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Detection of different groups of phytoplasma in palm species of Puerto Rico[1,2]

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ABSTRACT

Palm trees play important cultural, ecological, and economic roles in the tropics. Native palm species such as the royal palm, *Roystonea borinquena* O.F. Cook, are extensively planted in landscapes and urban areas of Hispaniola, Puerto Rico and the Virgin Islands. Worldwide, palms are affected by a disease commonly known as lethal yellowing, linked to the presence of phytoplasmas. Coconut Lethal Yellowing (CLY) is the most devastating disease of palms, associated with the 16SrIV phytoplasma group in the Americas. In Puerto Rico, palms displayed symptoms such as leaf

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chlorosis or yellowing, inflorescence and fruit necrosis, and eventual death. The objective of this research was to identify and characterize phytoplasmas in palms and their potential insect vectors, focusing on a native palm, *R. borinquena*. To fulfill this objective, 69 palms belonging to the species: *Cocos nucifera* (n=15), *Gaussia attendata* (n=1), *Leucothrinax morrisii* (n=1), *Pseudophenix sargentii* (n=1), *Roystonea borinquena* (n=50) and *Washingtonia robusta* (n=1) were sampled within three transects across the island. Ninety percent of palms sampled displayed typical lethal yellowing symptoms. In addition, 12 different insect species (Auchenorrhyncha: Fulgoroidea) that could act as potential phytoplasma vectors were sweep-collected from palms and grasses near the study area. The 16S ribosomal region of phytoplasmas was amplified using nested polymerase chain reaction (PCR) and subjected to restriction fragment length polymorphism (RFLP) analyses to allow their ribosomal grouping. Overall, 17 palm samples were positive to phytoplasmas. These are *C. nucifera* (n = 1), *L. morrisii* (n = 1) and *R. borinquena* (n = 15). Phytoplasmas detected belong to six ribosomal groups: 16SrII, 16SrIII, 16SrIV, 16SrVI, 16SrIX and 16SrX. The most frequent phytoplasma detected (59%) is enclosed in the 16SrII group. Samples from nine royal palms (*R. borinquena*) and one key thatch palm (*L. morrisii*) were positive to the 16SrII-related group. Only one insect specimen, the cixiid *Haplaxius crudus* collected from a phytoplasma positive *R. borinquena* palm, was positive for a 16SrII-related group phytoplasma. Another significant epidemiological finding was the detection of a phytoplasma related to CLY in the 16SrIV group in one individual of *R. borinquena*. This native species is the most abundant palm on the island. To our knowledge this is the first report of a phytoplasma related to the 16SrII group in *R. borinquena* in Puerto Rico and the world. Detection for the first time of other phytoplasmas related to 16Sr groups -III, -IX and -X in palms deserves further studies.

Keywords: phytoplasmas, Nested PCR, 16S rRNA gene

RESUMEN

Detección de diferentes grupos de fitoplasmas en especies de palmas de Puerto Rico

Las palmas juegan roles culturales, ecológicos y económicos importantes en los trópicos. Especies de palmas nativas como la palma real, *Roystonea borinquena* O.F. Cook, se utilizan extensamente en el diseño paisajista y se siembran en áreas urbanas de la Española, Puerto Rico y las Islas Vírgenes. Mundialmente las palmas son afectadas por enfermedades comúnmente llamadas amarillamientos letales asociados a fitoplasmas. El amarillamiento letal del cocotero (ALC) es la enfermedad más devastadora de las palmas asociada a un fitoplasma perteneciente al grupo 16SrIV. Alrededor de la isla de Puerto Rico las palmas muestran síntomas de clorosis o amarillamiento en las ramas, necrosis de las inflorescencias y frutos, y eventual muerte. El objetivo de esta investigación fue identificar y caracterizar fitoplasmas en palmas e insectos vectores potenciales, enfatizando la palma nativa, *R. borinquena*. Para lograr este objetivo, se muestrearon 69 palmas pertenecientes a las especies *Cocos nucifera* (n=15), *Gaussia attendata* (n=1), *Leucothrinax morrisii* (n=1), *Pseudophenix sargentii* (n=1), *Roystonea borinquena* (n=50) y *Washingtonia robusta* (n=1) utilizando tres transectos a través de la isla. El 90% de las palmas muestreadas mostraban síntomas típicos de fitoplasmas. Además, 12 especies diferentes de insectos (Auchenorrhyncha: Fulgoroidea) que podrían actuar como vectores potenciales de fitoplasmas se recolectaron utilizando la técnica de barrido en palmas y gramas cercanas al área de estudio. Se amplificó la región 16S

del gen ribosomal de los fitoplasmas utilizando PCR anidado y se sometió a análisis de RFLP, lo cual permitió separarlos por grupo ribosomal. En total, 17 muestras de palmas fueron positivas a fitoplasmas: *C. nucifera* (n = 1), *L. morrisii* (n = 1) y *R. borinquena* (n = 15). Los fitoplasmas detectados pertenecen a seis grupos ribosomales diferentes: 16SrII, 16SrIII, 16SrIV, 16SrVI, 16SrIX y 16SrX. El fitoplasma más frecuentemente detectado (59%) está asociado al grupo 16SrII. Nueve palmas reales (*R. borinquena*) y una muestra de palma de escoba (*L. morrisii*) fueron positivas al grupo 16SrII. Solo un especímen, el cíxiido *Haplaxius crudus,* fue positivo al fitoplasma relacionado con el grupo 16SrII, coleccionado de una palma de *R. borinquena* positiva al fitoplasma. Otro hallazgo epidemiológicamente significativo de esta investigación fue la detección de un fitoplasma relacionado con el ALC perteneciente al grupo 16SrIV en un individuo de *R. borinquena*. Esta especie de palma nativa es la palma más abundante en la isla. Para nuestro conocimiento este es el primer reporte de fitoplasmas relacionados al grupo 16SrII en *R. borinquena* en Puerto Rico y el mundo. La detección por primera vez de otros fitoplasmas relacionados a los grupos 16SrIII, -IX y -X merece estudios adicionales.

Palabras clave: fitoplasmas, PCR anidado, gen 16S rRNA

INTRODUCTION

In Puerto Rico, as well as in many tropical countries of the Caribbean region, coconut and other endemic palm species are cultural, touristic, aesthetic, agro-ecological and economic icons. Nevertheless, palm trees are threatened by diverse biotic and abiotic factors that disrupt their sustainable production for landscaping and ecotourism. The lack of adaptive varieties, low productivity, hurricanes, droughts and the presence of pests and disease have decreased palms production and their establishment (Batugal, 1999). Production of ornamental palms for landscaping has decreased dramatically, reduced to a minor role in the agricultural production sector of the island's economy. According to the 2012 Census of Agriculture, there were 255 palm tree farms, with annual revenues of about US $6 million, and 408 hectares under production (USDA-NASS, 2012). In 2018, the island produced 119,000 coconut fruits on 48 palm tree farms covering approximately 24 hectares, a drop of 81% in farms and 94% in hectares over six years (USDA-NASS, 2020).

