A REAL-TIME PCR ASSAY FOR DETECTION OF COCONUT LETHAL YELLOWING PHYTOPLASMAS OF GROUP 16SrIV SUBGROUPS A, D AND E FOUND IN THE AMERICAS

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SUMMARY

Lethal yellowing (LY) is a fatal disease that affects coconut and other palm species in the Americas. Phytoplasmas associated with this disease belong to group 16SrIV. Reliable detection of group 16SrIV phytoplasmas is important for diagnostic purposes and to increase understanding of pathogen-plant-vector pathosystems. The present study describes the development of a TaqMan/real-time PCR assay for detection and quantification of selected 16SrIV subgroups affecting palms in the Americas. The specificity of the assay was assessed on DNA extracts from LY-infected palms in the Americas, coconut lethal phytoplasma-associated diseases in Africa and other phytoplasma-infected plants. Successful amplification was obtained only with DNA extracts from palms infected by LY phytoplasmas that were sampled in the Americas, belonging to the 16SrIV group, subgroups A, D and E. No amplification was obtained from DNA of palms sampled in Africa and phytoplasma-infected plants of other 16Sr groups. The assay was compared with conventional nested-PCR on DNA extracts from 36 palms. The real-time PCR assay showed higher sensitivity as phytoplasmas were detected in several nested-PCR negative and in all the nested-PCR positive samples. The assay was also used to evaluate accumulation of LY phytoplasma DNA in different tissues of palms showing LY symptoms. The highest concentration was found in the trunk, followed in decreasing order by primary root apex, mature inflorescences -1 and -2, inflorescences -3 to -7, spear leaf, flag leaf and mature leaf. The present TaqMan/real-time PCR assay represents a new alternative for LY phytoplasmas detection and quantification, offering high specificity and improvements in sensitivity.

*Key words***:** palms, mollicutes, TaqMan probe, real-time PCR, diagnosis.

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INTRODUCTION

Lethal yellowing (LY) is a fast spreading, fatal disease that affects coconut (*Cocos nucifera* L.) and numerous other palm species in the Americas (Harrison and Oropeza, 2008). Since the 1950s, LY has killed millions of coconut palms mostly of the Atlantic Tall ecotype, adversely impacting the livelihood of coconut farmers in affected regions. Phytoplasmas are the accepted cause of LY (McCoy *et al*., 1983). These unculturable, phloem-inhabiting bacteria (Class: Mollicutes) are transmitted to palms by the planthopper *Haplaxius* (*Myndus*) *crudus* (Hemiptera: Cixiidae) (Ceotto *et al*., 2008), the only known insect vector of the disease (Howard *et al*., 1984).

As LY progresses and becomes more severe, infected coconut palms exhibit visual symptoms, such as premature nut drop (stage 1); inflorescence necrosis (stage 2 to 3); leaf chlorosis and senescence (stages 4 to 6); and spear leaf death (stage 7-8); before dying (stage 9), usually within 3-6 months (McCoy *et al*., 1983). Phytoplasmas associated with coconut and other palms displaying these typical LY symptoms have been characterized as a group 16SrIV, subgroup A (i.e. 16SrIV-A) strain according to the 16S rRNA gene RFLP classification system devised by Lee *et al.* (1998). Phytoplasmas belonging to additional subgroups 16SrIV-B (Harrison *et al*., 2002b), C (Harrison *et al*., 2002c), D (Harrison *et al*., 2002a; Vázquez-Euan *et al*., 2011), E and F (Martínez *et al*., 2008 and Harrison *et al*., 2008, respectively) have been identified in various palm species with symptoms resembling those of LY in non-palm hosts (Thomas, 1979) and planthoppers (Brown *et al*., 2006) in localities affected by LY, and in declining palms growing within localities with no prior history of the disease (Harrison *et al*., 2002b; Oropeza *et al*., 2011; Vázquez-Euán *et al*., 2011).

Reliable detection of group 16SrIV phytoplasmas is important for diagnostic purposes and to increase understanding of pathogen-plant-vector pathosystems to assist in development of effective disease control and prevention strategies. In particular, PCR assays incorporating primers designed upon ribosomal rRNA gene operon sequences

have widely been used to detect phytoplasma DNA in palms, preferably utilizing immature phloem-rich tissues of the palm stem apex (Harrison and Oropeza, 2008). However, as young palms rarely contract LY disease, most affected palms are often large in stature and must be felled to readily access and remove these tissues. This sampling approach is rarely feasible in urban and suburban landscape settings and often impractical in field situations too when multiple diseased palms are involved. The enhanced sensitivity achieved by nested-PCR assays (Gundersen and Lee, 1996) has made reliable detection of phytoplasmas possible in small amounts of tissue from the interior basal stem (trunk) of palms thus providing for efficient, non-destructive sampling method for palms (Harrison *et al*., 2002a, 2002b; Oropeza *et al*., 2011; Vázquez-Euán *et al*., 2011). However, nested-PCR requires two amplification reactions and post amplification processing, either RFLP or sequence analysis of resulting products, to precisely determine phytoplasma identity.

