Identification and Molecular Characterization of Pigeon Pea Witches'-Broom Phytoplasma in Plants and its Potential Vectors in Puerto Rico

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ABSTRACT

Few studies have determined the presence of phytoplasma from important crops in Puerto Rico. Disease symptoms resembling those caused by phytoplasma were observed in different plant species such as pigeon pea (*Cajanus cajan*), periwinkle (*Catharanthus roseus*), tabebuia (*Tabebuia heterophylla*), Spanish lime (*Melicoccus bijugatus*), ixora (*Ixora coccinea*), mango (*Mangifera indica*), cactus (*Opuntia* spp.), citrus trees (*Citrus* spp.), and coffee (*Coffea arabica*). Sixty-two plant samples from these species were tested using end point PCR with universal and specific primers (i.e., nested PCR) that prime amplification of the 16S rDNA and ribosomal protein genes (*rpIV-rpsC*). Fifty-one percent of the samples tested corresponding to periwinkle, pigeon pea, citrus, coffee and tabebuia were positive for phytoplasmas with amplicons of 0.8 and 1.2kb, respectively, depending upon the primers used in PCRs. In both cases the DNA sequences showed 99% of identity with pigeon pea witches'-broom phytoplasma (PPWB) and by restriction patterns (RLFP) obtained from these samples belonged to group 16SrIX. Due to the lack of studies of potential insect vectors, common auchenorrhyncha species were sweep-collected from pigeon pea and citrus and tested for phytoplasma. Of nine insect genera collected, *Empoasca kraemeri*, *Melornemis antillarum* and *Colpoptera maculifrons* were positive for PPWB phytoplasma based on results from conventional PCR and DNA sequence analysis. These findings indicate that these insects fed upon the aforementioned plant species, and may act as potential phytoplasma vectors in the field. Finally, specific primers were designed for qPCR assay to amplify a 102-bp region of the 16S rDNA gene from samples with low level infections of phytoplasma. By the SYBR® Green method, the melting temperature (Tm) recorded in positive samples was 82.3^{o}C. These primers amplified and

identified DNA of phytoplasma belonging to the groups and subgroups 16SrV-A, 16SrIII-H, 16SrII-D, 16SrV-C, 16SrII-C, 16SrVI-A, 16SrXII-A and 16SrIX-C.

RESUMEN

Pocos estudios han determinado la presencia de fitoplasma de cultivos importantes en Puerto Rico. Se observaron síntomas de fitoplasmas típicos en diferentes especies de plantas como el guandul (*Cajanus cajan*), playera (*Catharanthus roseus*), roble (*Tabebuia heterophylla*), quenepa (*Melicoccus bijugatus*), cruz de Malta (*Ixora coccinea*), mangó (*Mangifera indica*), cactus (*Opuntia* spp.), cítricos (*Citrus* spp.) y café (*Coffea arabica*). Sesenta y dos muestras de plantas de estas especies fueron analizadas mediante PCR convencional con cebadores universales y específicos (para PCR anidada) que amplifican los genes de 16S ADNr y *rpIV-rpsC*. Cincuenta y uno por ciento de las muestras analizadas correspondientes a muestras de playera, gandul, cítricos, café y roble resultaron ser positivas para la presencia de fitoplasmas produciendo amplicones de 0,8 y 1,2kb, respectivamente. En ambos casos, las secuencias de ADN y los patrones de restricción polimórfica (RLFP) obtenidos a partir de estas muestras mostraron un 99% de identidad con el fitoplasma del gandul perteneciente al grupo 16SrIX. Debido a la falta de estudios sobre potenciales insectos vectores de fitoplasmas, fueron colectadas especies comunes de insectos auchenorrhyncha por medio de una red de barrido en plantas de gandul y cítricos; mismos que fueron analizados para la presencia del fitoplasma. De los nueve géneros de insectos recolectados, únicamente *Empoasca kraemeri, Melornemis antillarum* y *Colpoptera maculifrons* fueron positivos para la presencia del fitoplama del gandul mediante PCR convencional y el análisis de secuencias de ADN. Estos resultados indican que estos insectos pueden actuar como potenciales vectores del fitoplasma en el campo. Por último, sobre la base de gen 16S rDNA, un par de cebadores específicos fueron

diseñados para amplificar una región de 102pb por medio de ensayos de PCR en tiempo real (RT PCR) en muestras con bajos niveles de infección del fitoplasma. Por el método de SYBR® Green, la temperatura de disociación (Tm) registrada en las muestras positivas fue 82.3^{o}C. Estos cebadores amplificaron e identicaron ADN de fitoplasmas pertenecientes a los grupos y subgrupos 16SrV-A, 16SrIII-H, 16SrII-D, 16SrV-C, 16SrII-C, 16SrVI-A, 16SrXII-A and 16SrIX-C.

DEDICATION

This work is dedicate

to **God** (My Lord and my Savior)

to my parents
Jorge Patricio Caicedo Villafuerte and Jennifer Edith Chávez
who raised me to be the person I am today,

to my brother,
Vladimir Fernando Caicedo Chávez

and very specially,
to my beautiful, my partner, my friend and my most illusion
María José Paca Moreno

Thanks for everything

"*He gives strength to the weary and increases the power of the weak*"
(Isaiah 40:29) NIV

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The Lord bless thee, and keep thee: The Lord make his face shine upon thee, and be gracious unto thee: the Lord lift up his countenance upon thee, and give thee peace.

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1. INTRODUCTION

Phytoplasmas are associated with diseases in several hundred plant species, including many important vegetable and fruit crops, ornamental plants, and timber and ornamental trees. Phytoplasmas, formerly called mycoplasmalike organisms (MLOs), are cell wall-less bacteria that inhabit phloem of plants and are transmitted between plants by phloem-feeding insects. Currently, the list of diseases caused by phytoplasmas continues to grow, as newly emerging diseases, including diseases having uncertain etiologies and diseases with diverse geographic distributions, have been identified as being associated with plant infections by phytoplasmas (Lee and Davis, 1986). For example, in China Huanglongbing (HLB) disease affecting *Citrus* spp. such as tangerine (*Citrus reticulata* Blanco), sweet orange (*C. sinensis* [L.] Osbeck) and pomelo (*C. maxima* [Burn.] Merrill) has been associated with aster yellows-related phytoplasmas (16SrI) (Chen *et al*., 2008). In Brazil sweet orange trees with symptoms characteristic of HLB has been found to be infected by pigeon pea witches'-broom-related phytoplasma strains (16SrIX) (Teixeira *et al*., 2009). These are only some of the most recently described diseases on the list associated with phytoplasma.

In Central America and the Caribbean Region, one devastating disease caused by phytoplasma is Coconut Lethal Yellowing (CLY). This disease is not only destructive on coconut, but on at least 30 other species of palms (Harrison *et al.,* 1999). CLY is particularly aggressive on tall palm varieties that are grown almost exclusively in the Caribbean Region, and which dominated plantations prior to the 1970 to 80's when dwarf varieties began to be introduced as resistant hybrids, especially in Jamaica, Cuba and the

Dominican Republic (Bruner and Boucle, 1943). CLY has not yet been reported in Puerto Rico, but a palm dieback observed in *Roystonea* spp., *Caryota mitis* and *Carpentaria acuminata* has been associated with a phytoplasma (Rodrigues *et al.,* 2010). In Central America, the relentless spread of the fatal disease throughout the coconut growing areas has had a serious economic impact on many vulnerable communities (Myrie *et al.,* 2006). It is estimated that close to 1.2 million coconut trees have been destroyed in the past 15 years, and therefore effective management of CLY spread is required (Myrie *et al*., 2006).

In Puerto Rico the first report of witches'-broom symptoms were observed in branches of tabebuia (*Tabebuia pentaphylla* [L.] Hemsl) (Cook, 1938). This early study indicated that inoculations made during periods of active tree growth result in symptoms developing within three to four months after budding. In grafting infestations, brooms usually appear at the nodes just above the points where the bud was inserted. After the formation of the first broom, other brooms will develop in other parts of the tree. These new brooms may develop on the branch in which the bud was inserted, or on other branches, indicating that the infectious agent could travel through the plant (Cook, 1938). Erroneously, the author suggested a virus as the infectious agent.

The second phytoplasma-related disease reported in Puerto Rico was pigeon pea witches'-broom (PPWB) (Rodríguez *et al.,* 1979). This disease of pigeon pea (*Cajanus cajan*) is characterized by terminal bud suppression, and by profuse secondary branching. Infected plants become stunted and unproductive. The authors identified phytoplasma DNA sequences seemingly unique to the 16S rDNA (16S ribosomal DNA region) gene group associated with PPWB. Specificity of the primer pair B32f1/B32r1 (primers unpublished) for use in PVR to detect this disease was verified by screening DNAs derived from various phytoplasma infected plants. PPWB-specific Polymerase Chain reaction (PCR) was then used to test insect species collected from pigeon pea fields showing PPWB, at various localities in Puerto Rico (Rodríguez *et al.*, 1979).