Coconut Lethal Yellowing disease (CLY) is one of the most destructive and serious coconut palm diseases. It has been recorded in over 30 palm species in the state of Florida and several Caribbean countries, associated with lethal decline and causing severe economic losses. CLY and lethal yellowing (LY) diseases in general are considered one of the major limiting factors in palm production worldwide (Harrison and Elliot, 2015; Hemmati et al., 2020). Although CLY is associated with the 16SrIV group phytoplasmas,

LY are associated to different groups of phytoplasmas (Hemmati et al., 2020; Hegde et al., 2023). Phytoplasmas are plant pathogenic bacteria that live as obligate intracellular phloem-parasites and are transmitted mainly by phloem-feeding insects (Bertaccini, 2022; Weintraub and Beanland, 2006).

Worldwide phytoplasmas are associated with thousands of serious plant diseases in economically important crops, such as vegetables, fruits, ornamental plants and trees (Bertaccini, 2023). In Puerto Rico, very limited information exists about diseases associated with phytoplasmas. The first report of a mycoplasma-like organism or MLO (viz. phytoplasmas) was based on meticulous observation of ultrathin sections of pigeon pea (*Cajanus cajan* (L.) Millsp.) foliage of plants severely affected with witches' broom disease and bushy canopy (Licha-Baquero, 1980). In 2015, a comprehensive study of phytoplasmas detected the 16SrIX group in periwinkle (*Catharanthus roseus*), tabebuia (*Tabebuia heterophylla),* Spanish lime (*Melicoccus bijugatus*), citrus species (*Citrus* spp.), coffee (*Coffea arabica*) and pigeon pea (*Cajanus cajan*) (Caicedo et al., 2015). The same phytoplasma was also detected in the leafhopper *Empoasca kraemeri* and the fulgoroids *Melormenis antillarum* and *Colpoptera maculifrons* (Caicedo et al., 2015).

Throughout the island of Puerto Rico, typical LY symptoms, such as dieback and eventual death are commonly observed in palm species, but information associated with these conditions has been limited to a report by Rodrigues et al. (2010a) of a 16SrIV-phytoplasma in royal palm (*Roystonea* sp.), fishtail palm (*Caryota mitis*) and carpentaria palm (*Carpentaria acuminata*). They also reported a 16SrIV-phytoplasma in the derbid planthopper, *Cedusa inflata* (Hemiptera: Derbidae) (Rodrigues et al. (2010b). A year later, a report of CLY insect vector, the cixiid *Haplaxius crudus,* was published (Segarra and Otero, 2011). More recently, Agosto (2021) detected a 16SrIV-D phytoplasma group in two palm species: *Aphaines minima* and *Dypsis lutescens*.

Currently, there are serious concerns about the possible spread of CLY from the Caribbean region into Puerto Rico. Given the economic importance and ecological diversity of endemic, native, and introduced palms, Puerto Rico appears vulnerable to outbreaks of CLY disease. The surveillance for CLY had been hampered by a lack of systematic molecular detection of phytoplasmas in the tissue samples of symptomatic palms. Therefore, this work was conducted with the dual objectives of detecting the presence and determining the identity of phytoplasmas in symptomatic palms and in their potential insect vectors.

MATERIALS AND METHODS

Collection of palm and insect samples. From October 2013 to May 2016, vascular tissue samples were collected from 69 mature palms showing LY symptoms, such as leaf chlorosis, inflorescence and fruit necrosis, and eventual death. Samples were collected at three transects oriented along and across the island of Puerto Rico (Figure 1). Palms species: *Cocos nucifera* (n=15)*, Gaussia attenuata* (n=1), *Leucothrinax morrisii* (n=1), *Pseudophenix sargentii* (n=1), *Roystonea borinquena* (n=50) and *Washingtonia robusta* (n=1) were sampled four times, using an electric drill and a sterile bit. About 3 g of tissue was removed from the interior basal trunk (10 to 15 cm) of the palm, according to the methodology described by Harrison et al. (2013). Palm shavings were placed in clean plastic bags in a cooler until their transport to the plant pathology laboratory for processing. Petri dishes containing sterile filter paper (Whatman no. 40) were used to dry samples overnight.

For potential insect vectors identification, a sweep collection from palms and grasses near the study areas was carried out. Insect population diversity was not homogenous among the three transects because they were located at different geographical positions throughout the island. Insects were identified by sorting according to gross morphology under a stereomicroscope and comparing them to specimens from the insect collection of the Luis F. Martorell Insectarium at the University of Puerto Rico - Mayagüez.

DNA extraction and PCR/RFLP phytoplasma identification. Genomic DNA was extracted from palm samples using the FastDNA

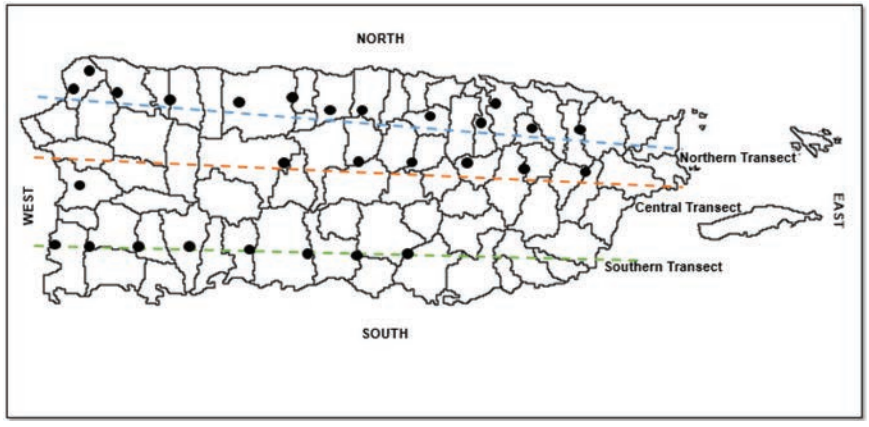

FIGURE 1. Map showing three sampling transects through the northern (blue), central (orange) and southern (green) regions of Puerto Rico.

SPIN Kit for soil[7] (Qbiogene Inc., Carlsbad, CA) according to manufacturer's instructions with the following modifications: 0.6 g of tissue was weighed and mixed with 978 µl sodium phosphate buffer and 122 µl MT buffer inside a Lysis Matrix A Tube (Model 6910-100, MP Biomedicals, Germany). The tissue was macerated using a FastPrep homogenizer (Model FP120A-115, Thermo Fisher Scientific, HQ - Waltham, MA) at a speed of 4.5 m/s with four cycles of 45 seconds.