Real-time PCR coupled with TaqMan probe technology for detection and quantification of various phytoplasmas has been reported (Angelini *et al*., 2007; Baric *et al*., 2006; Bianco *et al*., 2004; Christensen *et al*., 2004; Crosslin *et al*., 2006; Herath *et al*., 2010; Hodgetts *et al*., 2009; Hren *et al*., 2007). The benefits of expanding this application to diagnosis of phytoplasma diseases are short analysis time with high reproducibility and no need for post-PCR processing thus reducing the risk of carry-over contamination and false positive results. Lower limits of phytoplasma detection by real-time PCR assays are comparable to (Angelini *et al*., 2007; Baric *et al*., 2006; Crosslin *et al*., 2006) or may exceed those attainable by nested PCRs (Hren *et al*., 2007). Assays can also provide quantitative estimates of phytoplasmas in host tissues (Marzachí and Bosco, 2005). Collectively, these attributes increase the likelihood of phytoplasma detection in arborescent monocots such as palms with LY disease, in which primary infection is followed by a protracted latent (incubation) phase prior to overt symptom development (Dabek, 1975).

The present study describes the development of real-time PCR and TaqMan technology for detection and quantification of subgroup 16SrIV-A, 16SrIV-D and 16SrIV-E phytoplasmas affecting palms in the Americas.

MATERIALS AND METHODS

LY symptom development. Plants studied were naturally infected coconut palms (*Cocos nucifera* L.) showing stages 1 to 4 of LY symptom development, as defined by McCoy *et al.* (1983): nut fall (1), appearance of necrotic inflorescences (2), yellowing of lower (older) leaves (3), middle leaves (4).

Tissue sampling for PCR analysis. A first batch of tissue samples was collected from trunk of 41 mature Atlantic Tall variety coconut palms (5 for DNA dilution analysis and 36 for field testing) showing LY symptoms at stages 1 to 4 (McCoy *et al*., 1983). The palms were located in LY-infected coconut groves on the northern coast of Yucatan State, Mexico (Chelem 21°16'N, 84°44'W; Chuburna 21°15'N, 89°48'W; Celestun 20°51'N, 90°23'W; Sisal 21°09'N, 90°01'W, Dzilam 21°23'N, 88°53'W). A second batch was collected from other three palms with LY symptoms at stages 3 and 4 of disease development (McCoy *et al*., 1983) from a grove in San Crisanto (21°22'N, 89°00'W). Parts sampled were: the more accessible unemerged non-chlorophyllic leaf, the youngest emerged yet unopened leaf (spear leaf), the first open leaf (flag leaf), mature leaves, stem apex, inflorescences at developmental stages –1 to –7 (0 represents the most mature unopened inflorescence), primary root apex and tissues in the lower stem. Leaf samples consisted of leaflet lamina only. A third batch was collected from the trunks of 15 healthy coconut palms of Malayan Yellow Dwarf variety in a LY-free coconut grove in Telchac Puerto (21°21'N, 89°16'W). A fourth batch was collected from the flag leaves of 10 healthy Tabasco tall coconuts growing inside insect proof enclosures in Merida (21°1'N, 89°38'W). These palms were part of a separate study that will be reported elsewhere. DNA extracts from these healthy palms in batches 3 and 4 were used as negative controls.

All coconut tissue samples were obtained as previously described (Oropeza *et al*., 2011). Trunk tissue samples were obtained using a portable electric drill fitted with a 6.5 inch long bit (5/16th diameter) and the sawdust was collected into a clean, sealable plastic bag. To prevent cross-contamination of samples, the drill bit was washed first with alcohol 70%, then with a 0.6% NaClO solution and finally rinsed with sterile distilled water prior to sampling the next palm. The fine sawdust tissue obtained was mixed directly with cetyltrimethyl ammonium bromide (CTAB) buffer for DNA extraction (see below). Harvested tissues from all parts were stored on ice immediately after collection for transport to the laboratory and stored at -80°C prior to DNA extraction and PCR analysis.

DNA extraction. A protocol described by Harrison *et al*. (1994) was used with minor modification to extract DNA from 100 mg samples of palm tissue. Briefly, each sample was pulverized in liquid nitrogen with a mortar and pestle, mixed with 500 µl of hot (65°C) 2% CTAB buffer. In the case of trunk, the samples contained tissue already disrupted by the effect of drilling and therefore, skipping the use of liquid nitrogen, they were mixed directly with hot (65°C) 2% CTAB buffer. Samples were then incubated at 65°C for 30 min and cooled to room temperature. The resulting extracts were emulsified with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) and centrifuged at 14,000 *g* for 5 min. Total nucleic acids were precipitated from the upper aqueous phase by addition of cold isopropanol and pelleted by

centrifugation, as before. Nucleic acid pellets were dried briefly, resuspended in 100 ml of TE buffer (1 mM Tris, 0.1 mM EDTA, pH 8) and incubated with RNase for 1 hr at 37°C. DNA samples were quantified using a DNA Quantitation kit and VersaFlour fluorometer (Bio-Rad, USA) according to the manufacturer's instructions.