A year later, Licha (1980) stated that symptoms linked to witches' broom in pigeon peas were caused by toxins injected by leafhoppers of the genus *Empoasca*, but a complex set of symptoms, that includes a pale mosaic, was also observed. Plants affected with pale mosaic symptoms (i.e., witches'-broom-free, bushy canopy-free) consistently yielded rhabdovirus. Similarly, transmission electron microscopy (TEM) provided evidence of MLO in fairly consistent association with symptoms described as bushy canopy and witches'-broom. Thus a combined action of a mycoplasmas like organism (MLO) and a rhabdovirus likely occurred in the pigeon pea plant tissues.

In Puerto Rico economically important crops, such as coffee, mango, Spanish lime, citrus, pigeon pea, ornamental plants and trees, among others are affected by serious diseases that are not yet adequately described, and whose causal agents are still poorly understood or unknown. For example, citrus HLB caused by '*Ca.* Liberibacter asiaticus' has been reported causing considerable losses to the citrus industry (Alvarado, 2009). In Brazil citrus trees showing HLB symptoms were also infected with pigeon pea witches'-broom phytoplasma (Teixeira *et al.*, 2009). Therefore it is necessary to investigate identity, symptomatology and behavior, spread by vectors, host range, and impact of diseases of unknown etiology in crops of economic importance in Puerto Rico.

1.1. The Pigeon Pea Witches'-Broom (PPWB) phytoplasma group

Cajanus cajan is an important grain legume crop of rained agriculture in the semi-arid tropics, where they are used as a food crop (dried peas, flour, or green vegetable peas) and a forage/cover crop. Phytoplasma strains belonging to the PPWB group (16S rDNA gene RFLP group IX) has a broad host range which includes herbaceous plants, fruit trees and conifers. Harrison *et al.* (1991) described for the first time PPWB phytoplasma, subgroup IX-A on symptomatic pigeon pea plants (*Cajanus cajan*). Later Khan *et al.* (2007) reported the presence of phytoplasmas within the same group, classified in the subgroup IX-C, affecting herbaceous plants in the field such as bristly oxtongue (*Pichris echioides* L.) and field scabious (*Knautia arvensis* L.). The diseases were described as *Pichris echioides* yellows (PEY) and *Knautia arvensis* Phyllody (KAP). The phyllody symptom caused by phytoplasmas is characterized by the formation of leaf-like structures in place of flowers (Bertaccini, 2007).

In southern Italy a phytoplasma described from *Dimorphotheca sinuata* DC. (Cape marigold) was identified by restriction fragment length polymorphism (RFLP) analysis as a member of the PPWB group (Marcone *et al.*, 2000). It is related to the strain of *Pichris echioides* yellows (PEY) above mentioned. In the United States, in Oregon, Washington, Idaho, Nevada and California, a phytoplasma-causing Juniper witches'-broom phytoplasma on western junipe (*Juniperus occidentalis* H.) was identified and classified in 16S rDNA subgroup IX-E (Davis *et al.*, 2010).

In gliricidia (*Gliricidia sepium* [Jacq.] Kunth ex Walp), a phytoplasma closely related to the PPWB phytoplasma was found in Honduras causing little leaf symptoms. Orange trees (*Citrus sinensis* [L.] Osbeck) with HLB characteristic symptoms in a region of São Paulo state, Brazil, were negative for '*Ca*. Liberibacter' species ('*Candidatus* Liberibacter asiaticus', '*Candidatus* Liberibacter africanus' and '*Candidatus* Liberibacter americanus') infection, but were found positive for a phytoplasma highly related with PPWB phytoplasma of group 16SrIX (Teixeira *et al*., 2008).

By sequencing of 16S rDNA genes, other 16Sr-IX group members have been described. Based on gene sequence comparison, among them are *Lactuca serriola* phytoplasma from Iran, *Knautia arvensis* phyllody phytoplasma, Iranian almond witches'-broom, *Pichris echioides* yellows phytoplasma, Honduran *Gliricidia* little leaf phytoplasma, '*Ca*. Phytoplasma phoenicium' and Florida *Rhynchosia* little leaf phytoplasma (Al Subhi *et al*., 2007). In several Oman locations, plants of Senegal senna (*Cassia italica*) exhibiting symptoms like witches'-broom on the branches, resulted from infection caused by a phytoplasma belonging to PPWB phytoplasma (16SrIX), sharing a 93 to 97% sequence similarity (Khan *et al*., 2007).

2. LITERATURE REVIEW

2.1. Background on phytoplasmas

For decades prior to the mid-1960s, a large group of plant diseases, called yellows-type, were believed to be caused by viruses in the view of their infective spread, their symptomatology, and the fact that they were transmitted by insects (reviewed by Lee and Davis, 1986). Forty-seven years ago Doi *et al.* (1967), demonstrated for the first time that the etiological agents that caused the yellowing symptoms were prokaryotes lacking a cell wall rather than viruses. Thus, after the discovery of this new group of plant pathogens related to bacteria, structural analysis based on microscopy TEM studies revealed the presence of pleomorphic prokaryotes with no cell wall occurring in the phloem of many plant species affected with yellows-type diseases. Doi *et al.* (1967) used for the first time the term MLO's for these microorganisms, due to their morphological and ultrastructural similarity to mycoplasmas in humans. MLOs and mycoplasmas taxonomically belong to the *Mollicutes* Class, since they are prokaryotes without cell walls.

Phytoplasmas are obligate parasites of insects and plant phloem tissues (Lee and Davis, 1986). According to Gundersen *et al.* (1994) and Lim and Sears (1989), these microorganisms probably diverged from Gram-positive bacteria, and belong to the genus '*Candidatus* Phytoplasma'. These pleomorphic bacteria have diameters less than 1µm, and relatively small genomes (680–1,600kb) when compared with those of their ancestors, walled bacteria of the *Bacillus*/*Clostridium* group. They lack several pathways for the synthesis of compounds (such as: amino and nucleotide sugar, glyoxylate, and dicarboxylate metabolism) necessary for their survival (Tran-Nguyen *et al.*, 2008). It is hypothesized that these compounds must then be obtained directly from their hosts (Bai *et al.,* 2006).

Phylogenetically phytoplasmas descended from an acholeplasma-like ancestor (Lee *et al.,* 1998b). In other prokaryotes, including mycoplasmas and spiroplasmas (*Spiroplasma citri* in citrus species causes the Citrus stubborn disease), the amino acid tryptophan (*trp*) is coded by UGA, while phytoplasmas use UGA as a stop codon. Phytoplasmas are genetically distinguishable from mycoplasmas because they have a spacer region (about 300 bp) between the 16S and 23S ribosomal regions, which codes for isoleucine transfer RNA ($tRNA^{Ile}$) and part of the sequences for alanine transfer RNA ($tRNA^{Ala}$) (Lee *et al.,* 2004b).

Phytoplasmas also have a genome with a low G+C content, sometimes as little as 23%, which is thought to be the threshold for a viable genome (Dikinson, 2003) and this feature is common to all members of the class *Mollicutes* (Martini *et al.,* 2007). Phytoplasmas contain two rRNA operons, and the heterogeneity of these operons has been demonstrated for some phytoplasmas (Schneider and Seemüller, 1994). Phytoplasma genomes contain large numbers of transposon genes and insertion sequences that can be unique to these organisms. Genetic variability among phytoplasmas within the same group can depend (inter alia) of the presence of genomic islands termed sequence variable mosaics (SVMs) account for differences in genome size, which enable phytoplasmas to survive in diverse environments in plant and insect tissues, and they produce the marked heterogeneity of phytoplasma genome sizes (Jomantiene *et al.,* 2007). Similar studies have revealed that phytoplasma genomes contain clustering of genes, pseudogenes, mobile genetic elements, intergenic repeat units, and repetitive extragenic palindromes that occur in multiple, homologous clusters in some phytoplasma genomes (Jomantiene and Davis, 2006), known as sequence-variable mosaics (SVMs). The conclusions of these works suggested that the SVMs likely formed through recurrent and targeted mobile element attack and recombination at an early stage in their evolution, but the nature and origin of the hypothetical mobile element(s) remained obscure until further studies revealed SVMs as genomic islands composed of prophage genomes (Wei *et al.*, 2008). Although important features of phytoplasma genome architecture and gene complement thus have discovered and analyzed, the occurrence and potential role of prophages in shaping phytoplasma genomes has not been elucidated. In common bacteria, prophages have been identified as major contributors of laterally acquired genes encoding virulence factors (Wei *et al.*, 2008; Brüssow *et al.*, 2004). Interestingly, the alignments of the genome sequences from closely related bacterial strains revealed that in some cases all major genome differences can be attributed to prophage sequences (Canchaya *et al.*, 2003). Nearly half of the completely sequenced bacterial genomes possess prophage sequences that can constitute a sizable part (10–20%) of a bacterial genome (Canchaya *et al.*, 2003). In agreement with Srividhya *et al.* (2007), Wei *et al.* (2008) concluded that the prophages represent a major element of bacterial genomes and a significant driving force for bacterial strain diversification. Recent surveys focusing on prophage and other mobile-DNA elements in completely sequenced genomes of bacteria with and without cell walls, including obligated parasitic intracellular bacteria, set the stage for a resurgence of research on microbial mobile elements in host-restricted and other bacteria.

