes the classic symptoms of LY, such as nut falling, inflorescence necrosis, and young leaf yellowing, eventually progresses to all fronds and causes death (Harrison and Oropeza 2008). In palms with strains 16SrIV-B and 16SrIV-C, nut falling and necrosis inflorescence was absent. The syndrome associated with 16SrIV-E was not described by Martínez et al. (2008). In *Sabal Mexicana*, two strains of LY-phytoplasma were also

detected and included 16srIV-A and 16SrIV-D each in separated individual palms; however, the symptoms were similar for palms associated with each type of strain: discoloration, necrosis of leaves and inflorescences and eventually death (Vázquez-Euán et al. 2011).

Determining whether the manila palms are associated with phytoplasma belonging to a LY group is very important from an epidemiological point of view because manila palms co-exist with other palms and could function as an additional source of inoculum of these pathogens, thus threatening socioeconomical important palms such as the coconut palm.

This is the first report of the presence of two strains of phytoplasma belonging to subgroups 16SrIV-A and 16SrIV-D in the manila palm. The presence of the subgroup 16SrIV-A correlates with symptoms previously reported, and subgroup 16SrIV-D with spear leaf necrosis and early opening.

**Table 4** Association between syndromes presented in manila palms and phytoplasma strain

Syndromes	No. GeneBank accession	Group and Subgroup
Leaf browning	KU714843	16SrIV-A
	KU714844	
Spear leaf necrosis	KU714837	16SrIV-D
	KU714838	
Spear leaf necrosis	KU714839	16SrIV-D
	KU714840	
Spear leaf opening	KU714841	16SrIV-D
	KU714842	

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