ORIGINAL ARTICLE

Simultaneous detection of coconut lethal yellowing phytoplasmas (group 16SrIV) by real-time PCR assays using 16Sr- and GroEL-based TaqMan probes

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Abstract

Lethal yellowing (LY) represents a serious threat to coconut in Mexico, Central America and the Caribbean islands. It is caused by a phytoplasma of group 16SrIV, and particularly subgroups -A and -D have been the major phytoplasmas affecting coconut palms grown in the southeast of Mexico. Therefore, is important to reliably detect the 16SrIV group of phytoplasmas not only for diagnosis purposes but also to improve the understanding of pathogen-plant-vector pathosystems. The 16S ribosomal operon genes have been particularly used as the targets for probes and primers for universal phytoplasma detection. However, its conservative nature has imposed the search of non-ribosomal genes to support a finer characterization and differentiation of phytoplasmas, particularly those closely related and one alternative has been the *GroEL* gene for the specific detection of subgroups 16SrIV-A and 16SrIV-D. Sensitive, rapid and reliable singleplex and duplex assays were developed based on real-time PCR (TaqMan) targeting both the *GroEL* gene and 16S rRNA. The assay performed with higher sensitivity compared to conventional nested-PCR when analysing DNA extracts obtained from LY symptom bearing palms. The LY 16S TaqMan probe assays amplified DNA from both subgroups, while the LY GroEL TaqMan probe assay only amplified DNA from subgroup 16SrIV-A. The duplex TaqMan probe assay is a novel option for the simultaneous detection and quantification of LY phytoplasmas of 16Sr subgroups -A and -D, which are the phytoplasmas that predominantly affect palms in Mexico.

Keywords *Cocos nucifera* L. · Palms · Diagnosis · Singleplex and duplex assays

Introduction

Lethal yellowing (LY) is a serious disease that affects coconut palms and at least other 35 palm species in the American continent (Harrison and Oropeza 2008). It is caused by phytoplasma and the infected nut bearing coconut palms exhibit visual symptoms, beginning with an early fall of the nut (stage 1), necrosis of the inflorescences (stages 2-3), followed by leaf chlorosis and leaf senescence (stages 4–6), death of the spear leaf (stages 7-8), and finally palm death (stage 9), normally between 3 to 6 months after the symptoms first appear (McCoy et al. 1983). The only vector of LY phytoplasmas, identified so far, is the leafhopper *Haplaxius crudus* (Hemiptera: Cicadellidae) (Howard et al. 1984). LY epidemics, affecting mostly the Atlantic tall coconut palm, has been associated with the strain of phytoplasma belonging to the subgroup A of group 16SrIV, (i.e. 16SrIV-A) (Oropeza et al. 2005). Additional subgroups of phytoplasmas are 16SrIV-B, D, E and F (Harrison et al. 2008). In the southeast of Mexico, the predominant subgroups are 16SrIV-A and 16SrIV-D (Cordova-Lara et al. 2017).

The reliable and early detection of LY phytoplasma is essential to distinguish infected from healthy coconut palm populations, additionally it is important to discriminate LY phytoplasmas from others that are also responsible for yellowing symptoms in coconut palm trees. However, phytoplasma diagnosis in palm species is hindered due to its low

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titre in the plant phloem and its irregular distribution throughout the palm (Harrison and Oropeza 2008). To address this issue, reliable and sensitive molecular techniques have been developed to detect and differentiate LY phytoplasmas (Harrison et al. 1999). Currently a nested-PCR method is used to detect LY phytoplasmas in palms or tissues (Harrison and Oropeza 2008). It is important to note, however, that nested-PCR greatly increases the likelihood of contamination and of false positive results, mainly due to the opening of the first-round reaction vessel, from which an aliquot is removed, diluted and used in a second reaction. Additionally, the nested-PCR procedure also requires more labour and PCR components. To overcome these issues, real-time PCR methods have been developed.

In real-time PCR the accumulation of amplified products during the reaction is measured by detecting a fluorescent signal. It is faster, more accurate, has an increased high-throughput capacity and lacks post-PCR manipulation which could, in turn, be a source of carryover contamination (Mumford et al. 2006). Moreover, different fluorogenic dyes can be used enabling the detection of several DNA targets simultaneously in a multiplex format assay. In the case of phytoplasma detection, there are two approaches that have been mostly used: DNA intercalating dyes (i.e.: SYBR Green I or Eva-Green) and TaqMan probes (Linck et al. 2017). Even more, the use of TaqMan probes technology can improve the specificity of the gene targets as it has been reported for phytoplasmas infecting *Rubus* species (Linck et al. 2017) and *Vitis vinifera* (Hren et al. 2007).

Real-time PCR has been used to detect various groups of phytoplasmas (Linck et al. 2017). For phytoplasma associated with coconut, the development of TaqMan real-time PCR assays based on the 23S rRNA gene, for the universal phytoplasma group including the group 16SrIV, was reported by Hodgetts et al. (2009). Another TaqMan PCR assay was reported by Nejat et al. (2010) for the detection of the coconut yellow decline in Malaysia. Manimekalai et al. (2011) and Nair et al. (2016) used a real-time PCR assay to detect coconut root (wilt) phytoplasma in India. Additionally, a TaqMan probe method targeting the 16S ribosomal DNA of LY phytoplasma affecting coconut plantations in Mexico was developed by Córdova et al. (2014). The latter assay showed a higher sensitivity for amplification of coconut LY phytoplasma when compared to conventional nested-PCR. Recently, Bahder et al. (2017) utilized Eva-Green as an intercalating DNA agent combined with, and high-resolution melting analysis to detect and differentiate phytoplasma of the subgroups 16SIV-A and 16SIV-D in Florida (USA), using primers that amplified the 16Sr RNA gene.