Many important crops worldwide are affected by diseases associated with phytoplasmas, although individual phytoplasmas may be limited in their host range or distribution. Actually, hundreds of plant genera are affected by more than 300 distinct diseases associated to phytoplasma infection (Hoshi *et al.*, 2007). Phytoplasmas are the primary limiting factors of growth and photosynthesis for many herbaceous and woody plants all over the world (Bertaccini, 2007). Diseases caused by phytoplasmas include, Coconut Lethal Yellowing, peach X-disease, grapevine yellows and apple proliferation (Bertaccini, 2007). Diseases can be lethal to herbaceous plants severely affected by highly virulent strains (Hoshi *et al.,* 2007).

Plants infected with phytoplasmas suggest a severe disturbance in the normal balance of growth regulators showing symptoms such as: virescence/phyllody, sterility of flowers, proliferation of axillary buds resulting in witches'-broom symptoms, abnormal internode elongation, and generalized stunting (Bertaccini, 2007). For example, aster yellows phytoplasma causes major economic losses in vegetable crops including lettuce, carrot, and celery; and in ornamental plants including gladiolus, hydrangea, China aster, and purple coneflower in North America and Europe.

Phytoplasmas are spread by insect vectors in the Order Hemiptera, Suborder Auchenorrhyncha. Insects belonging to the Cicadellidae (leafhoppers), Fulgoroidea (planthoppers) and Psyllidae (psyllids) become infected by feeding on the phloem tissues of diseased plants. According to Severin (1946), the phytoplasma-insect relationship can be beneficial, deleterious, or neutral in terms of its impact on the fitness of the insect host, affecting the insect populations. Moreover, recent reports by Beanland *et al.* (2000) showed that the exposure to one strain of AY increases both the lifespan and fecundity of female *Macrosteles quadrilineatus*. Thus, the host-plant range of phytoplasmas is strongly dependent upon the feeding habits of its insect vectors, and plants infected with phytoplasmas may modify the insect's behavior, or influence vector fertility (Sugio *et al.*, 2011). Phytoplasmas possess a major antigenic protein that makes up the majority of their cell surface proteins, and that has recently been shown to interact with microfilament complexes of insect intestinal muscles (Suzuki *et al.*, 2006; Hoshi *et al.*, 2007). This protein is believed to be important for both transmission and infection. Few studies have corroborated the specific interaction between host plants, phytoplasmas and insect vectors.

Phytoplasmas can be transmitted from infected to healthy plants through the parasitic plant dodder (*Cuscuta* spp.). Experimental transmission of a phytoplasma by healthy dodders is one of the main ways by which phytoplasma infection is achieved under artificial conditions (Cordova *et al.*, 2003; Marcone *et al.*, 2007). Recently, natural transmission of phytoplasmas through infected seed has been reported (it although has not been fully explained). This type of transmission was first suspected in the spread of CLY (Cordova *et al.*, 2003). In Oman, studies with alfalfa (*Medicago sativa* L.) severely affected by phytoplasmas showed evidence of seed transmission. Similarly, seeds from phytoplasma-infected lime (*Citrus aurantiaca* L.), and from tomato (*Lycopersicum esculentum* Mill.) from Oman and Italy respectively were allowed to germinate under sterile conditions, and tested at different growth stages. Some of these seeds were infected with phytoplasmas belonging to ribosomal groups 16SrI, 16SrXII and 16SrII (Khan *et al.*, 2002; Botti and Bertaccini, 2006). Phytoplasmas can also be spread via vegetative propagation, such as grafting of infected plant tissues onto healthy plants, propagation through cuttings, micropropagation, and any other methods used to multiply plant material.

2.2. Phytoplasma Detection, Identification and Classification

General identification and classification of several strains of phytoplasma are based on molecular tools to such as PCR/RFLP and nested-PCR of the conserved 16S rDNA gene (Lee *et al.*, 1998a; Seemüller *et al.*, 1998). These studies conformed their classification in 16Sr groups, showing consistent clades defined by phylogenetic analysis of near-full-length 16SrRNA gene sequences. These facts indicate that RFLP-based 16Sr groups are phylogenetically valid. This approach using RFLP analyses of direct and nested PCR of the 16S rDNA gene amplification provides a simple, reliable and rapid means to differentiate and identify known phytoplasmas.

For a finer differentiation of phytoplasmas, additional genetic markers such as ribosomal protein (*rp*), a protein traslocate subunit (*secY*), elongation factor Tu (*tuf*) genes and the 16S-23SrRNA intergenic spacer region have been used as supplementary identification tools (Smart *et al.*, 1996; Schneider *et al.*, 1997; Martini *et al.*, 2002; 2007; Lee *et al.*, 1994, 2004a, 2004b, 2006a). Finer subgroup delineation could be achieved by combining RFLP analyses of 16S rDNA with RFLP analysis of the *rp* gene sequences. In fact, the subgroups recognized by this method were consistent with the subclusters identified by analyzing the phytoplasma genomes with techniques of Dot and Southern hybridizations, using a number of cloned phytoplasma DNA probes (Lee *et al.*, 1992; Gundersen *et al*., 1996; Martini *et al*., 2007).

Success of PCR to detect phytoplasams from tissue samples collected in the field largely depends of the quality of total nucleic acid preparations enriched with phytoplasma DNA (Firrao *et al.*, 2007). These procedures are somewhat difficult because of sample compounds that directly inhibit PCR. The amount of phytoplasma DNA is less than 1% of total DNA extracted from certain tissue (Bertaccini, 2007). Different protocols have been studied for total DNA extractions in order to detect these plant pathogens. The aim of each protocol has been to concentrate and purify phytoplasma DNA reducing plant inhibitory enzymes and compounds such as polyphenolic and polysaccharide molecules. Almost all protocols to extract phytoplasma DNA generally have been designed by including an enrichment step in the nucleic acid extraction procedure such as DNA extraction using modified DNeasy Plant Mini Kit from Qiagen with mercaptoethanol to CTAB extraction buffer (Green *et al.*, 1999).

Nested-PCR has been designed to increase sensitivity and specificity of the PCR assay. This approach is necessary to amplify phytoplasma DNA from samples having unusually low titers or inhibitors that may interfere with PCR efficacy (Gundersen *et al.*, 1994). Nested-PCR assays are performed by preliminary amplification using a universal primer pair followed by a second PCR amplification using a second universal primer pair. However, higher sensitivity of Nested-PCR relies on the use of a universal primer pair followed by PCR primed by a group-specific or phytoplasma-universal primer pair. The assay can detect phytoplasmas present in mixed infections, such as symptomatic tissue samples infected with both viruses and phytoplasmas (Lee *et al.*, 1994; 1995).

A major breakthrough in the detection, identification, and classification of phytoplasma strains has been through the application of bioinformatics tools. PCR primer pairs have been designed based on conserved sequences of genetic regions such as the 16S rDNA gene, rp gene operon (*rpIV* (*rpl22*) and *rpsC* (*rps3*)), *tuf* and *secY* (Gundersen *et al.*, 1996; Schneider *et al.*, 1997; Marcone *et al.*, 2000; Martini *et al.*, 2002, 2007; Wei *et al.*, 2004a). Putative phytoplasmas are routinely differentiated on the basis of the 16S rDNA gene by means of RFLP analysis of PCR-amplified DNA sequences (Lee *et al.*, 1998a; 1998b). Because the RFLP patterns characteristic of each phytoplasma are conserved, unknown phytoplasmas can be identified by comparing their 16S rDNA RFLP patterns with available RFLP patterns of known phytoplasmas, without the need to analyze all the representative reference phytoplasmas (Zhao *et al.*, 2009).