Insect DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD), according to manufacturer's instructions with the following modifications: insect bodies and heads from the same species were placed in a 1.5 ml Eppendorf microcentrifuge tube containing three sterile ceramic beads. Then, 180 µl of ATL buffer and 20 µl of proteinase K were added to each microtube. Samples were mixed in a vortex for 10 min and incubated at 56° C until their lysis was complete. Later, 50 µl of AE buffer was added to the center of the column membrane and microtubes were incubated for 1 min at room temperature (24° C), and centrifuged for one minute at 8,000 rpm. The final DNA concentration was measured with a nanophotometer (IMPLEN, Westlake Village, CA) and samples were stored at 20° C.

Phytoplasma DNA was amplified by polymerase chain reaction (PCR) using universal phytoplasma primers P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995) followed by nested PCR using U5/U3 (Lorenz et al., 1995) or M1 (=16R758f)/U3 (Gibb et al., 1995) general primers and/or R16(X)F1/R1 specific primers for group 16SrX (Lee et al., 1995). For the nested PCR reactions, 1 µl from a 1: 30 µl dilution of amplicons was employed as template. Amplification of phytoplasma DNA was performed in 25 to 50 µl reaction volumes using AccuPrime High Fidelity Taq DNA polymerase (Thermo Fisher Scientific/Invitrogen, Waltham, MA) or REDTaq® DNA Polymerase (Sigma-Aldrich, St. Louis, MO) following manufacturer's instructions. PCR amplifications were performed in a thermocycler (Mastercycler® Pro S, Eppendorf, Hamburg, Germany) with initial denaturation at 94° C for 2 min, followed by 38 cycles of denaturation at 94° C for 30 s, annealing at 55° C for P1/P7 primers, 58° C for U5/U3 primer pair and 50° C for all the other primer pairs for 2 min, followed by an extension at 72° C for 3 min, with an additional final extension step at 72° C for 7 min. Negative controls were represented by tubes containing molecular grade water (HPLC grade, Sigma-Aldrich, St. Louis, MO) instead of DNA templates and were used in each direct and nested PCR assay. PCR

[7]Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the University of Puerto Rico.

products were separated by electrophoresis in a 1.2% agarose gel (OmniPurâ Agarose, MilliporeSigma, Burlington, MA) in 1 X TAE buffer containing 2.5 µl of Gel Red (Gel Redâ Nucleic Acid, Gel Stain, Biotium, San Francisco Bay Area, CA) and visualized using an UV transilluminator. The housekeeping gene RuBisCO was used as an internal control to verify the palm's DNA quality, and it was amplified with primer pair RbcLa (5'-ATGTCACCACAAACAGAGACTA-3')/ RbcLarev (5'-GTAAAATCAAGTCCACCRC G -3') (Kress et al., 2009).

Phytoplasma ribosomal group identification was carried out by restriction fragment length polymorphism (RFLP) analyses of fragments amplified with primers U5/U3 (890 bp), M1/U3 (650 bp), and R16(X) F1/R2 (1,100 bp) using the following restriction enzymes: *Tru1*I, *Rsa*I and *Ssp*I (Fermentas UAB, Vilnius, Lithuania) on 100 to 200 ng of DNA under the conditions indicated by the manufacturer. University of Bologna plant pathologist, Dr. Assunta Bertaccini, provided the following phytoplasma strains used to compare the restriction patterns: ash yellows (ASHY, subgroup 16SrVII-A); aster yellows (AY, subgroup 16SrI-B); Bermuda grass white leaf (BGWL, subgroup 16SrXIV-A), clover proliferation (CP-1, subgroup 16SrVI-A); crotalaria phyllody (CrP, subgroup 16SrII-C); elm yellows (EY, subgroup 16SrV-A); European stone fruit yellows (ESFY, subgroup 16SrX-B); *Pichris echioides* yellows (PEY, subgroup 16SrIX-C); poinsettia branch inducing factor (JR, subgroup 16SrIII-H); Suriname virescence (SuV, subgroup 16SrXV-A); tomato big bud (TBB, subgroup 16SrI-A) and X disease (CX, subgroup 16SrIII-A) (Bertaccini, 2023). University of Florida plant pathologist, Dr. Nigel Harrison, provided coconut lethal yellowing (CLY, group 16SrIV) phytoplasma strain to compare the restriction patterns. The rest of the samples were sequenced directly using nested products of PCR. Restriction products were separated by electrophoresis through a 6.7% polyacrylamide gel and stained in ethidium bromide. DNA bands were visualized with a UV transilluminator.

Sequencing and phylogenetic analyses. PCR products from selected nested PCR amplicons obtained with primers U5/U3, M1/U3 and R16(X)F1/R1 were purified with a QIAquick PCR Purification Kit (QIAGEN, Germantown, MD) and directly sequenced in both directions at commercial facilities (Psomagen, Rockville, MD). Phytoplasma DNA sequences were edited using Sequencher® 5.3 (Gene Codes, Ann Arbor, MI). Sequences showing 95% or higher identity with phytoplasma sequences were aligned, and a consensus sequence was obtained.

Phylogenetic analysis included 93 ingroup taxa of 16SrRNA phytoplasma groups -I, -II, -III, -IV, -V, -VI, -IX, -X, -XI, -XII, -XXI, -XXII (Table 1), with *Bacillus subtilis* reference strain AB042061 as outgroup. Sequences were aligned using SATé (Katoh and Standley, 2013) and

TABLE 1.—Accession numbers of the 16S rRNA gene sequences of phytoplasma groups used to construct the phylogenetic tree in this study.

16Sr group	'*Candidatus Phytoplasma*'-related	NCBI Accession No.
I-B	*Phoenix dactylifera* Phytoplasma	DQ913090
I-B	Aster yellows Phytoplasma	KF826615
I-B	*Candidatus* Phytoplasma asteris	M30790
II-A	Peanut witches-broom	L33765
II-B	*Candidatus* Phytoplasma aurantifolia	U15442
II-C	Faba bean phyllody Phytoplasma (FBP)	X83432
II-D	*Washingtonia robusta* Phytoplasma	MH157917
II-D	*Phoenix datylifera* Al-Wijam Phytoplasma	MH157916
II-D	*Candidatus* Phytoplasma australasia*	Y10096
II-E	*Picris echiodes* phyllody Phytoplasma	Y16393
II-F	Cotton phyllody Phytoplasma strain CoP	EF186827
III-A	*Candidatus* Phytoplasma pruni strain PX11Ct1	JQ044392
III-A	*Candidatus* Phytoplasma pruni strain PX11Ct1	JQ044393
III-B	Clover yellow edge Phytoplasma	AF173558
III-C	Pecan bunchy top Phytoplasma	GU004371
III-D	Goldenrod yellows Phytoplasma	GU004372
III-E	Spiraea stunt Phytoplasma	AF190228
III-F	Milkweed yellows Phytoplasma	AF510724
III-G	Walnut witches broom Phytoplasma	AF190226
III-G	Walnut witches broom Phytoplasma	AF190227
III-H	Poinsettia branch-inducing Phytoplasma	AF190223
III-I	Virginia grapevine yellows Phytoplasma VGYIII	AF060875
III-J	Chayote witches' broom Phytoplasma ChWBIII	AF147706
III-K	Strawberry leafy fruit Phytoplasma	AF274876
III-L	Cassava frogskin disease Phytoplasma	EU346761
III-M	Montana potato purple top Phytoplasma	FJ226074
III-N	Potato purple top PhytoplasmaPPT-AK6	GU004365
III-P	Dandelion virescence Phytoplasma	AF370119
III-P	Dandelion virescence Phytoplasma	AF370120
III-Q	Black raspberry witches-broom Phytoplasma clone BRWB7	AF302841
III-T	Cherry decline Phytoplasma	FJ231728
III-U	Cirsium white leaf Phytoplasma	AF373105
III-U	Cirsium white leaf Phytoplasma	AF373106
III-Z	Passion fruit witches-broom Phytoplasma PassWB-Br4	GU292082
IV-A	Coconut lethal yellowing Phytoplasma strain Jamaica LYJ-C8	AF498307
IV-A	Coconut lethal yellowing Phytoplasma strain Florida LYF-C5	AF498308
IV-A	*Phoenix caraniensis* Coconut lethal yellowing Phytoplasma strain RMCo	EU241517