Ribosomal operon genes have been commonly used as the platform to design probes and primers to be used for universal phytoplasma detection through real-time or quantitative PCR. However, in order to characterize and discriminate among closely related phytoplasmas, non-ribosomal genes have been explored; Galetto et al. (2005) used the nitroreductase gene on apple proliferation phytoplasma (16SrX), Wei et al. (2004) used the *tuf* gene on onion yellows phytoplasma. Specific *Sec Y* and *Stoll 11* (Hren et al. 2007) and *Map* (Pelletier et al. 2009) genes were employed to detect bois noir (BN) and flavescence dorée (FD) phytoplasmas in grapevine. Danet et al. (2010) used a *Map* gene for the detection of stolbur phytoplasma in strawberry. Recently, a rapid and sensitive real-time PCR assay was developed targeting the *secY* gene of 'Candidatus Phytoplasma rubi', a phytoplasma that infects *Rubus* species (Linck et al. 2017). An alternative for phytoplasma detection is the *GroEL* gene. This is a highly conserved gene, previously used for the characterisation of prokaryotic strains (Desai et al. 2009); furthermore, it has been proposed as a useful marker for the distinction of strains of phytoplasma (Mitrović et al. 2011).

As mentioned above the predominant subgroups of phytoplasmas affecting palms in the southeast of Mexico are 16SrIV-A and 16SrIV-D. Currently the identification of these subgroups is carried out by Restriction Fragment Length Polymorphism (RFLP) analysis of the 16S rRNA gene and sequencing of the nested-PCR amplification products (Vázquez-Euán et al. 2011). The employment of a primer/probe set, capable of discerning subgroups 16SrIV-A and -D in the same PCR reaction tube, could help to promptly identify the LY phytoplasma subgroup affecting palms in these areas and to better understand the epidemiology of LY in Mexico.

Therefore, in this work a rapid, reliable and sensitive real-time PCR (TaqMan) duplex assay, based on the *GroEL* gene, was developed. This new method is a useful tool for the detection and identification of 16SrIV phytoplasma subgroups -A and -D, and, contributes to improve LY management in Mexico.

Materials and methods

Plant samples The plant material used in this study were the trunks of coconut palms (*Cocos nucifera* L.), bearing typical LY symptoms. Three batches of trunk samples were collected from coconut palms as previously described by Oropeza et al. (2011). The first batch was obtained from thirty mature coconut palms of the Atlantic Tall (AT) variety showing LY symptoms (trunks of 25 palms were used for field testing and the remaining five used for sensitivity analysis). These were located on the northern coast of the State of Yucatan, Mexico and belonged to LY-infected coconut groves (Celestun 20°51'N, 90°23'W; Dzilam 21°23'N, 88°53'W; Chelem 21°16'N, 84°44'W; Sisal 21°09'N, 90°01'W; Chuburna 21°15'N, 89°48'W). The second batch corresponded to trunks from ten Malayan Yellow Dwarf (MYD) coconut variety palms that were used for field evaluation. All these individuals were asymptomatic at the time of sample collection and they

belong to a LY-free coconut grove in Telchac Puerto (21°21′N, 89°16′W). The third batch corresponds to the sampling of ten additional coconut palms of the Atlantic Tall variety, growing in LY-infected groves of Chuburna and Chelem. The second and third batches were utilized for the experiment shown in Table 5. Additionally, leaves of six *Adonidia merrillii* palms, showing LY-like symptoms located in different sites of Merida, Mexico (21°04'N, 89°39'W; 21°01 N, 89°38′; 21°00 N', 89°42'W), were also collected as described by Cordova-Lara et al. (2017) for specificity assays.

DNA extraction Trunk tissue samples were obtained using a portable electric drill, fitted with a 6.5-in-long bit and the sawdust was collected into a plastic bag. The drill bit was first washed with 70% alcohol, then washed again with a dilution of commercial bleach (0.6% sodium hypochlorite) and rinsed with sterile distilled water. The plant tissue samples were stored on ice immediately for transport to the laboratory and stored at −20 °C, prior to DNA extraction and PCR analysis.

Each sample was pulverised in liquid nitrogen and mixed with 500 μL of hot (65 °C) 2% cetyltrimethylammonium bromide (CTAB) buffer according to the protocol described by Harrison et al. (1994). For the trunk samples the liquid nitrogen step was eliminated and the samples were mixed directly with hot (65 °C) CTAB buffer during 30 min. The resulting extracts were mixed with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) and centrifuged at 14,000 g for 5 min. The DNA was precipitated from the upper aqueous phase by the addition of cold isopropanol and pelleted by centrifugationand was dried, resuspended in 100 μL of TE buffer (1 mM Tris, 0.1 mM EDTA, pH 8) and incubated with RNAse at 37 °C, for 1 h. DNA samples were quantified using a NanoDrop 2000c (Thermo Scientific, Wilmington, DE, USA) spectrophotometer.