Prior to the use of molecular techniques, specific detection of phytoplasmas in diseased plants was difficult. Initially, phytoplasma strains were differentiated and identified based on their biological properties, such as symptoms, plant hosts, and insect vector ranges. This approach was laborious and time-consuming, and often results were inconclusive due to symptoms variability in the field (Bertaccini and Duduk, 2009). In the 1980's serological diagnostic techniques (i.e. Enzyme-Linked ImmunoSorbent Assay ELISA) began to emerge although with inefficient results. Using cloned phytoplasma DNA fragments as molecular probes in DNA-DNA hybridization provided sensitive and specific detection of phytoplasmas in infected plant or insect tissues (Kirkpatrick *et al.,* 1987, 1989; Davis *et al.,* 1988, 1990b, 1991; Lee and Davis, 1988; Deng and Hiniki, 1990a). In the early 1990's, the application of PCR coupled with RFLP analysis of PCR products allowed accurate identification of different strains and species of phytoplasma. Furthermore, the application of antibiotics such as tetracycline to diseased plant stems promoted the disappearance of symptoms providing additional evidence of phytoplasmas are agents of plant diseases (Ishie *et al.*, 1967; Davis *et al*., 1968).

In 1990's, cloning of phytoplasma DNA and nucleic acid-based probes assays (randomly cloned DNA or its complementary RNA) were applied to detect and differentiate phytoplasmas in plants and vectors (Kirkpatrick *et al.*, 1987; Davis *et al*., 1990; Lee and Davis, 1988). During that time, probes based on cloned phytoplasma-specific chromosomal and extrachromosomal DNAs provided the first evidence of genetic differences in phytoplasma DNA. More specifically differences were detected between phytoplasma strains derived from different host plants or geographical locations. PCR assays, using primers based on cloned DNA fragments (non-ribosomal DNAs) specific to a given phytoplasma, provided sensitive and specific tools for phytoplasma detection. In contrast, PCR assays using generic or broad-spectrum primers based on conserved sequences (e.g. 16S rDNA, ribosomal protein, *tuf*, 16S-23S spacer above mentioned) allowed detection of a wide array of phytoplasmas associated with plants and insects.

Molecular data on phytoplasmas have provided considerable insight into their diversity and genetic interrelationships. This in turn has served as a basis for several comprehensive studies on phytoplasma phylogeny and taxonomy (Hogenhout *et al*., 2008). Some investigations, particularly those employing the sequence analysis of 16Sr DNA, have shown that phytoplasmas constitute a coherent, monophyletic and genus-level taxon (Gundersen *et al.*, 1994). Within the phytoplasma clade, groups and subgroups have been delineated, many of which are now considered species. A few remain under the provisional non-taxonomic status of '*Candidatus*' for incompletely described prokaryotes (Murray and Stackebrandt, 1995) (Table 1). Several provisional species have been described, and rules for future putative species delineation have been defined (IRPCM, 2004). The first comprehensive phytoplasma classification scheme was based on RFLP analysis of PCR-amplified 16S rDNA (Lee *et al.*, 1998a, 2000). This approach provided a reliable tool for broad differentiation among phytoplasmas. Currently this system has classified phytoplasmas into 30 groups and more than 40 subgroups, and has become the most comprehensive and widely accepted phytoplasma classification system worldwide (Lee *et al.*, 2004a, 2004b, 2006; Arocha *et al.*, 2005; Al-Saady *et al.*, 2008; Bertaccini and Duduk, 2009; Wei *et al.*, 2007). Although phytoplasmas have not yet been cultivated *in vitro*, phylogenetic analyses based on various conserved genes have shown that they represent a distinct, monophyletic clade within the class *Mollicutes* (Gundersen *et al.*, 1994).

Table 1. List of ‘*Candidatus* Phytoplasma’ species based on 16S rDNA gene sequences.

Strain name	GenBank no.	16S RFLP group and subgroup	Reference
‘*Ca*. Phytoplasma asteris’	M30790	16SrI-B	Lee *et al.* (2004a)
‘*Ca*. Phytoplasma aurantifolia’	U15442	16SrII-B	Zreik *et al.* (1995)
‘*Ca*. Phytoplasma australasiae’	Y10097	16SrII-D	White *et al.* (1998)
‘*Ca*. Phytoplasma ulmi’	AY197655	16SrV-A	Lee *et al.* (2004b)
‘*Ca*. Phytoplasma ziziphi’	AB052876	16SrV-B	Jung *et al.* (2003a)
‘*Ca*. Phytoplasma trifolii’	AY390261	16SrVI-A	Hiruki & Wang (2004)
‘*Ca*. Phytoplasma fraxini’	AF092209	16SrVII-A	Griffiths *et al.* (1999)
‘*Ca*. Phytoplasma phoenicium’	AF515636	16SrIX-D	Verdin *et al.* (2003)
‘*Ca*. Phytoplasma mali’	AJ542541	16SrX-A	Seemüller & Schneider (2004)
‘*Ca*. Phytoplasma pyri’	AJ542543	16SrX-C	Seemüller & Schneider (2004)
‘*Ca*. Phytoplasma spartii’	X92869	16SrX-D	Marcone *et al.* (2004a)
‘*Ca*. Phytoplasma prunorum’	AJ542544	16SrX-F	Seemüller & Schneider (2004)
‘*Ca*. Phytoplasma oryzae’	AB052873	16SrXI-A	Jung *et al.* (2003b)
‘*Ca*. Phytoplasma australiense’	L76865	16SrXII-B	Davis *et al.* (1997)
‘*Ca*. Phytoplasma japonicum’	AB010425	16SrXII-D	Sawayanagi *et al.* (1999)
‘*Ca*. Phytoplasma fragariae’	DQ086423	16SrXII-E	Valiunas *et al.* (2006)
‘*Ca*. Phytoplasma cynodontis’	AJ550984	16SrXIV-A	Marcone *et al.* (2004b)
‘*Ca*. Phytoplasma brasiliense’	AF147708	16SrXV-A	Montano *et al.* (2001)
‘*Ca*. Phytoplasma graminis’	AY725228	16SrXVI-A	Arocha *et al.* (2005)
‘*Ca*. Phytoplasma caricae’	AY725234	16SrXVII-A	Arocha *et al.* (2005)
‘*Ca*. Phytoplasma americanum’	DQ174122	16SrXVIII-A	Lee *et al.* (2006)
‘*Ca*. Phytoplasma castaneae’	AB054986	16SrXIX-A*	Jung *et al.* (2002); Wei *et al.* (2007)
‘*Ca*. Phytoplasma rhamni’	X76431	16SrXX-A	Marcone *et al.* (2004a); Wei *et al.* (2007)
‘*Ca*. Phytoplasma pini’	AJ632155	16SrXXI-A	Schneider *et al.* (2005); Wei *et al.* (2007)
‘*Ca*. Phytoplasma allocasuarinae’	AY135523	Not determined	Marcone *et al.* (2004a)
‘*Ca*. Phytoplasma lycopersici’	EF199549	Not determined	Arocha *et al.* (2007)

'*Ca*. Phytoplasma omanense'	EF666051	16SrXXIX-AD	Al-Saady *et al.* (2008)
'*Ca*. Phytoplasma tamaricis'	FJ432664	16SrXXX	Zhao *et al.* (2009)
'*Ca*. Phytoplasma solani'	AF248959	16SrXII-A	Quaglino *et al.* (2013)
'*Ca*. Phytoplasma pruni'	L04682	16SrIII-A	Davis *et al.* 2013
'*Ca*. Phytoplasma balanitae'	AB689678	16SrV-A	Win *et al.* (2013)
'*Ca*. Phytoplasma sudamericanum'	GU292081	16SrVI-I	Davis *et al.* (2012)
'*C*. Phytoplasma palmicola'	KF751387	16SrXXII-A	Harrison *et al.* (2014)
Reference strains of proposed potentially new or incidentally cited taxa			
– Phytoplasma Taxonomy Group (2004)			
'*Ca*. Phytoplasma palmae'	U18747	16SrIV-A	IRPCM Phytoplasma/Spiroplasma Working Team
– Phytoplasma Taxonomy Group (2004)			
'*Ca*. Phytoplasma cocostanzaniae'	X80117	Not determined	IRPCM Phytoplasma/Spiroplasma Working Team
– Phytoplasma Taxonomy Group (2004)			
'*Ca*. Phytoplasma vitis'	AF176319	16SrV-C	IRPCM Phytoplasma/Spiroplasma Working Team
– Phytoplasma Taxonomy Group (2004)			
'*Ca*. Phytoplasma luffae'	AF086621	16SrVIII-A	IRPCM Phytoplasma/Spiroplasma Working Team
– Phytoplasma Taxonomy Group (2004)			
'*Ca*. Phytoplasma cocosnigeriae'	Y14175	16SrXXII-A	IRPCM Phytoplasma/Spiroplasma Working Team
– Phytoplasma Taxonomy Group (2004); Wei et al. (2007)			
Mexican periwinkle virescence phytoplasma	AF248960	16SrXIII-A	IRPCM Phytoplasma/Spiroplasma Working Team
– Phytoplasma Taxonomy Group (2004)			
Chinaberry yellows phytoplasma	AF495882	Not determined	IRPCM Phytoplasma/Spiroplasma Working Team
– Phytoplasma Taxonomy Group (2004)			
Buckland valley grapevine yellows phytoplasma	AY083605	16SrXXIII-A	Wei *et al.* (2007)
Sorghum bunchy shoot phytoplasma	AF509322	16SrXXIV-A	Wei *et al.* (2007)
Weeping tea witches'-broom phytoplasma	AF521672	16SrXXV-A	Wei *et al.* (2007)
Sugar cane phytoplasma D3T1	AJ539179	16SrXXVI-A	Wei *et al.* (2007)
Sugar cane phytoplasma D3T2	AJ539180	16SrXXVII-A	Wei *et al.* (2007)
Derbid phytoplasma	AY744945	16SrXXVIII-A	Wei *et al.* (2007)