*Recently, 'Ca. Phytoplasma australasia' of group II was abolished (removed) from the 'Ca. Phytoplasma' species list by the International Research Programme on Comparative Mycoplasmology (IRPCM) because its 16S rRNA gene sequence shares 99.5% identity with that of 'Ca. Phytoplasma aurantifolia' and does not represent an ecologically separate population (Wei and Zao, 2022).

TABLE 1.—(Continued) Accession numbers of the 16S rRNA gene sequences of phytoplasma groups used to construct the phylogenetic tree in this study.

16Sr group	'*Candidatus Phytoplasma*'-related	NCBI Accession No.
IV-A	*Phoenix sylvestris* Phytoplasma	EU241516
IV-A	*Phoenix dactylifera* Phytoplasma strain BCT-C5	EU241514
IV-B	Coconut leaf yellowing Phytoplasma	AF500334
IV-B	Coyol palm decline palm Phytoplasma	DQ321818
IV-B	Coconut decline Phytoplasma	DQ321819
IV-B	Coconut lethal yellowing Phytoplasma	DQ631640
IV-D	Coconut lethal yellowing Phytoplasma	EU241513
IV-D	*Carludovica palmata* leaf yellowing Phytoplasma	AF237615
IV-D	Texas Phoenix palm Phytoplasma	AF434989
IV-F	*Phoenix dactylifera* Phytoplasma strain BCT	EU241515
IV-F	*Washingtonia robusta* Phytoplasma strain WRFP	EU241512
V-A	*Candidatus* Phytoplasma ulmi	AF122910
V-B	*Candidatus* Phytoplasma ziziphi	AY072722
VI-B	Fragaria multicipita Phytoplasma Strawberry multiplier disease (MC)	AF190224
VI-C	Illinois elm yellows EY-IL2	AF409069
VI-C	Illinois elm yellows EY-IL1	AF409070
VI-D	Periwinkle little leaf Phytoplasma	AF228053
VI-E	*Centaurea solstitialis* virescence Phytoplasma	AY270156
VI-F	Catharanthus phyllody Phytoplasma	EF186819
VI-H	Portulaca little leaf Phytoplasma strain PLL-Ind	EF651786
VI-I	*Candidatus* Phytoplasma sudamericanum strain PassWB-Br3	GU292081
XIV	Bermuda grass white leaf Phytoplasma	AJ550984
XIV	Malaysia coconut yellow decline Phytoplasma	EU328159
VI-A	*Candidatus* Phytoplasma trifolii	AY390261
IX-B	*Candidatus* Phytoplasmaphoenicium	AF515636
X-A	*Candidatus* Phytoplasma mali	AJ542541
X-B	*Candidatus* Phytoplasma prunorum	AJ542544
XI-A	*Candidatus* Phytoplasma oryzae	D12581
XI-A	Coconut root wilt disease	AB052873
XI-B	Coconut root wilt Phytoplasma clone kayankulam	GU947111
XII-B	*Candidatus* Phytoplasma australiense	L76865
XXI-A	*Candidatus* Phytoplasma pini	AJ632155
XXII-A	*Candidatus* Phytoplasma palmicola strain LYDM-178	KF751387
XXII-B	Cape St. Paul wilt Phytoplasma strain LDG	Y13912
XXII-B	Cape St. Paul wilt Phytoplasma strain CSPWB	JQ868442

MAFFT with RAxML for the tree estimator with 10 iterations. Maximum likelihood tree was constructed to infer the phylogeny of phytoplasmas using RAxML with GTRCAT as the default model, 25 gamma categories and the automatic bootstrap MRE implemented in CIPRES

Science Gateway portal (Miller et al., 2010; Stamatakis, 2006). Trees were visualized and edited in FigTree V.1.4.0 (Rambaut, 2013).

RESULTS

Detection and identification of phytoplasmas in palms

About 90% of the palms sampled showed symptoms such as leaf chlorosis or yellowing, inflorescences or fruit necrosis (Figure 2). Royal palms (*R. borinquena*) were strong and robust, but their inflorescences and fruits were often necrotic and showed floral abortion. Coconut palm (*C. nucifera*) and key thatch palm (*L. morrissi*) showed chlorosis in their mature leaves and necrosis in their nuts and inflorescences (Figure 2).

After a three-year survey, phytoplasmas were detected in 17 out of 69 (24.6%) palms sampled using universal primer pairs P1/P7 (Fig-

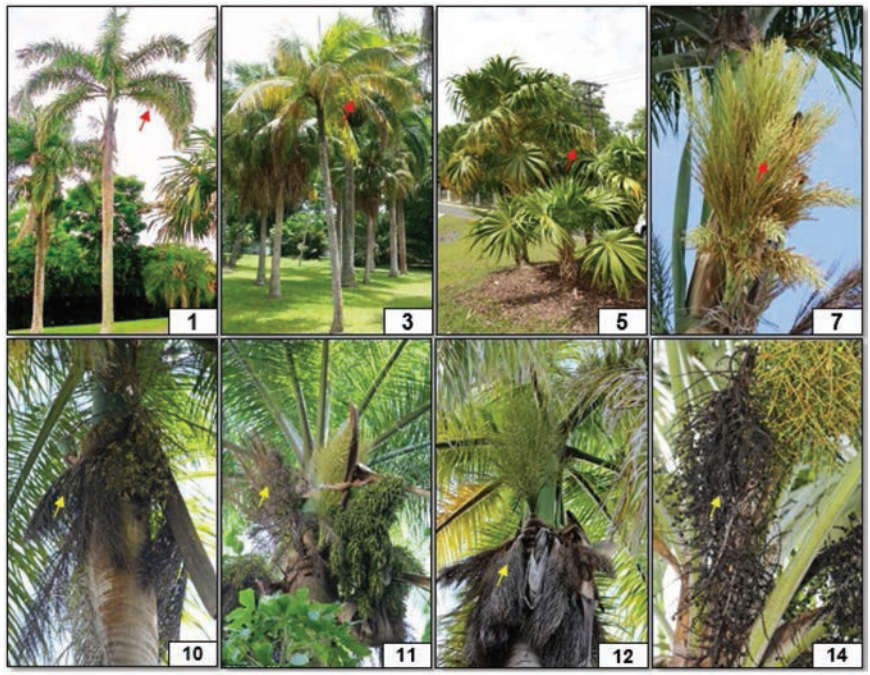

FIGURE 2. Symptomatology of representative palms positive for phytoplasmas in Puerto Rico. *R. borinquena* (Royal palm) samples 1, 7, 10, 11, 12 and 14; *C. nucifera* (Coconut palm) sample 3; and *L. morrisii* (Key thatch palm) sample 5. Palms 1, 3, and 5 showed leaf yellowing; palm 7, inflorescense chlorosis (red arrows). Palms 10, 11, 12 and 14 showed inflorescence and fruit necrosis (yellow arrows).