Conventional PCR assays. Amplifications were done in 25 µl reaction volumes, each containing a 1 µl sample of DNA (50 ng of DNA), 50 ng of each primer, 125 µM of each dNTP (Invitrogen, USA), 1 U Taq DNA polymerase (Invitrogen, USA) and standard PCR buffer containing 1.5 mM $MgCl_2$. PCR was run for 35 cycles in a iCycler IQ PCR detection system (Bio-Rad, USA) using phytoplasma-universal rRNA primer pair P1 (Deng and Hiruki, 1991) and P7 (Smart *et al*., 1996) with the following parameters: denaturation for 60 sec at 94°C; annealing at 54°C for 50 sec and extension at 72°C for 1 min (10 min for final cycle). The products of the initial P1/P7-primed PCR were diluted to 1:40 with sterile ultrapure water and re-amplified for 35 cycles using LY-group 503f/LY16Sr primer pair, as described by Harrison *et al.* (1999) or phytoplasma universal 16S rRNA gene primer pair R16F2n/R16R2 (Gundersen and Lee, 1996).

Positive and negative control samples (healthy plant DNA extract; ultrapure water in the place of DNA) were included in all PCR assays. Nested-PCR aliquots (10 µl) were electrophoresed through 1% agarose gels, stained with ethidium bromide 0.1%, visualized by UV transiluminador and photographed Gel Documentation by GelDoc 2000 (Bio-Rad, USA).

TaqMan/real-time PCR Assay. Reactions were performed in 20 µl volumes each containing 10 µl of TaqMan Universal PCR master mix with AmpErase UNG (uracil N-glycolase) (Applied Biosystems, USA), 1 µl of primer mix containing 900 nM of each primer, probe (250 nM) and 50 ng of DNA or as indicated in the text. Amplification was performed with a CFX96 real-time PCR System (Bio-Rad,USA). PCR was initiated with two steps: 2 min at 50°C to activate AmpErase UNG, 10 min at 95°C to activate AmpliTaq Gold DNA polymerase followed by 40 cycles at 95°C for 15 sec and 1 min at 61°C. 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 by CFX manager software IQ (Bio-Rad, USA). A TaqMan LY16S primer pair / probe set for real-time PCR was designed and details and presented in the results section. PCR products amplified with the TaqMan LY16S primer pair/probe set were cloned and sequenced to validate the specificity of the amplification products.

Specificity. The specificity of the real-time PCR assay was assessed using DNA of 16 other phytoplasma strains. These included elm yellows (EY), Mexican periwinkle virescence (MPV), strawberry green petal (SGP), Jujube witches' broom (JWB), peach western X (PWX), coconut lethal disease Mozambique, coconut lethal decline Tanzania (LD), Bermuda grass white leaf (BGWL), ash yellows (ASHY), pigeon pea witches' broom (PPWB), coconut LY-Florida (CLY-FL), coconut LY-Dominican Republic. DNAs from healthy palms were included as a negative control. The 16Sr group affiliations of the various controls samples are shown in Table 1.

Sensitivity. DNA extracts from five diseased palms were serially diluted (10-fold) from 300 ng/µl to 0.03 ng/µl and 1 µl of each dilution was evaluated in duplicate as template to compare the sensitivity of real-time PCR and nested-PCR assays. DNA from 25 healthy palms was also evaluated. Moreover samples from 36 field palms were used to compare both techniques.

Standard curve for phytoplasma DNA assay. An rRNA gene amplicon (869 bp) obtained by nested PCR employing LY-group specific primer pair 503f/LY16Sr was purified using a QIAquick gel extraction kit (Qiagen, USA) and cloned into pGEM-T easy vector and *Escherichia coli* cells (DH5α-T1) according to the supplier's instructions (Promega, USA). Plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen, USA) according to the directions of the manufacturer. Purified recombinant plasmid DNA was quantified with a Fluorescent DNA quantitation kit and Versa Fluor fluorometer (Bio-Rad, USA). To prepare a positive assay standard, plasmid DNA was diluted in ultrapure water alone or water containing DNA (20 ng) from healthy palm. Serial 10-fold dilutions (10^6 to 10^1 plasmid copies) were used as template to determine the lower limits of detection sensitivity by the real-time PCR assay. The plasmid DNA dilutions were tested in triplicate. The efficiency (E) and square correlation coefficient (R^2) of the real-time PCR assay were assessed. Detection efficiency was calculated by plotting Ct values against the log value of each DNA standard (copies) in the dilution series. The slope of the standard curve represented the efficiency $E=10^{-1/slope}$ of the assay.