Reference strains of additional 16Sr subgroups			
'*Ca*. Phytoplasma asteris'-related strain AYWB	NC_007716	16SrI-A	Bai *et al.* (2006); Lee *et al.* (1998)
Clover phyllody phytoplasma CPh	AF222065 (rrnA) AF222066 (rrnB)	16SrI-C	Dally, E.L., Bottner, K.D. & Davis, R. E.; Lee *et al.* (1998)
'*Ca.* Phytoplasma asteris'-related strain PaWB	AY265206	16SrI-D	Lee, I.-M., Gundersen-Rindal, D. E., Davis, R. E., Bottner, K. D., Marcone, C. & Seemuller, E.; Lee *et al.* (1998)
Blueberry stunt phytoplasma strain BBS3	AY265213	16SrI-E	Lee, I.-M., Gundersen-Rindal, D. E., Davis, R. E., Bottner, K. D., Marcone, C. & Seemuller, E.; Lee *et al.* (1998)
'*Ca.* Phytoplasma asteris'-related strain ACLR-AY	AY265211	16SrI-F	Lee, I.-M., Gundersen-Rindal, D. E., Davis, R. E., Bottner, K. D., Marcone, C. & Seemuller, E.; Lee *et al.* (1998)
Peanut witches'-broom phytoplasma	L33765	16SrII-A	Lee *et al.* (1998)
Cactus witches'-broom phytoplasma	AJ293216	16SrII-C	Cai *et al.* (2002); Wei *et al.* (2007)
Canada peach X-disease phytoplasma CX	L33733	16SrIII-A	Lee *et al.* (1998)
Clover yellow edge phytoplasma	AF189288	16SrIII-B	Jomantiene, R., Postman, J. D., Montano, H., Maas, J., Davis, R. E. & Johnson, K. B.; Lee *et al.* (1998)
Phytoplasma sp. LfY5(PE65)-Oaxaca	AF500334	16SrIV-B	Harrison *et al.* (2002a); Wei *et al.* (2007)
Carludovica palmata leaf yellowing phytoplasma	AF237615	16SrIV-D	Harrison *et al.* (2002b); Wei *et al.* (2007)
Alder yellows phytoplasma strain ALY882	AY197642	16SrV-C	Lee, I.-M., Martini, M., Marcone, C. & Zhu, S.F.; Lee *et al.* (1998)
'*Ca*. Phytoplasma ziziphi'-related strain JWB-Kor1	AB052879	16SrV-G	Jung *et al.* (2003a); Wei *et al.* (2007)
Pigeon pea witches'-broom phytoplasma	AF248957	16SrIX-A	Davis & Dally (2001); Lee *et al.* (1998)

*In the report by Jung *et al.* (2002), '*Ca.* Phytoplasma castaneae' was assigned to group VI according to DNA sequence similarity, rather than results from RFLP analysis. In accordance with the more widely accepted RFLP-based classification system, this phytoplasma was reassigned to group 16SrXIX by Wei *et al.* (2007).

DThe original reference (Al-Saady *et al.*, 2008) reported '*Ca.* Phytoplasma omanense' as the reference member of a new group designated group 16SrXIX. However, the group number 16SrXIX had been previously published (Wei *et al.*, 2007) to accommodate a different phytoplasma, '*Ca.* Phytoplasma castaneae'. Therefore, we assign 'Ca. Phytoplasma omanense' to a new group, 16SrXXIX, subgroup 16SrXXIX-A.

3. MATERIALS AND METHODS

3.1. Sample collection

Common phytoplasmas disease symptoms such as virescence/phyllody, mottling yellow of the leaves, sterility of flowers, yellow mottling of the leaves, flower sterility, proliferation of axillary buds resulting in witches'-broom symptoms, abnormal internode elongation, and generalized stunting were observed in nine plant species (Table 2). A total of 62 samples were collected from August 2012 to June 2013 in eight locations of covering many agricultural regions on the island of Puerto Rico. GPS coordinates for and specific townships for sample collection sites were: Adjuntas (18°09'31"N; 66°48'06"W), Cabo Rojo (18°5'14"N; 67°8'48"W), Corozal (18°20'28"N; 66°190'01"W), Isabela (18°30'03"N; 67°01'28"W), Juana Díaz (18°03'09"N; 66°30'24"W), Las Marías (18^o15'7.833"N; 66^o59'30.4296"W), Mayagüez (18^o12'10.0902"N; 67^o7'54.3144"W) and San Sebastián (18^o16'56.946"N; 66^o55'12.2808"W) (Figure 1 and 2) (Appendix 1).

At collection sites, samples were harvested, labeled and deposited in individual plastic bags and were transported in a cooler to the laboratory for further processing. In the laboratory, petioles and leaf midribs were disinfected with 10% commercial bleach solution (sodium hypochlorite), washed with deionized double-distilled water and stored at -20^oC until their use. Total nucleic acids were extracted from 3 g of petioles and leaf midribs following the protocol described by Thompson and MacKenzie (1999).

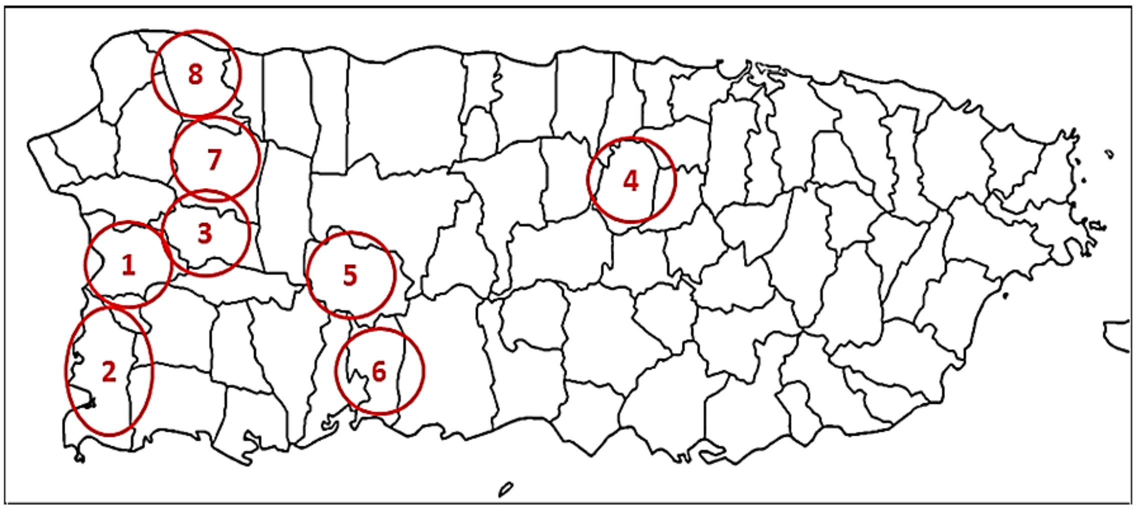

Figure 1. Samplings locations in Puerto Rico: **A.** Mayagüez: periwinkle (*Catharanthus roseus*), Ixora (*Ixora coccinea* L.), tabebuia (*Tabebuia pallida* L.) and mango (*Mangifera indica* L.); **B.** Cabo Rojo: Spanish lime (*Melicoccus bijugatus* Jacq.) and cactus (*Opuntia* spp.); **C.** Las Marías: orange (*Citrus sinensis* L.); **D.** Corozal: lemon (*Citrus limon* L.); **E.** Adjuntas: orange (*Citrus sinensis* L.) and coffee (*Coffea arabica* L.); **F.** Juana Díaz: orange (*Citrus sinensis* L.) and pigeon pea (*Cajanus cajan* L.); **G.** San Sebastián: orange (*Citrus sinensis* L.); **H.** Isabela: *Citrus* sp. (lemon) and pigeon pea (*Cajanus cajan* L.).