ure 3A). PCR products showed expected amplification size fragments of 1,800 bp. RuBisCO gene used as control verifies the amplification of genomic DNA in all samples (Figure 3B). The maximum likelihood phylogenetic tree derived from the analysis of the partial 16S rRNA gene sequences allowed the clustering of phytoplasmas detected in three palm species: *C. nucifera, L. morrisii* and *R. borinquena* (Figure 4). Phytoplasmas detected clustered with phytoplasmas enclosed in ribosomal groups 16SrII, 16SrIII, 16SrIV, 16SrVI, 16SrIX and 16SrX, some of which are reported for the first time in *C. nucifera*, *L. morrisii* and *R. borinquena* palms in Puerto Rico.

The predominant phytoplasma detected was enclosed in the 16SrII group and identified in ten palms (59%) [one *L. morrissi* and nine royal palms (*R. borinquena*)] (Table 2; Figure 4). This phytoplasma related group was dispersed around the island in the towns of Coamo, Juana Díaz, Lajas, Mayagüez, Río Piedras and Trujillo Alto. An important finding was the detection of a phytoplasma with 100% identity with the

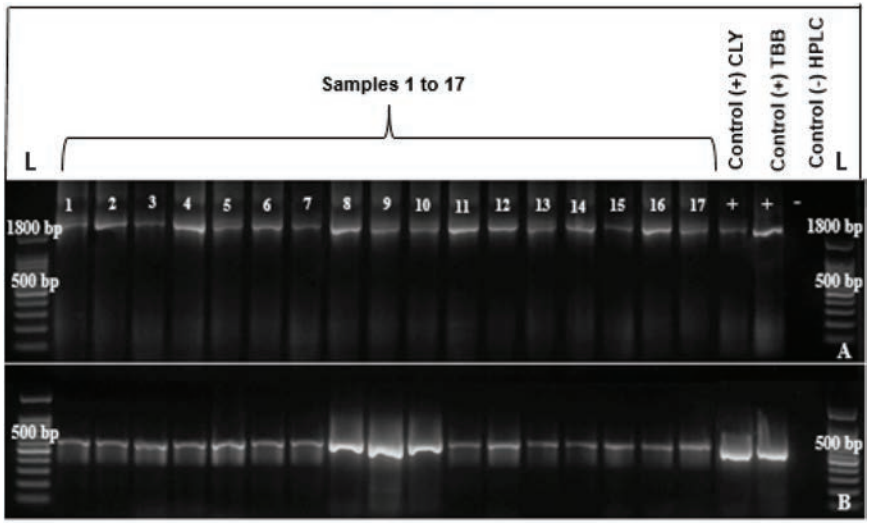

FIGURE 3. Direct PCR amplicons of A.) 16S rRNA gene (1,800 bp) of palm samples amplified with P1/P7 universal pair primers on 1.2% agarose gel. Lanes 1 - 3: *R. borinquena* palms at Río Piedras; lanes 4, 5, 6, 8, 9, 12, 13, 14, 15, 16: *R. borinquena*, *C. nucifera* and *L. morrisii* palm specimens collected in northern, central and southern Puerto Rico and at Mayagüez; lane 7: *R. borinquena* located in the southern transect at Sabana Grande; lanes 10 and 11: *R. borinquena* located in the center transect at Juncos and Naguabo; lane 17: *R. borinquena* located in southern transect at Cayey. B.) Amplification of RuBisCO gene used as internal positive control (790 bp). Positives control lanes for coconut lethal yellowing (CLY, group 16SrIV), American tomato big bug (TBB, subgroup 16SrI-A), and negative control lane (HPLC water). Ladder (L): Gel A = 1.8 kb; Gel B = 0.5 kb (Promega).

TABLE 2.—*Phytoplasmas detected in palms of Puerto Rico; identification, location, and accession number of the 16S rRNA gene sequences.*

Sample no.	16Sr group	'Candidatus Phytoplasma'-related	Host plant	Location	NCBI Accession No.
1	16SrIV-A	CLY[a]	*R. borinquena*	Río Piedras[b]	MF134654
2	16SrIX-B	'*Ca.* P. phoenicium'	*R. borinquena*	Río Piedras[b]	MF141841
3	16SrIII-A	'*Ca.* P. pruni'	*C. nucifera*	Río Piedras[b]	MF163185
4	16SrII[c]	'*Ca.* P. aurantifolia'	*R. borinquena*	Río Piedras[b]	MF185735
5	16SrII-D	'*Ca.* P. aurantifolia'	*L. morrisii*	Muñoz Marín Foundation, Trujillo Alto	MF185741
6	16SrII	'*Ca.* P. aurantifolia'	*R. borinquena*	Lajas	MF185736
7	16SrX-A	'*Ca.* P. mali'	*R. borinquena*	Sabana Grande	MF189568
8	16SrII	'*Ca.* P. aurantifolia'	*R. borinquena*	Juana Díaz	MF185737
9	16SrII	'*Ca.* P. aurantifolia'	*R. borinquena*	Coamo	MF185738
10	16SrX-B	'*Ca.* P. prunorum'	*R. borinquena*	Juncos	MF189569
11	16SrX-B	'*Ca.* P. prunorum'	*R. borinquena*	Naguabo	MF189570
12	16SrII	'*Ca.* P. aurantifolia'	*R. borinquena*	Mayagüez	MF185739
13	16SrII	'*Ca.* P. aurantifolia'	*R. borinquena*	Mayagüez	MF185740
14	16SrII	'*Ca.* P. aurantifolia'	*R. borinquena*	Mayagüez	MF185742
15	16SrII-D	'*Ca.* P. aurantifolia'	*R. borinquena*	Mayagüez	MF185743
16	16SrII-D	'*Ca.* P. aurantifolia'	*R. borinquena*	Mayagüez	MF185744
17	16SrVI-A	'*Ca.* P. trifolii'	*R. borinquena*	Cayey	MF189571
18	16SrII-D	'*Ca.* P. aurantifolia'	*Haplaxius crudus*	Río Piedras[b]	MN 276039

[a]CLY = Coconut Lethal Yellowing group
[b]UPR Botanical Garden, Agricultural Experiment Station, Río Piedras
[c]16SrII = Peanut witches' broom group.