Standard curve for plant DNA assays. For the plant DNA standard curve a series of 10-fold dilutions (300, 30, 3, and 0.3 ng) of a DNA extract from a healthy plant, were used as templates for real-time-PCR assays with a TaqMan probe for eukaryotic 18S rRNA (Applied Biosytems, USA), carried out by triplicate. Absolute quantification of plant DNA was achieved by plotting the means of the CTs for each dilution of DNA from the healthy palm versus the logarithm of each corresponding concentration.

Quantification of phytoplasmas. The quantification of phytoplasmas was done according to Christensen *et al.* (2004), based on two standard curves, one for plant DNA

quantification and one for phytoplasma quantification, determined as described above. The amount of input sample DNA and the corresponding copy number could be calculated using the plant and the phytoplasma standard curves. The number of phytoplasma cells would be half of the calculated copy number for each sample, because two copies of the 16Sr gene exist in phytoplasmas (Schneider and Seemüller, 1994). The phytoplasma cell amount divided by the amount of plant DNA in each sample yields the number of phytoplasma cells per µg of plant DNA.

Statistical analysis. Data were subjected to analysis of variance (one-way ANOVA) and the procedure for mean comparisons (LSD test at $P \leq 0.05$) was performed with OriginPro software.

RESULTS

Design of the real-time PCR assay. Primer pair LY16S-LSF (5'-GCTAAAGTCCCCACCATAACGT-3') / LY16S-LSR (5'-CGTGTCGTGAGATGTTAGGTTAAGT-3') and probe (FAM-CCCCTGTCGTTAATTG-NFQ) for specific detection of group 16SrIV phytoplasmas were designed from a sequence of the 16S rRNA gene of LY phytoplasma (GenBank accession No. AY919862). The primers anneal in the region (5'-265-287-3' and 5'-323-348-3' respectively) or its complement. The probe anneals in the region 5'-291-307-3'. The amplification product was cloned and sequenced and the resulting base sequence (83 bp) was the expected one with 100% identity.

***In silico* analysis of the LY 16S primer and probe set.** The sequences of the forward primer (LY16SFLS) and probe (LY16S probe) were tested together *in silico* for homology to sequences of phytoplasmas and other prokaryotic using BLAST (NCBI) (Altschul *et al*., 1997). The results showed shared 100% identity, with coverage of 100%, with the first 100 hits (E value 0.049) corresponding to sequences derived from rRNA gene reported as coconut LY-related and sourced in countries of the Americas. Only two derived from sugarcane are not reported as LY-related and were sourced in Mauritius. The following hits 101-250 had 100% identity with coverage of 52-57%. In these cases a base sequence (or part of it) corresponding to that of the forward primer was present, but not that corresponding to the probe sequence. In this case, only two of all the entries were reported as LY related. The reverse primer was not included in this analysis since preliminary BLAST testing showed that it had no homology with any sequences reported as LY-related.

LY 16S TaqMan / real-time PCR assay specificity. The primer pair and TaqMan probe, from here on will be referred to as the "LY 16S primers/probe set"; and the assay as the "LY 16S TaqMan/real-time PCR assay". The specificity of the LY 16S primers/probe set was assessed on a series of DNA extracts from LY-infected palms from the Americas, from phytoplasma-associated lethal diseases from Africa, from plants of non-palm species infected by non-LY phytoplasmas and from healthy coconut palms (Table 1). The infection or non-infection status of the sourced palms was determined by nested-PCR using universal or LY-group primer sets.

Using the LY 16S TaqMan/real-time PCR assay successful amplification was obtained only with DNA extracts from palms affected by LY phytoplasmas that were sampled in the Americas; with no amplification detected (ND) for DNA of palms sampled in Africa, healthy palms sampled in the Americas and Africa or from DNA of plants affected with other diseases (Table 1).

The DNAs that could be amplified were of phytoplasmas of the 16SrIV group A, D and E found in the Americas. These have been reported for coconut in Florida, USA (16SrIV-A) (Tymon *et al*., 1998) and Dominican Republic (16SrIV-E) (Martínez *et al*., 2008); and for other plant species in Mexico, such as *Sabal mexicana* (16SrIV-D), *Pseudophoenix sargentii* (16SrIV-D) and *Pritchardia pacifica* (16SrIV-D) (Vázquez-Euán *et al*., 2011).

The LY 16S primers/probe set did not amplify DNAs of phytoplasmas (associated with coconut) of the 16SrIV subgroups B and C from Africa (Table 1). Also no amplification was obtained from DNAs of phytoplasmas of other groups: 16SrI, 16SrIII, 16SrV, 16SrVII, 16SrXIII-A, 16SrIX-A, 16SrXIV, and 16SrXV (Table 1). Similar results were obtained by nested-PCR using the LY-group primer pairs. In contrast, all the DNAs could be amplified by nested-PCR using universal primer pairs (Table 1).