Table 2. Potential phytoplasma hosts, tissues samples and symptoms observed in Puerto Rico.

Plants	Tissue sample	Symptoms
Coffee	Petioles and young leaves (leaf midrib)	Witches'-broom (Galvis *et al.*, 2007).
Orange trees	Petioles and young leaves (leaf midrib)	Stunting of tree, shortened stem internodes, leaves small and yellow mottling (Texeira *et al.*, 2008)
Pigeon pea	Petioles and young leaves (leaf midrib)	Witches'-broom and bushy stunt (Licha, 1980).
Periwinkle	Petioles and young leaves (leaf midrib)	Phyllody, big bud and virescent flowers (Nejat *et al.*, 2012).
Tabebuia	Petioles and young leaves (leaf midrib)	Witches'-broom (Cook, 1938).
Spanish lime	Petioles and young leaves (leaf midrib)	Fasciation and Malformation
Ixora or Flame of the woods	Petioles and young leaves (leaf midrib)	Witches'-broom and chlorotic variegation on the leaves.
Mango	Petioles and young leaves (leaf midrib)	Fasciation and malformation (Om-Hashem M. and S.H. El-Deeb, 2007).
Cacto	Young cladode	Dense clusters and highly proliferating cladodia. (Hong *et al.*, 2008).

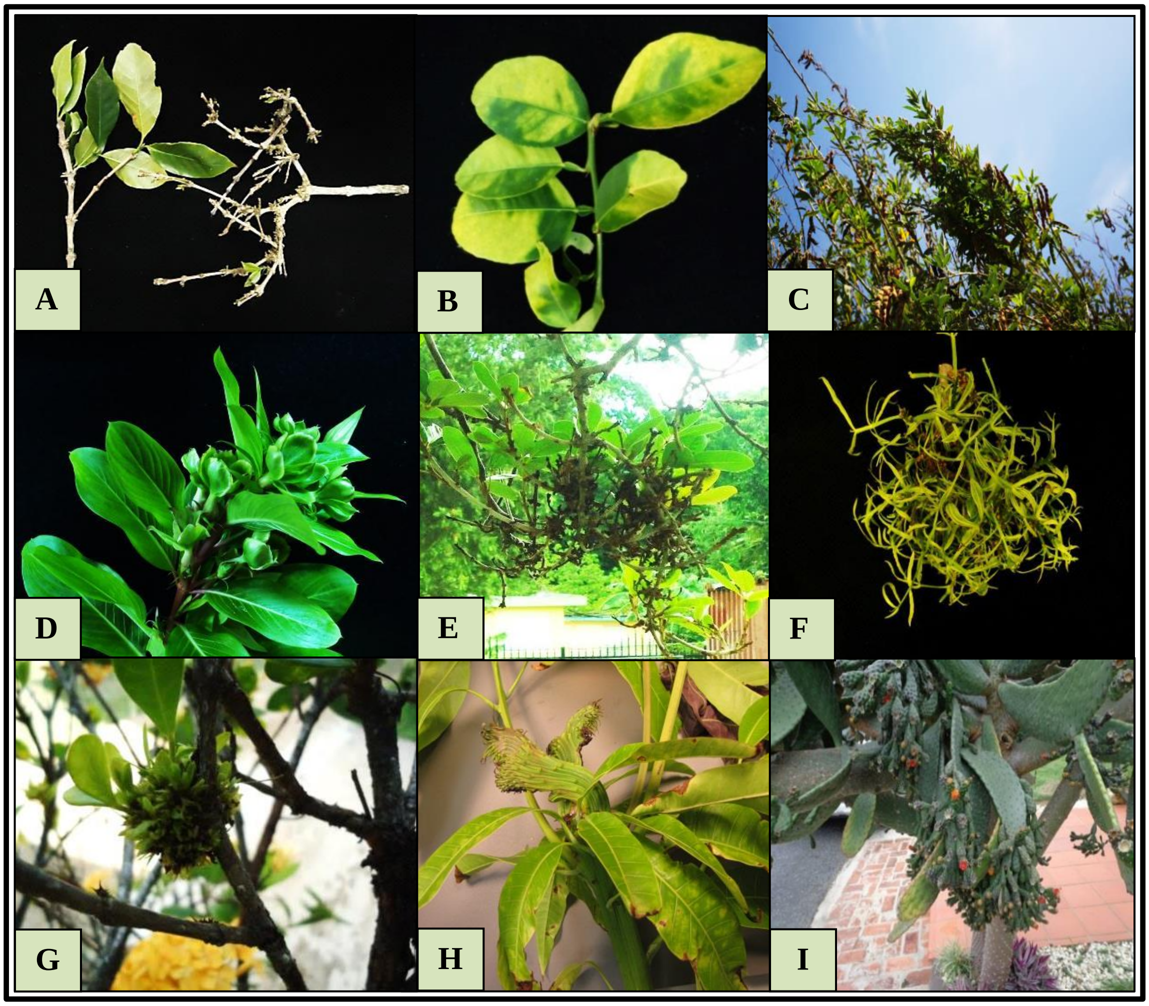

Figure 2. Symptoms commonly observed in plants sampled for phytoplasmas infection in Puerto Rico. **A.** Witches'-broom in coffee (*Coffea arabica*). **B.** Leaves with blotchy mottling in citrus trees. **C.** Witches'-broom in pigeon pea (*Cajanus cajan*). **D.** Phyllody, big bud and virescent flowers in perinwinkle (*Catharanthus roseus*). **E.** Witches' broom in tabebuia (*Tabebuia pallida*). **F**. Witches'-broom in Spanish lime (*Melicoccus bijugatus*). **G.** Witches'-broom in Ixora (*Ixora coccinea*)**. H.** Fasciation and malformations at tip of mango (*Mangifera indica*) **I.** Cladode proliferation (witches'-broom) in cactus (*Opuntia* spp.)

3.2. Plant DNA Extraction

Total DNA extraction was carried out using a modified protocol of Quiagen's DNeasy Plant Mini Kit (Qiagen, Germantown, MD) (Green and MacKenzie, 1999). Briefly, buffer AE was prewarmed to 65 ^{o}C. A FastPrep FP120 Machine (MP BioMedicals, Cleveland, OH) was used to grind 3 g of fresh or frozen plant tissue placed in a Fast Prep 2.0 ml tube (lysing matrix A) containing 1 ml of cetyl trimethylammonium bromide (CTAB) extraction buffer with 0.2% (v/v) 2-mercaptoethanol. Samples sat at room temperature for one to two hours. To homogenize samples a Fast Prep instrument was set at 6.5 speeds for 45 seconds. Samples were placed on ice for one minute, or until tube was cool. This last step was repeated using a FastPrep FP120 (MP BioMedicals, Cleveland, OH) machine and ice incubation until samples were completely homogenized to a maximum of three cycles. After processing, tubes were placed on ice for five minutes and tubes were briefly spun in microcentrifuge.

Using a large-bore pipette tip, 0.5 ml of supernatant was transferred to a 1.5 ml microcentrifuge tube. Four μl of 100mg/ml of RNaseA (Qiagen, Germantown, MD) was added and mixed by inversion. Samples were incubated at 65°C for approximately 35 min. Then 162.0 μl of buffer AP2 from the DNeasy kit was added, mixed by inversion and placed on ice for 5 min. The entire contents of the tube were placed onto a QIAshredder column with a 2 ml collection tube and centrifuged at maximum speed (20,000 x g; 14,000 rpm) for 2 min. Column flow-through (450 μl) was transferred to a new 1.5 ml tube without disturbing the cell debris pellet. To the sample 675 μl of buffer AP3/E was added and mixed by inversion. An additional 650 μl of the mixture was added to a QIAshredder column (white) and centrifuged for 1 min. at 8,000 rpm. The flow-through was discarded and the centrifugation was repeated with the remaining sample. The column was transferred to a clean 2 ml microcentrifuge tube. Five hundred μl of buffer AW (Qiagen) were added and centrifuged for 1 min. at 8,000 rpm. The flow-through was discarded and an additional 500 μl of buffer AW was added. Sample was centrifuged at maximum speed for 2 min. The column was transferred to a new 1.5 ml tube and 100 μl of pre-warmed (65 °C) buffer AE was added. Samples were placed at room temperature for 10 min. DNA was eluted by centrifuging for 2 min. at 10,000 rpm. DNA quality and concentration was determined using Implen's NanoPhotometer (Implen, Westlake, Village, CA).