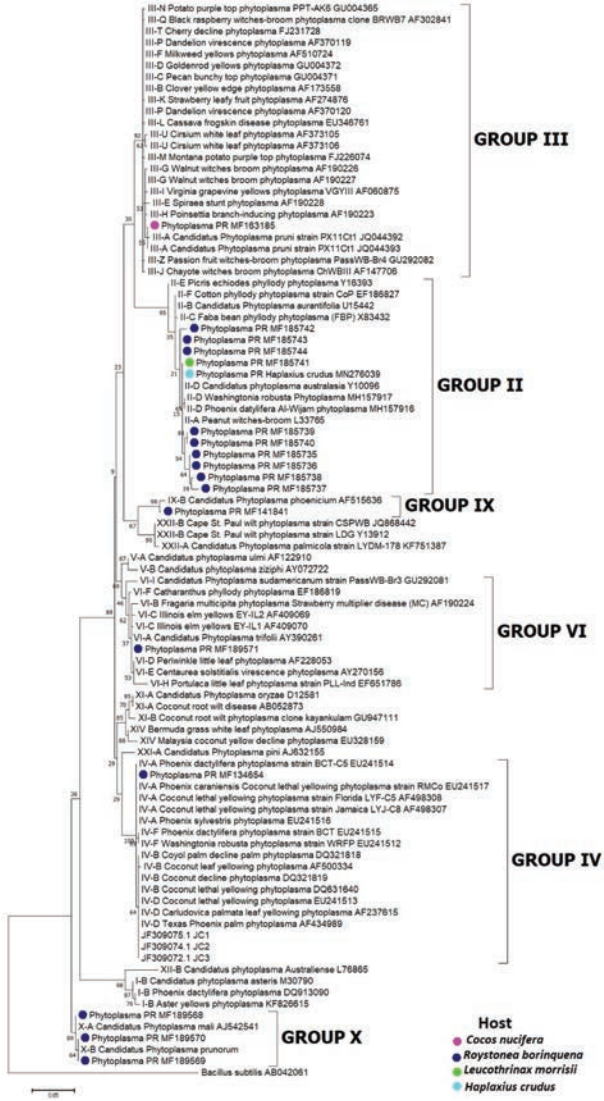

FIGURE 4. Maximum likelihood phylogenetic tree derived from analysis of partial 16S rRNA gene sequences of phytoplasmas detected in three palms species (*Cocos nucifera* in magenta, *Roystonea borinquena* in navy blue and *Leucothrinax morrisii* in green) and the insect, *H. crudus* (in sky blue) in Puerto Rico, and representative phytoplasmas in the coconut lethal yellowing (16SrIV) group and other phytoplasma groups. Bootstrap support values are shown at the nodes. The tree was rooted to the outgroup *Bacillus subtilis*. Recently, '*Ca*. Phytoplasma australasia' of group II was abolished from the '*Ca*. Phytoplasma' species list by the International Research Programme on Comparative Mycoplasmology (IRPCM) because its 16S rRNA gene sequence shares 99.5% identity with that of '*Ca*. Phytoplasma aurantifolia' and does not represent an ecologically separate population (Wei and Zao, 2022).

16SrIV phytoplasma group (CLY group) in the sample of *R. borinquena* from UPR Botanical Garden at the Agricultural Experiment Station in Río Piedras (Table 2; Figure 4). At the same location, *R. borinquena* samples were positive to 16SrII group (peanut witches' broom group) and to a 16SrIX phytoplasma group with 100% identity to pigeon pea witches' broom phytoplasma (GenBank Accession number EF186824). One coconut palm sample was positive to 16SrIII phytoplasma group (X-disease group) (Table 2; Figures 3 and 4).

A royal palm located in Cayey (east-central Puerto Rico) was positive to 16SrVI phytoplasma group showing 100% identity with clover proliferation phytoplasma strains ('*Ca.* P. trifolii') (Table 2; Figures 3 and 4). Another, three royal palms were positive to 16SrX phytoplasma group (apple proliferation group). Within this 16SrX phytoplasma group, two samples located at the eastern region of Puerto Rico (i.e., municipalities of Juncos and Naguabo) showed homology with '*Ca.* P. prunorum', and one from Sabana Grande, located in southwestern Puerto Rico, was similar to '*Ca.* P. mali' (Table 2; Figures 3 and 4).

RFLP analysis of phytoplasma sequences

Furthermore, positive results were confirmed by nested PCR using primer pairs U5/U3, M1/U3 and R16(X)F1/R1 (data not shown) and RFLP analysis on U5/U3 amplicons (Figure 5). RFLP analysis confirmed the presence of a 16SrIV phytoplasma group (CLY) in sample 1 and a 16SrIX in sample 2 of *R. borinquena* palms located at the UPR Botanical Garden in Río Piedras. Also, RFLP confirmed a 16SrIII phytoplasma in *C. nucifera* (sample 3) at the UPR Botanical Garden in Río Piedras and a 16SrVI-A phytoplasma group in sample 4 (original sample no. 17 in Table 2) in *R. borinquena* at Cayey (Figure 5).

Insect identification during the survey of phytoplasmas in palms

A total of 435 insect specimens belonging to eleven species of Hemiptera Auchenorrhyncha were collected during the survey (Figure 6). The most collected species was *Haplaxius crudus* (Cixiidae), representing 33.33% of the specimens. This species was present at UPR Botanical Garden at Río Piedras and in Trujillo Alto. Two additional cixiids, *Bothriocera undata* (5.75%) and *Oliarus complectus* Ball (14.94%), were also identified on palms. Other fulgoroid species collected in lesser proportions were the derbids *Cedusa inflata* Ball (4.83%), *Neocenchrea* sp. (4.14%), and *Omolicna puertana* Caldwell (3.45%). Only 2.76% of the specimens collected belonged to the flatid genus *Petrusa* spp. (Figure 6). Other non-fulgoroid Auchenorrhyncha collected were: *Chlorotettix minimus* (5.75%), *Hortensia similis* Walker (13.79%) *Graminella* sp.