LY 16S TaqMan/real-time PCR assay sensitivity. The assay was tested with a range of 1-10^6 copies of a plasmid containing a fragment of 16S rRNA gene of LY-phytoplasma in ultrapure water and with DNA from healthy palm (20 ng). No amplification was obtained when one copy was included in the reaction mixture, but successful amplification could be obtained with ten copies or more (Table 2, left panel). Comparative sensitivity was achieved also using DNA from field samples obtained from five palms previously confirmed as infected with LY-phytoplasma using LY-group specific nested-PCR. The DNA dilutions of LY-infected palms (batch 1) showed that amplification was positive in both assays for all samples down to the 3 ng of DNA dilution (Table 3). For the last two dilutions, the LY16S TaqMan/real-time PCR assay showed a higher sensitivity than the LY-group specific nested-PCR, 5 to 3 positive detection, respectively, with 0.3 ng of DNA, and 5 to 1 positive detection, respectively, with 0.03 ng of DNA (Table 3). All Ct values ranged from 19 to 36.3.

At the same time DNA samples from 25 symptomless coconut plants showed no amplification (ND) or Ct values equal or higher than 37 (Table 4). After samples were taken from sampled plants 1-15 (mature bearing plants

Table 1. PCR amplification specificity for DNA samples of different types of phytoplasmas using three different PCR assays.

Phytoplasma strain/16Sr RNA group	Conventional nested-PCR: universal primers P1/P7-R16F2n/R16R2	Conventional nested-PCR: LY-group primers P1/P7-503f/LY16Sr	16STaqMan/real-time PCR: LY 16S primers / probe set (Ct)*
Non-LY group phytoplasmas			
Strawberry green-petal (SGP),16SrI	+	–	ND
Peach Western X (PWX),16SrIII	+	–	ND
Elm Yellows (EY), 16SrV-A	+	–	ND
Ash Yellows (ASHY),16SrVII	+	–	ND
Mexican Periwinkle virescence (MPV), 16SrXIII-A	+	–	ND
Pigeon pea witches' broom (PPWB), 16SrIX-A	+	–	ND
Bermuda grass white leaf (BGWL), 16SrXIV	+	–	ND
Jujube witches' broom (JWB), 16SrXV	+	–	ND
LY group phytoplasmas			
Coconut LY, Florida, 16SrIV-A	+	+	22.5
Coconut LY, México, 16SrIV-A	+	+	25.5
Coconut lethal disease , Mozambique, 16SrIV-B	+	–	ND
Coconut lethal decline, Tanzania (LDT), 16SrIV-C	+	–	ND
Sabal mexicana lethal decline, Mexico, 16SrIV-D	+	+	28.3
Pseudophoenix sargentii lethal decline, Mexico, 16SrIV-D	+	+	28.6
Pritchardia pacifica lethal decline, Mexico, 16SrIV-D	+	+	13.4
Coconut LY, Dominican Republic, 16SrIV-E	+	+	23.9
Uninfected palms			
Healthy coconut (Mexico)	–	–	ND
Healthy coconut (Mozambique)	–	–	ND

ND, No amplification detected within the working range of the program with a maximum of 40 cycles. * Tested in duplicate.

Table 2. Analysis with LY 16S TaqMan / real-time PCR assay of serial dilutions of a solution of a plasmid containing a fragment of the 16S gene sequence of LY phytoplasma (16SrIV subgroup A) prepared in water or in a DNA extract solution of healthy palm (left panel), and linear regression analysis (right panel).

Plasmid copy number	Ct value	
	In water	In DNA solution
One copy	ND	ND
10 copies	35.41 ± 0.58	35.30 ± 0.26
10^2 copies	33.11 ± 0.23	33.50 ± 0.17
10^3 copies	30.57 ± 0.32	30.53 ± 0.11
10^4 copies	27.01 ± 0.02	27.03 ± 0.06
10^5 copies	23.10 ± 0.70	23.13 ± 0.06
10^6 copies	19.66 ± 0.17	20.23 ± 0.06

	Linear regression		
	Slope	R^2	% E
In water	–3.21	0.99	104.9
In DNA solution	–3.14	0.99	108.1

ND, No amplification detected within the working range of the program with a maximum of 40 cycles. (R^2) Average square regression coefficient. (% E) Percentage efficiency amplification.

planted in open environment, batch 3) none of the plants developed any LY symptoms within 6 months of observation. Similarly, sampled plants 16-25 (young non-bearing plants kept in insect-proof enclosures, batch 4) did not develop any LY symptoms within 12 months of observation (Table 4). No further observation was carried after these periods of time.