Conventional nested-PCR assays The PCR reactions were performed in a 25 μL volume, containing 125 μM of each dNTP (Invitrogen, Carlsbad, CA, USA), 1 μM of each primer, 1 μL DNA (50 ng DNA), 1 U Taq DNA polymerase (Invitrogen) and standard PCR buffer with 1.5 mM MgCl₂. The PCR was run for 35 cycles in a CFX96 (Bio-Rad, Hercules, CA, USA), utilizing phytoplasma-universal rRNA primer pair P1 (Deng and Hiruki 1991) and P7 (Smart et al. 1996), with the following parameters: denaturation for 60 s at 94 °C; annealing for 50 s at 54 °C and extension at 72 °C for 1 min (10 min for final cycle). The PCR products of the initial P1/P7 primers were diluted to 1:40 with sterile ultrapure water and re-amplified for 35 cycles utilizing LY-group 503f/LY16Sr primer pair, according to Harrison et al. (1999) or universal phytoplasma 16S rRNA gene primer pair R16F2n/R16R2 as described by Gundersen and Lee (1996).

Negative (symptomless PCR negative plant DNA extract and ultrapure water in the place of DNA) and positive control samples (palm with symptoms PCR positive) were included in all PCR assays. Nested-PCR aliquots (10 μL) were electrophoresed through 1% agarose gels, stained with 0.1% ethidium bromide and photographed using the Gel Doc 2000gel documentation system (Bio-Rad).

Design of the LY GroEL primers/ TaqMan probe TaqMan probes and primers were designed using primer express software (Applied Biosystems, Foster City, CA, USA). Twelve sequences from the GenBank which showed higher homologies (76–74% identity) with the LY GroEL sequence (16SrIV-A subgroup; GenBank accession number KY779619.1) were aligned. A divergent nucleotide zone was used to design the GroEL TaqMan probe and primers (Fig. 1). The following GenBank accessions were used: 'Onion yellows phytoplasma' (AB124808.1, AB124807.1 and AP006628.2); 'Marguerite yellows phytoplasma' (AB242235.1); 'Paulownia witches-broom phytoplasma' (AB124810.1); 'Potato purple top phytoplasma' (AB167357.1); 'Phytoplasma sp. AYBG (AB124811.1); 'Candidatus Phytoplasma solani' (KF991242.1); 'Brassica napus phytoplasma'(KF430807.1); 'Sesame phyllody phytoplasma' (KM272591.1); 'Rhus yellows phytoplasma' (AB738741.1); 'Lettuce yellows phytoplasma' (AB242233.1). The alignment was constructed utilizing the CLUSTALW program.

The TaqMan probe and primers were synthesized by Thermo Fisher Scientific Company (Applied Biosystems, Foster City, CA, USA). The TaqMan probe was labelled with 6-carboxyfluorescein (FAM) at the 5′ end and a non-fluorescent quencher (NFQ), with minor groove binder (MGB), at the 3′ end in the singleplex assay. The specific primers sequences and TaqMan probe were the following: LY GroEL-LSF 5′-CTACTACTGCAACTGTTTTA GCTCAGT-3′/ LY GroEL-LSR 5′-AATTCCTTCTCTAA GCAGAACAGCTTT-3′ primers and probe 5′-FAM-CCGGATTCAACAAATTT-NFQ-3′, for specific detection of group 16SrIV phytoplasmas.

Real-time PCR reactions Real-time PCR reactions were performed according to Córdova et al. (2014). The reactions were carried out in a 20 μL volume, containing 1 μL of primer mix with 900 nM of each primer, probe (250 nM) and 50 ng DNA of sample, 10 μL of TaqMan Universal PCR master mix with AmpErase uracil N-glycosylase UNG (Applied Biosystems). Real-time PCR reactions were performed with a CFX96 real-time PCR system (Bio-Rad). PCR was initiated for 2 min at 50 °C, to activate AmpErase UNG, then, 95 °C for 10 min, to activate AmpliTaq Gold DNA polymerase, followed by 40 cycles for 15 s at 95 °C and at 61 °C for 1 min. All DNA samples, including controls, were assessed in duplicate or triplicate as described in text. The baseline was automatically calculated by using Bio-Rad CFX Manager software IQ, and the fluorescence threshold was set manually to intersect with

Fig. 1 Alignment of the *GroEL* gene sequences within aligned nucleotide sequences from the GenBank. The empty boxes show the position of the primers and gray box the position of the probe LY GroEL. AB124808.1, AB124807.1 and AP006628.2 'Onion yellows phytoplasma'; AB242235.1 'Marguerite yellows phytoplasma'; AB124810.1 'Paulownia witches-broom phytoplasma'; AB167357.1 'Potato purple top phytoplasma'; AB124811.1 'Phytoplasma sp. AYBG; KF991242.1 '*Candidatus* Phytoplasma solani'; KF430807.1 '*Brassica napus* phytoplasma'; KM272591.1 'Sesame phyllody phytoplasma'; AB738741.1 'Rhus yellows phytoplasma'; AB242233.1 'Lettuce yellows phytoplasma'. The alignment was constructed utilizing the CLUSTALW program

the linear part of the amplification curves, resulting in the final cycle crossing point (Cq).

The assay including the GroEL primers/probe set and real-time PCR conditions will henceforth be referred to as the "LY GroEL TaqMan probe assay".