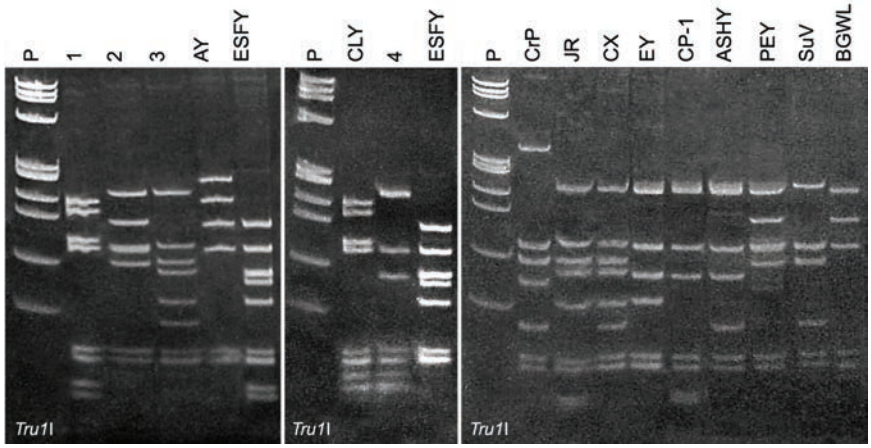

FIGURE 5. RFLP analyses in polyacrylamide gels of samples (1 to 4) amplified with nested PCR using primers U5/U3 and digested with *Tru1*I restriction enzyme. Palm samples: 1. 16SrIV (CYL group) in *R. borinquena* at Río Piedras; 2. 16SrIX ('*Ca*. P. phoenicium') in *R. borinquena* at Río Piedras; 3. 16SrIII ('*Ca*. P. pruni') in *C. nucifera* at Río Piedras and 4. 16SrVI-A ('*Ca*. P. trifolii') in *R. borinquena* at Cayey. Lanes for reference phytoplasmas: AY, aster yellows (16SrI-B); ESFY, European stone fruit yellows (16SrX-B); CLY, coconut lethal yellows (16SrIV-A); CrP, crotalaria phyllody (16SrII-C); JR, poinsettia branch inducing factor (16SrIII-H); CX, X disease (16SrIII-A); EY, elm yellows (16SrV-A); CP-1, clover proliferation (16SrVI-A); ASHY, ash yellows (16SrVII-A); PEY, *Pichris echioides* yellows (16SrIX-C); SuV, Suriname virescence (16SrXV-A); BGWL, Bermuda grass white leaf (16SrXIV-A); Ladder P, marker phiX174 digested with *Hae*III, fragment sizes (bp) from top to bottom of 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72.

(5.06%) and *Agalliopsis pepino* (6.24%) (Cicadellidae). These are likely to be associated with surrounding weed species.

Using nested PCR with primer pairs U5/U3 and M1/M2, amplicons were obtained for only one specimen of *H. crudus* collected near a *R. borinquena* palm. The sequence clustered with those of the16SrII phytoplasma group (Table 2; Figure 4), the same phytoplasma prevalent in *R. borinquena* around the island.

DISCUSSION

Detection of different phytoplasma belonging to different ribosomal groups in palms has been reported globally (Hegde et al., 2023), inclulding the Caribbean region (Myrie et al., 2019; Simbaña, 2019). The results of this study confirm the presence of phytoplasmas enclosed in six ribosomal groups within three transects across the island of Puerto Rico. All palm species sampled showed symptomatology consisting of inflorescences and fruit necrosis, and foliage chlorosis or yellowing,

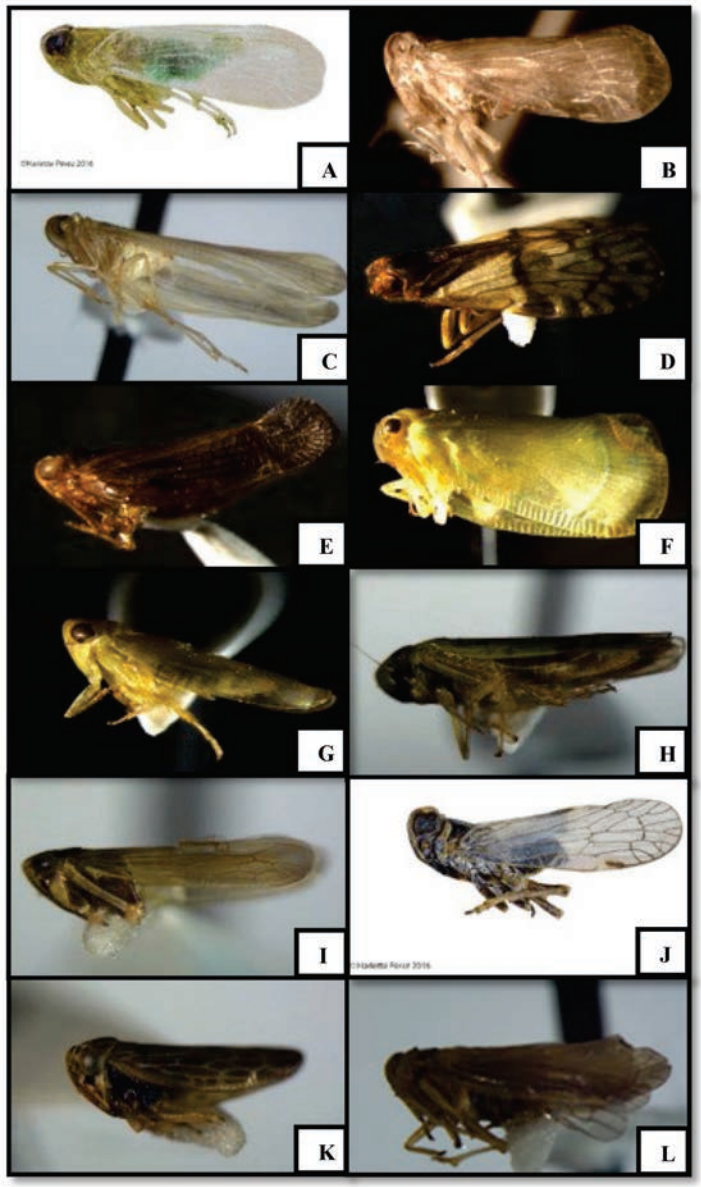

FIGURE 6. Potential insect vectors sweep-collected from palms and grasses near the study sites. Morpho-species A. *Haplaxius crudus* van Duzee (Fam. Cixiidae); B. *Cedusa inflata* Ball (Fam. Derbidae); C. *Neocenchrea pallida* (Fam. Derbidae); D. *Bothriocera undata* (Fam. Cixiidae); E and F. *Petrusa* spp. (Fam. Flatidae); G. *Chlorotettix minimus* (Fam. Cicadellidae); H. *Hortensia similis* Walker (Fam. Cicadellidae); I. *Graminella* sp. (Fam. Cicadellidae); J. *Oliarus complectus* Ball (Fam. Cixiidae); K. *Agalliopsis pepino* (Fam. Cicadellidae); L. *Omolicna puertana* Caldwell (Fam. Derbidae).

and necrosis. According to Harrison and Elliot (2015), the symptoms observed in palms are not key indicators to diagnose and identify palm phytoplasma associated diseases. Thus, the lack of correlation between the prevalence of LY symptoms and palm location within the transects indicates that the symptomatology expressed by the palms does not determine the presence of a specific phytoplasma strain.