In addition, a total of 36 mature bearing coconut palms (batch 2) showing symptoms of LY (stages 3 or 4) were sampled at different locations from the northern coast of the Yucatan state, (Mexico) (Table 5) and analyzed for the presence of LY-phytoplasma using the LY-group specific nested-PCR and LY 16S TaqMan/real-time PCR assays. The results showed that, with the LY 16S TaqMan/real-time PCR assay, positive amplification with Ct values ranging from 14.4 to 34.7, was obtained for 29 of 36 samples (81%). In the case of negative detection, the Ct values were 37.0 or above or ND. With the LY group-specific nested

Table 3. Comparative sensitivity evaluation of LY 16S TaqMan / real-time PCR (RT-PCR) assay using LY 16S primers/probe set and nested-PCR assay using primer pairs P1/P7 and 503f/LY16Sr. DNA samples were obtained from stem tissues of 5 coconut palms showing LY symptoms.

Palm	Dilution factor of original sample (DNA content in tube)									
	10^0 (300 ng)		10^{-1} (30 ng)		10^{-2} (3 ng)		10^{-3} (0.3 ng)		10^{-4} (0.03 ng)	
	RT PCR (Ct)*	nested PCR	RT PCR (Ct)*	nested PCR	RT PCR (Ct)*	nested PCR	RT PCR (Ct)*	nested PCR	RT PCR (Ct)*	nested PCR
1	22.6	+	26.2	+	24.9	+	32.4	+	36.0	-
2	21.4	+	24.1	+	26.4	+	29.0	+	32.2	+
3	20.1	+	23.1	+	26.3	+	29.1	+	32.4	-
4	19.0	+	21.7	+	25.6	+	28.9	-	32.4	-
5	24.0	+	26.9	+	30.0	+	34.3	-	36.3	-

* Tested in duplicate.

Table 4. Evaluation of LY 16S TaqMan / real-time PCR assay using LY 16S primers/probe set. DNA samples were obtained from 25 healthy coconut palms.

Sample	Ct*	Conventional nested-PCR: universal primers P1/P7-R16F2n/R16R2	Conventional nested-PCR: LY-group primers P1/P7-503f/LY16Sr	Type of sample
1	37.0	–	–	Samples taken from mature bearing plants planted in an open environment exposed to surrounding wild insects. After the samples were taken, the plants were observed for six months and did not develop any LY symptoms.
2	37.5	–	–	
3	37.0	–	–	
4	37.5	–	–	
5	ND	–	–	
6	ND	–	–	
7	38.1	–	–	
8	ND	–	–	
9	38.0	–	–	
10	ND	–	–	
11	38.4	–	–	
12	ND	–	–	
13	ND	–	–	
14	37.4	–	–	
15	38.8	–	–	
16	ND	–	–	Samples taken from young non-bearing coconut plants kept within an insect proof cage that was free of insects. After the samples were taken, the plants were kept for 12 months within the cages and did not develop any LY symptoms.
17	ND	–	–	
18	ND	–	–	
19	ND	–	–	
20	38.2	–	–	
21	38.2	–	–	
22	38.7	–	–	
23	ND	–	–	
24	38.1	–	–	
25	38.7	–	–	

* Tested in duplicate. ND, No amplification detected within the working range of the program with a maximum of 40 cycles. (–) indicates no DNA amplification.

PCR assay, amplification was obtained in 12 of the 36 samples (33%) (Table 5). All nested PCR-positive samples were detected by the real-time PCR assay. Also a greater percentage of detection with the LY 16S TaqMan/real-time PCR assay was consistently obtained for the samples from each site (Table 5).

Quantification of phytoplasma in different plant parts. The LY 16S TaqMan/real-time PCR assay was used to evaluate the accumulation of LY phytoplasma DNA in tissues of different plant parts of three palms showing LY symptoms, corresponding to stages 3-4 (as defined by McCoy *et al.* 1983). Absolute quantification of coconut palm DNA was achieved by plotting the mean CTs of four dilutions of healthy coconut palm DNA versus the logarithm of each concentration. Correlation coefficient for the regression lines was 0.99 for coconut palm DNA and for LY phytoplasma DNA. Slopes of the regression lines were –3.20 for coconut palm DNA and –3.41 for LY phytoplasma DNA.

The ANOVA results for significant differences at $P< 0.05$ showed four groups of tissues with significant differences among them (Fig. 1). These are in decreasing order: (a) the trunk with the highest concentration

Table 5. Comparative detection of LY phytoplasma DNA with LY 16S TaqMan / real-time PCR assay using LY 16S primers / probe set and LY-group specific nested-PCR assay using primer pairs P1/P7-503f/LY16Sr. DNA samples were obtained from trunk tissues of coconut palms exhibiting LY-like symptoms in different locations on the northern coast of Yucatan State, Mexico.

Site of sampling	Palms sampled *per* site and amplification					
	LY-group specific nested-PCR			LY16S TaqMan Real-time-PCR		
	Sampled	Positive	%	Sampled	Positive	%
Chelem	5	2	40.0	5	5	100.0
Chuburna	5	0	0.0	5	5	100.0
Celestun	5	0	0.0	5	1	20.0
Sisal	7	5	71.5	7	6	85.7
Dzilam	14	5	35.7	14	12	85.7
Total	36	12	33.3	36	29	80.6

Samples were considered positive if the Ct value was < 37 in duplicate samples.