Specificity The LY GroEL Taqman probe assay specificity was assessed using DNA of 13 other phytoplasma strains. These included: ash yellows (ASHY), strawberry green petal (SGP), peach western X (PWX), elm yellows (EY), pigeon pea witches' broom (PPWB), beet leafhopper-transmitted virescence agent (BLTVA), Mexican periwinkle virescence (MPV), Bermuda grass white leaf (BGWL), Jujube witches' broom (JWB), coconut LY-Florida (CLY-FL), coconut lethal decline Tanzania (LD), *A. merrillii* phytoplasma and coconut lethal disease (Mozambique). As negative control, DNAs from symptomless palms were included. In Table 1 are shown the 16Sr group affiliations of the phytoplasma DNA samples.

Robustness of the LY GroEL TaqMan probe assay The robustness of the assay was evaluated by applying the LY GroEL TaqMan probe assay at two different days on random DNA samples of ten infected palms from the three batches mentioned above. A paired t-test was carried out using the average

Cq values of the ten palms at day 1 and compared to the Cq values obtained at day 2, with a significance level of 0.05.

Duplex GroEL TaqMan probe assay development Samples from different symptomatic and non-symptomatic palms were simultaneously analysed, using the LY GroEL TaqMan probe assays developed in this study and LY 16S TaqMan probe assays developed by Córdova et al. (2014), labelled with different fluorophores. The 16S TaqMan probe was labelled with FAM, while GroEL was labelled with 4,5,6,7-tetrachlorofluorescein (TET). The assays and the PCR conditions were the same as those mentioned above for LY GroEL TaqMan probe/primers assays and for LY 16S, as reported by Córdova et al. (2014). The CFX96 real-time PCR system (Bio-Rad) was programmed to record the readings at 515 nm for the FAM labelled probe and 536 nm for the TET labelled probe. The Cq values of each PCR reaction were calculated automatically by CFX Manager software IQ (Bio-Rad).

Sensitivity of single and duplex assays DNA extracts from AT palms bearing necrosis of the inflorescences (stages 2–3), were diluted (10-fold) from 300 ng/µL to 0.03 ng/µL with ultrapure water and used to compare the sensitivities of the

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

Type of phytoplasma/source	Conventional nested-PCR with universal primers: P1/P7-R16F2n/R16R2	Conventional nested-PCR With LY group primers: P1/P7-503f/LY16Sr	TaqMan/Real-time PCR with LY GroEL primers/ probe set (Cq)[a]
Non-LY group phytoplasmas			
Peach western X (PWX), 16SrIII 'Candidatus Phytoplasma pruni'	+	ND	ND
Elm yellows (EY), 16SrV-A.' Candidatus Phytoplasma ulmi'	+	ND	ND
Jujube witches' broom (JWB), 16SrV-B. 'Candidatus Phytoplasma ziziphi'.	+	ND	ND
Beet leafhopper-transmitted virescence agent (BLTVA), 16SrVI-A	+	ND	ND
Ash yellows (ASHY), 16SrVII-A 'Candidatus Phytoplasma fraxini'	+	ND	ND
Pigeon pea witches' broom (PPWB), 16SrIX-A	+	ND	ND
Mexican periwinkle virescence (MPV), 16SrXIII-A 'Candidatus Phytoplasma hispanicum'	+	ND	ND
Strawberry green-petal (SGP), 16SrXIII-B	+	ND	ND
Bermuda grass white leaf (BGWL), 16SrXIV-A. 'Candidatus Phytoplasma cynodontis'	+	ND	ND
LY group phytoplasmas			
Coconut LY, Florida, 16SrIV-A 'Candidatus Phytoplasma palmae'	+	+	21.6
Coconut LY, México, 16SrIV-A 'Candidatus Phytoplasma palmae'	+	+	19.5
Coconut lethal decline, Tanzania (LDT), 16SrIV-C	+	ND	ND
A. merrillii, Mexico, 16SrIV-D 'Candidatus Phytoplasma palmae'	+	+	ND
Coconut lethal disease, Mozambique, 16SrXXII-A 'Candidatus Phytoplasma palmicola'	+	ND	ND
Uninfected palms			
Symptomless coconut palm (Mexico)	–	ND	ND
Symptomless coconut palm (Mozambique)	–	ND	ND

Cq quantification cycle, LY lethal yellowing, ND not detected, + positive detection

[a] tested in duplicate

nested-PCR and real-time PCR assays. DNA samples were used in triplicate for real-time PCR assays. The correlation coefficient (R^2) and Efficiency (E) of the real-time PCR assays were also assessed. The efficiency of detection was calculated by plotting the obtained Cq versus the log values of each concentration of DNA. from the serial dilution. The efficiency of the assay corresponds to the slope of the obtained standard curve ($E = 10^{-1/slope}$).

Results

In silico analysis of the LY GroEL TaqMan probe/primer

The sequence of LY GroEL gene consists of 1629 bp (GenBank accession No. KY779619.1). Primers anneal in the region 5′-259-286-3′ and 5′-327-354-3′ and its complement respectively, while the probe anneals in the region 5′-306-323-3′. An amplification product of 95 bp was obtained. The sequences of reverse primers and probe were tested together on GenBank (NCBI) in search of in silico homology to sequences of phytoplasmas using BLAST (Altschul et al. 1997). The results showed homology only with the LY GroEL sequence of this study with 100% of identity, 100% coverage and E value of 3×10^{-15}. Oppositely, the forward primer presented 100% identity with the LY GroEL sequence and other unrelated sequences of procaryotes.