The 16SrII phytoplasma group previously undetected in palms in the Americas (Hegde et al., 2023) was the phytoplasma most commonly detected in Puerto Rico. In the Middle East, the detection of 16SrII-D in LY symptomatic leaves of date palms (*Phoenix dactylifera* L.) and Mexican fan palm (*Washingtonia robusta*) in Oman and Saudi Arabia has been reported (Alhudaib et al., 2017; Hemmati et al., 2020). These authors reported palms showing LY symptoms, including yellow streaks, chlorosis, and stunting. They reported a 30% decrease in date fruit size and fruit stalk as the most common symptoms, concurring with a very short palm lifespan and eventual death. In addition, Alhudaib et al. (2017) detected a 16SrII group phytoplasma in *Ocimum basilicum* L. and *Medicago sativa* L. collected around symptomatic date palms. More recently, this phytoplasma has been reported in *Areca catechu* showing symptoms of yellow leaf disease in China (Lin et al., 2023). The 16SrII phytoplasma group appears to be distributed worldwide in several important crops, such as lime, sesame, sugar beet, tomato and papaya (Zreik et al., 1995; Arocha et al., 2005, 2006; Al-Sakeiti et al., 2005; Esmailzadeh-Hosseini et al., 2007; Mirzaie et al., 2007; Alhudaib and Rezk, 2014). In Cuba, the same phytoplasma group has been reported in papaya (Arocha et al., 2006). The 16SrII phytoplasma group also appears to be present in common weeds, such as *Cleome viscosa, Crotalaria pallida* and *Tephrosia purpurea* (Li et al., 2014), as well as in many weeds and herbaceous crops in the Americas, Africa, Asia, New Zealand, Southern Europe and Australia (Garnier et al., 1991; Ghosh et al., 1999; Leyva-Lopez et al., 2002; Tolu et al., 2006; Tran-Nguyen et al., 2003; Zamora et al., 2015).

The detection of a 16SrII phytoplasma in a *H. crudus* specimen does not imply that the insect is vectoring the phytoplasma. Interestingly it is not surprising that this phytoplasma, the most detected in our survey, might be carried by the known insect vector of LY reported in Mexico and Florida (Oropeza et al., 2009).

Other phytoplasmas identified in palms are enclosed in groups 16Sr -III, -IV, -VI, -IX and -X. A phytoplasma in the 16SrIII group detected in one sample of *C. nucifera* is reported for the first time in palms; curiously, phytoplasmas in the same group were detected in poinsettias and stone fruits (Hegde et al., 2023; Bertaccini, 2022; Wei and Zhao, 2022). Remarkably, phytoplasmas of 16SrIV group (CLY) were

detected in only one royal palm sample. After years of observation, the specimen remains alive, perhaps indicating the presence of sub-lethal LY strains or a tolerance to this phytoplasma by the native royal palm species.

A 16SrVI phytoplasma group was detected in one sample of *R. borinquena* located in Cayey, a town in east-central Puerto Rico. The ribosomal sequence of this sample showed 100% identity with a 16SrVI group phytoplasma reported in India (Khasa et al., 2016), where it was associated with a severe leaf yellowing in giant milkweed, *Calotropis gigantean* (Priya et al., 2010). A congener, *C. procera* is a very common weed in Puerto Rico (Martorell, 1976). In addition, this phytoplasma has often been reported infecting *Hibiscus rosa-sinensis* and *Saponaria officinalis* in India (Khasa et al., 2016). Phytoplasmas belonging to this group have been detected in *Phoenix dactilifera* (date palms) showing streak yellowing in Iran (Hemmati et al., 2020). This group has been reported in cabbage, beans, and potatoes (Salehi et al., 2007; Lee et al., 2004; Cheng et al., 2011), generally associated with severe damage and deformation of infected plants.

The detection of the 16SrIX phytoplasma group in one sample of royal palm at the UPR Botanical Garden confirms previous observations from Caicedo et al. (2015) who reported this phytoplasma group in several plants species widespread in Puerto Rico. These authors also detected 16SrIX phytoplasma in several Auchenorrhyncha planthoppers and leafhoppers. In this study, only one royal palm was infected with the 16SrIX phytoplasma group despite its prevalence in several localities on the island (Caicedo et al. 2015). These authors reported *E. kraemeri, M. antillarum and C. maculifrons* as potential insect vectors of group 16SrIX phytoplasmas, but these insect species were not collected near palms or in the grass areas sampled. This phytoplasma group has previously been reported in palms (Hegde et al., 2023)

Three royal palm samples were positive to phytoplasmas in group 16SrX; one sample, clustered with a 16SrX-A phytoplasma was detected in a palm located in Sabana Grande in western Puerto Rico. Another two samples, clustered with 16SrX-B, were collected in the eastern-central towns of Juncos and Naguabo. Even though the palms showed the same symptomatology as LY, to date none has died. Phytoplasmas in this ribosomal group were previously reported alone in date palms or in mixed infection with 16SrI and 16SrII in Saudi Arabia (Alhudaib et al., 2019).

Currently, 66 of the 69 symptomatic palms surveyed continue to be alive and productive. The three exceptions, located in the northwestern region of the island (i.e., Aguadilla), were negative to phytoplasmas. Thus it was hypothesized that phytoplasma strains infecting palms in Puerto Rico may somehow prevent, or are incapable of, a lethal expres-

sion of the symptoms. Perhaps a phytoplasma tolerance phenomenon is being observed in Puerto Rico, where palms in the pathosystem do not present a sudden death, despite being infected.

Remarkably, even after several years since LY phytoplasma was first detected in Puerto Rico by Rodrigues et al. (2010a, b), and after years of visual inspections, no massive or sudden palm death events have occurred on the island. Even though Gurr et al. (2016) point out in their studies that the species *C. nucifera and G. attenuate* are highly susceptible to this disease, the devastation caused by CLY in other regions of the Caribbean, such as Jamaica, has not been observed in Puerto Rico, most likely because of the predominant presence of different phytoplasmas on the island.

The findings of this survey contribute to the knowledge of palm phytoplasma diversity associated with yellowing and other symptoms observed in the Americas and especially in the Caribbean region. An unexpected finding was to identify a great diversity of phytoplasma groups in three palm species in Puerto Rico, however this concurs with other findings in palms in the area (Myrie et al., 2019). Six phytoplasma groups were detected in *R. borinquena* palms that showed yellowing symptoms, but these were never observed to be lethal during the period of monitoring and observation. The presence of diverse groups of phytoplasma in the same species is not unique. There are reports of various phytoplasmas affecting the same genus of plants. To our knowledge, 16Sr groups -III and -IX have not been previously reported in palms (Hemmati et al., 2020; Hegde et al., 2023).

Since four 16Sr phytoplasma groups (-II, -III, -IV and -IX) were detected at the UPR Botanical Garden, additional surveys should be conducted at this location to verify the presence of possible alternative host plants or reservoirs for those phytoplasmas in the area. To our knowledge this is the first report of a 16SrII group phytoplasma in *R. borinquena* in Puerto Rico and the world. The detection for the first time in Puerto Rico of other phytoplasmas in 16Sr groups -III, -VI, -IX and -X in palms deserves further study.

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