($1.61\times10^7 \pm 4.85\times10^6$ cells/µg DNA), (b) primary root apex ($1.76\times10^6 \pm 7.40\times10^5$ cells/µg DNA), (c) inflorescences -1 and -2 ($9.3\times10^5 \pm 8.19\times10^5$ and $5.12\times10^5 \pm 3.49\times10^5$ cells/µg DNA respectively), and (d) the rest of the tissues, inflorescences -3 to -7 ($5.99\times10^4 \pm 2.65\times10^4$; $4.53\times10^4 \pm 3.62\times10^4$; $3.12\times10^4 \pm 3.09\times10^4$; $3.47\times10^4 \pm 2.32\times10^4$; $1.51\times10^5 \pm 1.46\times10^5$ cells/µg DNA respectively), apex ($7.14\times10^4 \pm 4.17\times10^4$ cells/µg DNA), spear leaf ($4.84\times10^4 \pm 4.36\times10^4$ cells/µg DNA), flag leaf ($5.10\times10^4 \pm 4.94\times10^4$ cells/µg DNA) and mature leaf ($3.61\times10^3 \pm 2.96\times10^3$ cells/µg DNA) (Fig. 1).

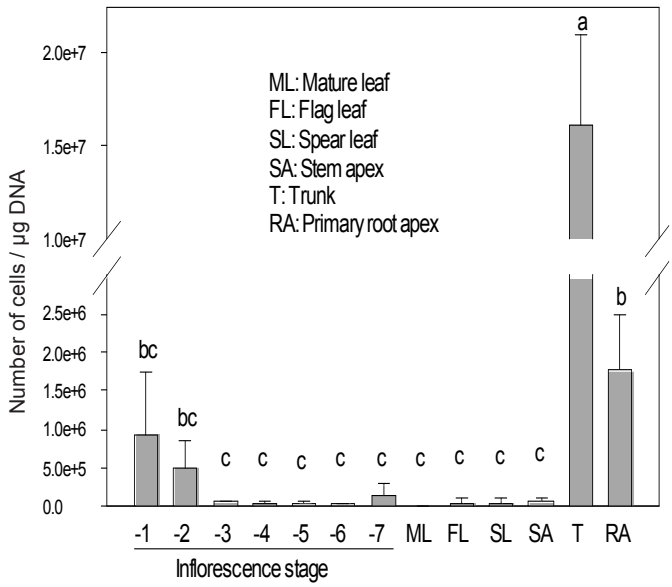

Fig. 1. Quantification with the LY 16S TaqMan/real-time PCR assay (LY 16S primers/probe set) of LY phytoplasma DNA in different plant parts of three LY symptomatic coconut palms (stage 3-4 of disease development). Data shown represents the mean ± standard deviation (SD) of three results, each from a sample of a different plant, testing each tissue sample by triplicate. Different letters denote significant differences among the tissues ($P \leq 0.05$).

DISCUSSION

Accurate and rapid methodology for the detection and quantification of LY phytoplasma DNA is required to obtain a deeper insight into plant-vector-pathogen interactions, but also it is a very important tool for the management of the LY disease to avoid or decrease its spread. One major problem in LY phytoplasmas detection is their uneven distribution and low concentration in some of the tissues of the palms affected by this disease (Harrison and Oropeza, 2008), particularly in palms at early stages of the disease or in symptomless palms (Oropeza *et al*., 2011). This limitation is usually overcome using nested-PCR, involving two amplification cycles. Unfortunately, this is a time consuming process and more prone to generate carryover contaminations, among other disadvantages. An alternative approach is the use of real-time PCR coupled with the use of TaqMan probe technology; as it has recently been developed for specific, accurate and highly sensitive assays for detection and quantification for other phytoplasmas (Christensen *et al*., 2004; Herath *et al*., 2010; Hren *et al*., 2007). Here we report the development of a TaqMan/real-time PCR assay for the detection and quantification of LY-phytoplasma DNA in coconut palms.

A real-time PCR assays based on the 23S rRNA gene for universal phytoplasma detection, including the group 16SrIV was recently developed (Hodgetts *et al*., 2009). These authors reported non-quantitative assays for subgroup 16SrIV-A phytoplasma in the Americas, and for both subgroup 16SrIV-C and group 16SrXXII phytoplasmas in West and East Africa, respectively, but did not include other palm-associated phytoplasmas present in the Americas. Another case is the detection of coconut wilt phytoplasma by real-time PCR using the SYBR green reported by Manimekalai *et al.* (2011), however quantification of the phytoplasma was not included.