Specificity of the LY GroEL TaqMan probe assay The specificity of the newly designed LY GroEL primers/probe set was evaluated using DNA extracts from palm and non-palm species, with and without LY phytoplasma (Table 1), The plants were assessed by nested-PCR with either universal or LY group specific primers targeting the 16S rRNA gene, which yielded amplifications for all samples tested, except for the

asymptomatic palms used as negative controls. Samples that showed amplification curves using the LY GroEL primers/ probe set corresponded to the 16SrIV subgroup A phytoplasmas, previously reported in coconut palms in Florida, USA (Tymon et al. 1998) and in Mexico (Oropeza et al. 2005). The newly designed GroEL primers/probe set did not yield amplification plots when used on DNA extracts of phytoplasmas of groups 16SrIV-C or 16SrXXII-A from African palms and 16SrIV-D from *A. merrillii* (Table 1). No amplification was neither obtained for other phytoplasma groups, including 16SrIII, 16SrV, 16SrVI, 16SrVII, 16SrIX, 16SrXIII, and 16SrXIV (Table 1).

LY GroEL TaqMan probe assay sensitivity The sensitivity of both, the nested-PCR and TaqMan probe real-time PCR assays, was compared using DNA samples extracted from four field palms which had been confirmed as positive for LY phytoplasma (Table 2). Samples were serially diluted (10-fold) with ultrapure water in order to determine the lower detection limits, by means of a real-time PCR assay, with a range of 300 ng – 0.003 ng of DNA. Both assays where able to detect the presence of LY DNA in all four samples in the dilutions ranging from 300 ng – 3 ng (Table 2). In one of the four palms (Palm 1), amplification was only obtained for up to three dilutions (300 to 0.3 ng) with the LY GroEL TaqMan probe assay, while no amplification could be obtained using the LY group-specific nested-PCR assay at 0.3 ng (Table 2). Additionally, in another of the tested samples (Palm 4), amplification with LY GroEL TaqMan probe assay could be obtained within the range of 300 to 0.03 ng, two additional dilutions, when compared to the LY group-specific nested-PCR assay. In the case of the samples of Palm 3 the nested-PCR amplification was obtained with one additional dilution (0.3 ng) when compared to the LY GroEL TaqMan probe assay. In the remaining case (Palm 2), the sensitivity was similar in both methods (Table 2). The PCR efficiency ranged from 93–107% and the correlation coefficient ranged from 0.99–1.0%, showing that the LY GroEL TaqMan assay, was adequate for the detection and quantification of LY phytoplasma DNA in palm samples (Table 2).

The robustness of the assay was evaluated by performing a comparative detection of ten LY infected palms using the LY GroEL TaqMan probe assay in two separate days. No difference was observed in Cq values of LY infected palms between the two days (Table 3).

Comparison of LY GroEL TaqMan probe assay and LY group-specific nested-PCR in field coconut palms Samples were collected at various locations along the coast of Yucatan from the trunks of a total of 25 coconut palms showing LY symptoms. The presence of LY phytoplasma in the samples was detected using nested-PCR and LY GroEL TaqMan probe assays mentioned previously. When the LY GroEL TaqMan probe assay was used, 20 out of the 25 samples (80%) showed positive amplification for LY phytoplasma; while the LY group-specific nested-PCR assay showed positive amplification for only 13 out of the 25 samples (52%). Furthermore, the samples that tested positive by nested-PCR assay were also positive by real-time PCR. The Cq values of the positive samples for the real-time PCR assay and negative for nested-PCR assay ranged from 28.4 to 32.3 and the Cq values for positive samples using both assays ranged from 16 to 28.2.

Comparison of LY GroEL and LY 16S TaqMan probe assays A duplex assay was developed using the LY 16S TaqMan probe assay, reported by Córdova et al. (2014), combined with the LY GroEL/real-time PCR assay for LY phytoplasma detection of this study to test if both assays could be performed in a single reaction tube. For this purpose, the probes were labelled with different fluorophores; the LY 16S TaqMan and the LY GroEL probes were labelled with FAM and TET, respectively. To compare the sensitivity of

Table 2 Comparison of sensitivity between the LY GroEL TaqMan probe assay and nested-PCR assay with primers P1/P7 and 503f/LY16Sr

Palm	Dilution factor of original sample (DNA content in tube)												Linear regression		
	10^0 (300 ng)		10^{-1} (30 ng)		10^{-2} (3 ng)		10^{-3} (0.3 ng)		10^{-4} (0.03 ng)		10^{-5} (0.003 ng)				
	RT-PCR (Cq)[a]	Nested-PCR	RT-PCR (Cq)[a]	Nested-PCR	RT-PCR (Cq)[a]	Nested-PCR	RT-PCR (Cq)[a]	Nested-PCR	RT-PCR (Cq)[a]	Nested-PCR	RT-PCR (Cq)[a]	Nested-PCR	R^2	%E	Slope
1	25.4	+	29.1	+	31.8	+	35	–	ND	–	ND	–	1.0	107.3	−3.16
2	25.5	+	28.6	+	32.0	+	36.0	+	ND	–	ND	–	1.0	93.0	−3.50
3	27.0	+	29.8	+	33.7	+	37.1	+	ND	–	ND	–	0.99	98.1	−3.37
4	21.5	+	23.2	+	27.2	+	30.9	–	35.8	–	37.9	–	0.98	88.3	−3.64