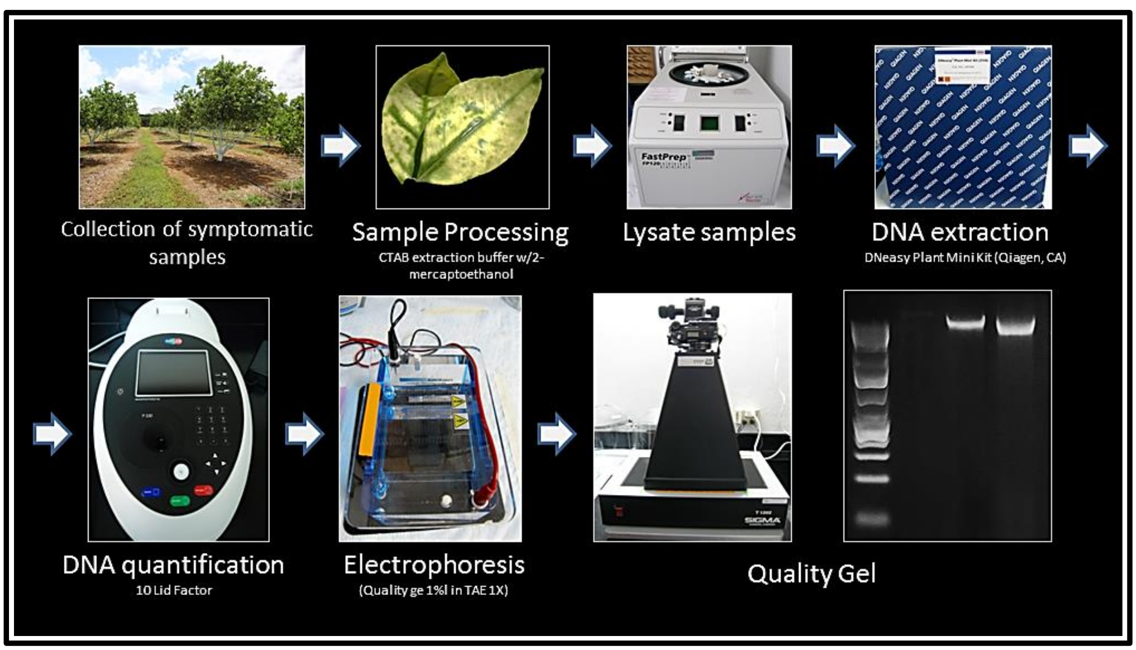

Figure 3. Diagrammatic representation of the DNA extraction protocol and quantification from symptomatic leaf samples corresponding to several plant species.

3.3. Direct and nested PCR protocol

Phytoplasma detection through direct and nested PCR was conducted using universal and specific primers (Figure 4). In addition to being tested for phytoplasmas, citrus samples were also tested for spiroplasmas using primer pairs ScR16F1/ScR16R1 for direct PCR and ScR16F1A/ScR16R2 for nested PCR. PCR master mix (or cocktail) components for both direct and nested amplification are described in Table 3 and 4. For both direct and nested PCR amplifications, 38 cycles were conducted in an automated termal cycler (Mastercycler® Pro S, Eppendorf, NY) with AccuPrime High Fidelity Taq DNA polymerase (Invitrogen, Carlsbad, CA). Cycling conditions were denaturation at 94^{o}C for 2 min; annealing at 50^{o}C for 2 min; and primer extension at 72°C for 3 min with a final extension of 7 min at 72 °C. A negative control (water) devoid of DNA template was included in amplification reactions. Amplification products (3 µl) plus 3 µl of tracking dye were electrophoresed in a 0.8% agarose gel in 1X TAE buffer. PCR amplification products were stained with 2.5 µl of GelRed™ nucleic acid stain (Biotium Hayward, CA) (10,000 X in water) and visualized with UV transillumunator (Figure 5).

Table 3. Master mix used for direct and nested PCR amplification based on a 50µl reaction.

Components	**AccuPrime HF[1]**
Water (Molecular Biology grade)	39.75µl
10X AccuPrime buffer II	5µl
Primer 1 (20 pmol/µl)	1µl
Primer 2 (20 pmol/µl)	1µl
AccuPrime™ High Fidelity Taq DNA Polymerase[1]	0.25µl
Template DNA (25ng/µl)	3µl

[1]Enzyme: AccuPrime High Fidelity DNA polymerase (Invitrogen® Carlsbad, CA).

Table 4. Pimers used to detect 16S rDNA and *rpIV* (*rpl22*)-*rpsC* (*rps3*) genes of phytoplasmas and *Spiroplasma citri* by direct and nested PCR

Name	Sequence (5'-3')	Length	References
Phytoplasmas (direct and nested PCR)			
16Sr DNA gene			
P1 (Direct PCR)	AAGAGTTTGATCCTGGCTCAGGATT	25	Deng, 1991
P7 (Direct PCR)	CGTCCTTCATCGGCTCTT	18	Schneider *et al*, 1995
P1A (Nested PCR)	AACGCTGGCGGCGCGCCTAATAC	23	Unpublished[¥]
16S-Sr (Direct or nested PCR)	GGTCTGTCAAAACTGAAGATG	21	Lee *et al.*, 2004b
R16F2n (Direct or nested PCR)	GAAACGGTTGCTAAGACTGG	20	Gundersen and Lee, 1996
R16R2 (Direct or nested PCR)	TGACGGCCGTGTGTACAAACCCCG	25	Gundersen and Lee, 1996
fU5 (Nested PCR)	CGGCAATGGAGGAAACT	17	Seemüller *et al.*, 1994
rU3 (Nested PCR)	TTCAGCTACTCTTTGTAACA	20	Seemüller *et al.*, 1994
***rpIV* (*rpl22*) and *rpsC* (*rps3*) genes**			
rpL2-F3 (Direct PCR)	WCCTTGGGGYAAAAAAGCTC	20	Matini *et al.*, 2007
rpF1C (Direct or nested PCR)	ATGGTDGGDCAYAARTTAGG	20	Matini *et al.*, 2007
rp(1)-R1A (Nested PCR)	GTTCTTTTTGGCATTAACAT	20	Matini *et al.*, 2007
Spiroplasma citri			
16Sr DNA gene			
ScR16F1 (Direct PCR)	AGGATGAACGCTGGCGGCAT	20	Lee *et al.*, 2006
ScR16R1 (Direct PCR)	GTAGTCACGTCCTTCATCGT	20	Lee *et al.*, 2006
ScR16F1A (Nested PCR)	GCATGCCTAATACATGCAAG	20	Lee *et al.*, 2006
ScR16R2 (Nested PCR)	ATCCATCCGCACGTTCTCGTAC	22	Lee *et al.*, 2006

[¥] Personal communication with Dr. Robert Davis (Virologist) and Ellen Dally (Microbiologist) at USDA-ARS, Molecular Plant Pathology Beltsville-Maryland.

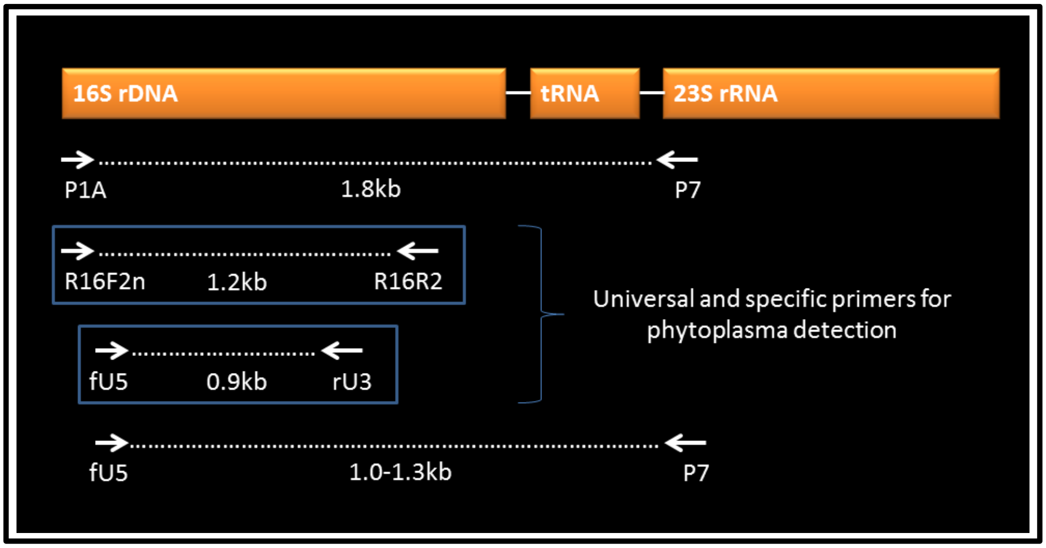

Figure 4. Diagrammatic representation of the 16S–23S rDNA operon, showing the position of some of the various universal primers that have been developed for phytoplasma PCR detection. Primer names are given under the arrows and the sizes of the expected amplicons are shown between the dotted lines.