In order to test the specificity of the primers/probe set designed in this study a blast analysis of the forward primer and the probe combination was made and the results suggested high specificity. Then the whole set LY 16S primers/probe set was tested experimentally for real-time

PCR amplification of samples of DNA of different phytoplasmas, from different 16Sr groups; successful amplification was obtained only with DNA of LY phytoplasmas (16SrIV group) that were sampled in the Americas (subgroups A, D and E), whereas no amplification was obtained from DNA of LY phytoplasmas from Africa (16SrIV subgroups B and C) or associated with other diseases and corresponding to other 16Sr groups. Therefore, the forward and reverse primers and the probe for the TaqMan/real-time PCR assay designed in this study showed the same high specificity as the state-of-the-art primers for LY phytoplasma subgroups present in the Americas (16SrIV-A, -D, and -E subgroups).

The LY-specific TaqMan/real-time PCR assay was also able to detect 10 copies of the target phytoplasma gene with a Ct of 35.3. Failure to amplify one copy of the target within the remaining 4.7 PCR cycles could be attributed to a stochastic effect as previously suggested by Hren *et al.* (2007), when less than 10 copies are present in the reaction mix.

Twenty five plants sampled as negative controls were symptomless at the moment of sampling and remained symptomless afterwards (for at least six months in the case of mature bearing plants in an open environment and twelve month in the case of young non- bearing plants kept within insect-proof and insect free enclosures). Nevertheless, 14 of these plants tested positive in real time PCR assay, although with a high Ct value (37 and above). Low levels of cross reactivity have been reported for other group-specific reagents in real-time diagnostic PCR (Herath *et al*., 2010; Chandelier *et al*., 2010). The low signal in some samples could be due to contamination with DNA from bacteria during sampling. For example Christensen *et al.* (2004) report slight cross reactivity in their TaqMan probe assay for a broad range of phytoplasma, with some bacteria unrelated with phytoplasma. In this study, as all healthy plants tested negative in LY-specific and universal nested PCR tests, a Ct value of 37 was set as threshold for positive LY detection. However is important to take into consideration that the limit of detection was below 10 copies per reaction and any Ct ranging from 35.3 to 36.9 should be considered uncertain. Establishing a clear-cut separation between the true signal from infected palms (Ct ≤ 35.3) and the signal noise obtained from healthy palms (Ct ≥ 37) would make the diagnostic system reliable.

When the two assays were then tested with DNA extracts obtained from trunk samples of 36 coconut palms showing LY symptoms (stage 1-4 as described by McCoy *et al*., 1983), results showed that the LY 16S TaqMan/real-time PCR assay (80.6% positive detection), with one amplification step, was more sensitive than the LY-group specific nested-PCR assay (33.3% positive detection), with two amplification steps.

The increase in sensitivity could be related to the low size of the amplicon designed for the real-time PCR assay compared to that of the conventional nested PCR (at least 10 times shorter than nested-PCR), increasing the efficiency of amplification, reducing sensitivity to DNA degradation (Hren *et al*., 2007), and reducing the effect of inhibitors more than for standard PCR, because they have a greater effect in the late cycles of PCR which are critical for product accumulation and product visualization by gel electrophoresis (Mumford *et al*., 2006). The LY16S TaqMan real-time PCR assay described above was then used to study the quantity of LY phytoplasma DNA in different parts of LY-affected palms. The phytoplasma titre found in the present study is within the range of those reported in other studies, such as for the branch-inducing phytoplasma in *Euphorbia pulcherrima* stems and petioles (Christensen *et al*., 2004), the peach yellow leaf roll phytoplasma in *C. roseus* shoots (Christensen *et al*., 2004) or the Chrysanthemum yellows phytoplasma in *Chrysanthemum carinatum* leaves and roots (Saracco *et al*., 2006).

The measured differences in phytoplasma titre among plant parts are consistent with the hypothesis proposed previously by Parthasarathy (1974) and Zimmerman (1979), that phytoplasmas move from photosynthate source tissues to sink tissues via the phloem as a result of a mass flow process. Therefore, as in the present case, phytoplasmas would not be detectable or would be less abundant in source tissues like mature and intermediate leaves whereas they would be more abundant and more easily detectable in sink tissues of expanding parts such as immature leaves, inflorescences and root apex, or in phloem-rich organs such as trunk. These results also agree with those recently reported by Oropeza *et al.* (2011) who, using nested-PCR, found that detection was more readily achieved in sink tissues than in source tissues, with the trunk as the organ with the highest frequency of detection of LY-phytoplasmas.

Similar patterns of distribution have been reported for phytoplasmas associated with other plant species such as Flavescence dorée phytoplasmas in *Vicia faba* (Lherminier *et al*., 1994) and dieback disease phytoplasmas in *Carica papaya* (Siddique *et al*., 1998). Herath *et al.* (2010) estimated the concentration of elm yellows phytoplasma in different tissues of *Ulmus americana*, but without normalization. They found that bark samples showed the highest pathogen load, and sprouts and leaves showed the lowest one.

In summary, the present LY16S TaqMan/real-time PCR assay represents a new alternative for LY phytoplasmas detection and quantification, offering high specificity and improvements in sensitivity. The assay was also used to perform quantitative analysis of LY phytoplasmas interaction with coconut plants in a more precise fashion.

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