DNA samples were obtained from trunk tissues of four coconut palms infected with LY phytoplasma

Cq quantification cycle, R^2 correlation coefficient, %E %efficiency, ND No amplification detected within the working range of the program with a maximum of 40 cycles. Samples were considered negative for LY GroEL TaqMan probe assay if the Cq value was ≥37

[a] Tested in triplicate

Table 3 Comparative detection of LY phytoplasma DNA using LY GroEL TaqMan probe assay in two different days

Coconut palms	Day 1 ($Cq \pm SD$)[a]	Day 2 ($Cq \pm SD$)[a]
Palm 1	28.9 ± 0	28.9 ± 0.28
Palm 2	29.2 ± 0.07	29.1 ± 0.28
Palm 3	31.2 ± 0	31.3 ± 0.07
Palm 4	30.9 ± 0.21	30.8 ± 0.28
Palm 5	25.5 ± 0.21	25.6 ± 0.57
Palm 6	30.1 ± 0	30.2 ± 0.07
Palm 7	24.9 ± 0.14	24.8 ± 0
Palm 8	30.2 ± 0.14	29.9 ± 0.57
Palm 9	29.3 ± 0.14	29.3 ± 0.49
Palm 10	27.2 ± 0	27.9 ± 0.07

DNA samples were obtained from trunk tissue of ten coconut palms infected with LY phytoplasma

Cq quantification cycle, SD standard deviation, Cq values of two different days did not show significance difference using paired t-test ($p = 0.66$)

[a] Tested in duplicate

the single and duplex assays, 10-fold dilutions of 300 ng total DNA, in a series of five steps were tested. The singleplex assays had a 12.2% difference in PCR efficiency (Table 4). When both probes were tested in the same tube, the PCR efficiency of the duplex assay for LY 16S with respect to that of the singleplex assay was similar (95.1 and 97.9%, respectively). However, the PCR efficiency of the duplex assay for LY GroEL decreased from 107 to 89% compared to that of the singleplex TaqMan probe assay. For both, the single and duplex assays, the coefficient of determination was 0.99 (Table 4). These

results indicate that single and duplex assays can be used to detect and quantify LY phytoplasma.

Comparison of LY GroEL and LY 16S TaqMan probe duplex assays on field samples The potential of the LY GroEL and LY 16S TaqMan probe duplex assay to detect LY phytoplasma in field palms was tested. For this purpose, 20 palms were sampled at the north coast of the Yucatan Peninsula and samples were analysed using the duplex assays. Ten palms showed visual symptoms of LY (stages 2-4) such as fallen nuts and necrotic inflorescences. The results showed that both assays were able to amplify the LY phytoplasma in symptomatic palms. The Cq values ranged from 16.1–30.8. The real-time PCR assays of DNA from ten symptomless palms of the Malayan Yellow Dwarf ecotype showed a high Cq for seven and two palms for the LY 16S and GroEL TaqMan assays respectively ($Cq \geq 37$ was considered to be a negative detection for LY according to Córdova et al. 2014) (Table 5).

Using a duplex TaqMan probe assay to detect two subgroups of LY phytoplasma In the results showed in Table 1, no amplification for phytoplasma subgroup 16SrIV-D using the TaqMan probe LY GroEL assay was detected, however, in a previous study the 16S TaqMan probe assay was able to detect this subgroup (Córdova et al. 2014). For this reason, the LY 16S and LY GroEL TaqMan probe singleplex assays were combined in a duplex assay to enable the simultaneous detection of the two strains of LY phytoplasma, namely Group 16SrIV, subgroups A and D that predominantly infect palms in Southeast Mexico. Phytoplasma DNA from subgroups 16SrIV-A and 16SrIV-D from *A. merrillii* infected palms used

Table 4 Comparison of sensitivity between single and duplex assays using LY phytoplasma DNA from trunk tissue of coconut palms

DNA amount (ng)	Single assay probe/primers LY 16S ($Cq \pm SD$)[a]	PCR parameters	Duplex assay probe/primers LY 16S ($Cq \pm SD$)[a]	PCR parameters
300	19.6 ± 0.14	$R^2 = 0.99$	20.4 ± 0.07	$R^2 = 0.99$
30	24.4 ± 0.09	Slope $= -3.45$	24.2 ± 0.08	Slope $= -3.37$
3	27.6 ± 0.26	PCR efficiency $= 95.1\%$	27.4 ± 0.25	PCR efficiency $= 97.9\%$
0.3	30.0 ± 0.11		30.4 ± 0.09	
0.03	34.1 ± 0.04		34.0 ± 0.08	
	Single assay probe/primers LY GroEL ($Cq \pm SD$)[a]		Duplex assay probe/primers LY GroEL ($Cq \pm SD$)[a]	
300	21.3 ± 0.17	$R^2 = 0.99$	21.2 ± 0.06	$R^2 = 0.99$
30	25.9 ± 0.36	Slope $= -3.15$	24.7 ± 0.23	Slope $= -3.60$
3	28.1 ± 0.09	PCR efficiency $= 107.3\%$	28.4 ± 0.33	PCR efficiency $= 89.5\%$
0.3	31.5 ± 0.02		31.2 ± 0.13	
0.03	34.3 ± 0.13		35.8 ± 0.02	

In the singleplex assay, LY 16S and GroEL TaqMan probes were labelled with FAM and for the duplex assay LY 16S probe was labelled with FAM and LY GroEL probe with TET

Cq quantification cycle, SD standard deviation, R^2 correlation coefficient, LY lethal yellowing

[a] Tested in triplicate

Table 5 Comparative detection of LY phytoplasma DNA using the duplex assays with LY 16S probe labelled with FAM and LY GroEL probe labelled with TET

Coconut palms	LY 16S TaqMan probe Duplex assay $(Cq \pm SD)$[a]	LY GroEL TaqMan probe Duplex assay $(Cq \pm SD)$[a]
Atlantic Tall Palms with symptoms		
1	16.1 ± 0.07	15.1 ± 0.07
2	18.1 ± 0.21	21.0 ± 0.07
3	19.1 ± 0.14	21.3 ± 0.07
4	24.8 ± 0.00	27.0 ± 0.00
5	27.7 ± 0.57	30.0 ± 0.35
6	26.8 ± 0.21	28.6 ± 0.35
7	17.5 ± 0.28	22.6 ± 0.71
8	23.4 ± 0.14	24.5 ± 0.28
9	20.4 ± 0.14	22.9 ± 0.07
10	28.2 ± 0.92	30.8 ± 1.41
Malayan Yellow Dwarf Palms Symptomless		
1	38.0 ± 0.90	ND
2	ND	ND
3	ND	ND
4	ND	ND
5	37.3 ± 1.20	ND
6	37.6 ± 0.73	ND
7	37.7 ± 0.60	ND
8	38.6 ± 1.30	ND
9	38.5 ± 0.92	37.3 ± 1.27
10	39.2 ± 1.60	38.7 ± 0.5

DNA samples were obtained from trunk tissues of palms with and without LY symptoms at different locations in Yucatan, Mexico

Negative controls (water) for LY TaqMan probe duplex assay (Cq): 38.5, ND

Negative controls (water) for LY GroEL TaqMan probe duplex assay (Cq): 39.7, ND

Cq quantification cycle, SD standard deviation, ND No amplification detected within the working range of the program with a maximum of 40 cycles, LY lethal yellowing, FAM 6-carboxyfluorescein; TET 4,5,6,7-tetrachlorofluorescein. Samples were considered negative if the Cq value was ≥37

[a] Tested in duplicate

Table 6 Comparative detection of LY phytoplasma DNA using the duplex assays on two strains of LY phytoplasma with LY 16S probe labelled with FAM and LY GroEL probe labelled with TET

Phytoplasma subgroup	LY 16S TaqMan probe Duplex assay $(Cq \pm SD)$[a]	LY GroEL TaqMan probe Duplex assay $(Cq \pm SD)$[a]
16SrIV-A		
Palm 1	24.8 ± 0.5	28.2 ± 0.6
Palm 2	28.0 ± 1.5	28.2 ± 0.2
Palm 3	26.6 ± 0.3	21.1 ± 0.1
16SrIV-D		
Palm 1	26.2 ± 0.4	ND
Palm 2	28.6 ± 1.2	ND
Palm 3	22.6 ± 0.2	ND

DNA samples were obtained from young leaf tissues of symptomatic *A. merrillii* palms at different locations in Yucatan, Mexico

[a] Tested in duplicate; Cq: quantification cycle; SD: standard deviation; ND: No amplification detected within the working range of the program, with a maximum of 40 cycles; LY: lethal yellowing; FAM: 6-carboxyfluorescein; TET: 4,5,6,7-tetrachlorofluorescein

in this assay was the same obtained from the study reported by Cordova-Lara et al. (2017). The results, presented in Table 6, indicate that the LY 16S TaqMan probe assay amplifies DNA from both subgroups, while the LY GroEL TaqMan probe assay exclusively amplifies DNA from subgroup A.

Discussion

A rapid and precise method for the detection of LY phytoplasma DNA is a very important tool for the management of the LY disease, in order to prevent or at least decrease its spread. The TaqMan probe technology, which has already been developed for the detection and quantification of other phytoplasmas (Linck et al. 2017), features these characteristics. Therefore, in a previous work, we used this technology to develop a TaqMan probe assay based on a ribosomal gene for the detection of LY phytoplasma which showed more sensitivity and rapidity when compared to nested-PCR assay (Córdova et al. 2014). An alternative target for phytoplasma detection is the *GroEL* gene. *GroEL* is a conserved gene already used for prokaryotic organism strain characterisation (Desai et al. 2009) and has also been proposed as an additional marker for differentiation of phytoplasma strains (Mitrović et al. 2011).

In order to have a specific LY GroEL TaqMan probe assay for LY phytoplasma detection an alignment with different GroEL sequences obtained from the NCBI GenBank was carried out. The probe and primers were designated in the regions with lesser homology among sequences. Moreover, a blast analysis of the primers and probe sequences was performed and the results suggested a high specificity of the LY GroEL TaqMan probe assay that was corroborated with the experimental results, showing no detection with strains of different groups of phytoplasmas, including some strains that affect coconut palms such as Coconut lethal decline (16SrIV-C) and Coconut lethal disease (16SrXXII-A).

The sensitivity of the LY GroEL TaqMan probe assay was evaluated using 10-fold dilutions of DNA from LY affected palms and used to compare the performance against the LY group-specific nested-PCR assay. The sensitivity of TaqMan probe assay was higher than that observed for conventional nested-PCR. This supports the feasibility of using the one-step amplification GroEL TaqMan probe assay versus the nested PCR assay that requires two steps of amplification. The percentage of PCR efficiency of four different palms with LY

phytoplasma ranged from 107.3 to 91.8 with a coefficient of variation of 7.3%. Similar variations have been reported in TaqMan probe assays for the detection of apple proliferation phytoplasma in apple trees and Flavescence dorée (FD) and Bois noir phytoplasmas in grapevine (Baric and Dalla-Via 2004; Hren et al. 2007). The sensitivity assays showed that LY GroEL TaqMan probe assay could be used for the quantification of the 16SrIV-A LY phytoplasma strain. This could be used to determine the number of LY phytoplasma during the progress of the disease or if ecotypes of coconut palm with different susceptibility to LY disease have different titre of phytoplasma when they are infected, as has been reported for Flavescence dorée phytoplasma in grapevine cultivars (Roggia et al. 2014).

In order to further test the reliability of the assay, ten palms of the Malayan Yellow Dwarf ecotype, that showed no symptoms during a 12 month observation period, were sampled as negative controls. This ecotype had previously been considered resistant to LY by Zizumbo-Villareal et al. (2008). The high Cq values observed for these samples were similar to those corresponding to no-template controls, indicating that the ten collected samples were LY phytoplasma free and that the LY GroEL Taqman probe assay could distinguish healthy from infected palms. Taking into account that a symptomless palm showed a Cq value of 37.3 and that the sensitivity assays showed that the higher limit of detection was 37.9 and according to the established Cq threshold for the 16Sr TaqMan PCR assay, results with a $Cq \geq 37$ were considered as negatives for this study.

The probes designed in our laboratory, LY 16S and LY GroEL, were labelled with different fluorophores to test their performance for the LY phytoplasma detection from coconut palms. Both TaqMan probe assays showed similar performance in linearity and capacity to detect LY phytoplasma from field coconut palms. However, the LY GroEL TaqMan probe assay showed a discrepancy in the PCR efficiency among the singleplex and duplex assays (107% and 89% respectively). This could be due to the two copies of the 16S ribosomal gene in the genome of phytoplasma (Schneider and Seemüller 1994) compared to a single copy of *GroEL* gene (Pérez-López et al. 2016). Therefore, the amplification of the 16S rRNA gene could be more efficient at the beginning of the reaction of the duplex assay, in turn affecting the efficiency of PCR amplification of the *GroEL* gene.

Regarding the specificity, both sets of primers and probes target the LY phytoplasma, but each singleplex assay is able to detect different subgroups. For example, the LY 16S primer probe set can detect both subgroups 16SrIV-A and -D, while the LY GroEL primer/ probe set is able to detect the subgroup 16SrIV-A. The latter has been found in *Thrinax radiata* and *Coccothrinax readii* (Narvaez et al. 2006), *Roystonea regia* and *Acrocomia mexicana* (Narvaez et al. 2016), while the subgroup 16SrIV-D has been detected in *Pseudophoenix sargentii* (Vázquez-Euán et al. 2011). *Sabal mexicana* and *A. merrillii* can harbour both subgroups in the southeast of

Mexico (Vázquez-Euán et al. 2011; Cordova-Lara et al. 2017). A TaqMan approach for detecting and discriminating LY-type disease of coconut was developed by Hodgetts et al. (2009). They used a TaqMan probe designed on the base of the 23S ribosomal gene and a different set of primers to discriminate between the subgroups 16SrIV-B and 16SrIV-C (actually16SrXXII-A) from Africa and 16SrIV-A from America, however the 16SrIV-D subgroup phytoplasma strain was not tested. In another study, Bahder et al. (2017) developed a high resolution melting (HRM) method to differentiate the subgroups 16Sr IV-A (LY) from the 16Sr IV-D (Texas Phoenix palm decline) which has been related to lethal decline of palms in Florida (USA) based on the 16S rRNA gene amplification, which did not involve a TaqMan probe. The use of HRM implies out a post run analysis and to determine the differences in the melting temperature of the corresponding amplified products.

The use of a primer/probe set that is able to discriminate subgroups 16SrIV-A and -D, in the same PCR reaction tube would enable a fast and prompt identification of LY phytoplasma subgroups affecting palms. This would not only reduce processing time but also increase resource efficiency by eliminating the steps required for running the nested-PCR products, their digestion with restriction enzymes and the determination of the digestion profiles in another gel, thereby avoiding a time-consuming protocol.

In conclusion, the present LY GroEL TaqMan singleplex assay is a novel alternative for the rapid and reliable identification of subgroup 16SrIV-A LY phytoplasma, in affected *C. nucifera* palms and other host palm species. Also, in combination with the 16S primer/probe set, the method developed herein represents an important diagnostic tool for the simultaneous detection of phytoplasma groups 16S IV-A and -D, showing its potential to reveal further knowledge related to the interactions that may be established between LY phytoplasmas and their most palm species, particularly *C. nucifera*.

Compliance with ethical standards

Conflict of interest I. Cordova declares that he has no conflict of interest. C. Oropeza declares that he has no conflict of interest. N. Harrison declares that he has no conflict of interest. S. Ku-Rodríguez declares that she has no conflict of interest. C. Puch- Hau declares that he has no conflict of interest. M. Narvaez declares that she has no conflict of interest. L. Sáenz declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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