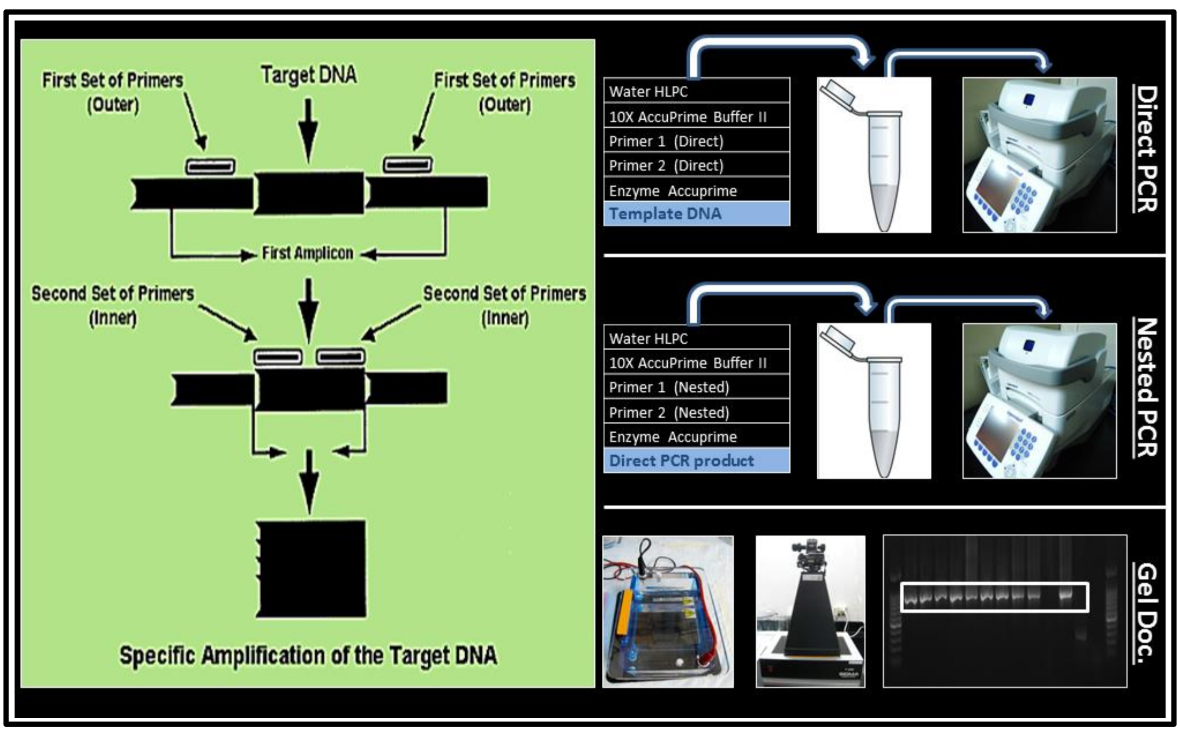

Figure 5. Diagrammatic representation of direct and nested PCR used for the Pigeon pea witches'-broom phytoplasma (PPWB) detection in several important crops in Puerto Rico.

3.4. PCR product purification

Amplicons from direct and nested PCR were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). PCR products corresponding to phytoplasmas were loaded and separated in 1% standard agarose or low-melt agarose gels in 1X TAE buffer. Briefly, DNA fragment was excised from the agarose gels using a sharp scalpel, minimizing the size of the gel slice by removing extra agarose. The gel slice was weighed in a 2.0 ml microfuge tube and three volumes of buffer QG were added per volume of gel. Tubes were incubated at 50 °C for 10 min or until the gel slice had completely dissolved. After the gel slice had dissolved completely, samples turned yellow, similar to buffer QG without dissolved agarose. Then, one gel volume of isopropanol was added to the sample and mixed. Next, samples were placed in a spin column with 2.0 ml collection tube. Samples were transferred to the column to bind DNA fragments, and centrifuge for 1 min at 8,000 rpm. The flow-through was discarded and the column was placed back in the same collection tube. Samples were washed with 0.75 ml of buffer PE that was added to column and centrifuge for 1 min at 8,000 rpm. The flow-through was discarded and the column was centrifuged for an additional 1 min at 14,000 rpm). The column was placed into a clean 1.5 ml microcentrifuge tube. To elute DNA, 30 µl of buffer EB or water (pH 7.0) was added to the center of the QIAquick membrane and centrifuge for 2 min at 14,000 rpm. DNA quality and concentration was determined using Implen’s NanoPhotometer (Implen, Westlake, Village, CA) (Figure 6).

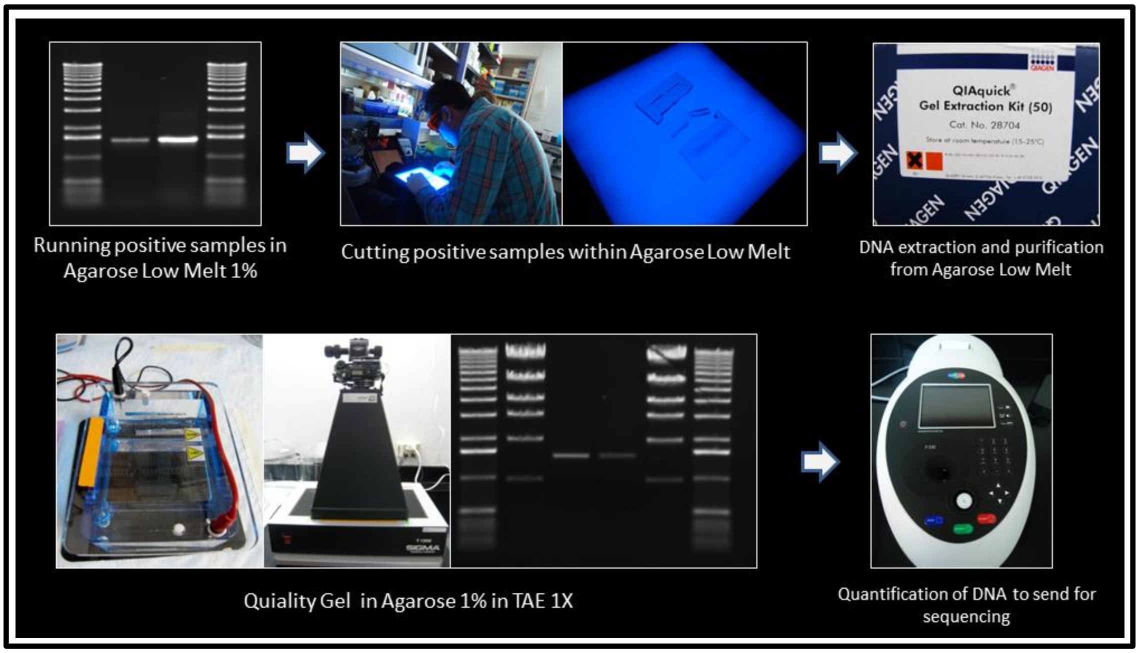

Figure 6. Diagrammatic representation of the PCR products purification of Pigeon pea witches'-broom phytoplasma from important crops in Puerto Rico.

3.5. Sequencing

Thirty microliters of PCR purified product at a final concentrations ranging from 15 to 50 ng/µl were sent to be sequenced at commercial facilities (Macrogen, Rockville, MD). Amplicons were sequenced in both directions with primer pairs P1/P7; R16F2n/R16R2; fU5/rU3 and fU5/P7, for 16S rDNA gene and rpL2-F3/rp(1)-R1A for *rplV* (*rpl22*) and *rpsC* (*rps3*) genes. Nucleotide sequences obtained from the 16S rDNA and rp genes were compared with reference nucleotide sequences using the Basic Local Alignment Search Tool (BLAST) in the GenBank sequence database hosted by the National Center of Biotechnological Information (NCBI) http://blast.ncbi.nlm.nih.gov/Blast.cgi.

3.6. Potential vectors of PPWB phytoplasma

In August 2013, potential insect vectors including leafhoppers of the order Homoptera, suborder Auchenorrhyncha, were collected. Leafhoppers belonging to families Cicadellidae, Flatidae, Nogodinidae, Cixiidae, Derbidae and Psyllidae were collected at four locations: Isabela (18°30’03” N; 67°01’28” W), San Sebastián (18^o16’56.946” N; 66^o55’12.2808” W), Adjuntas (18°09’31” N; 66°48’06” W) and Juana Díaz (18°03’09” N; 66°30’24” W), Puerto Rico. Locations were selected based on positive PPWB phytoplasma samples from citrus and pigeon pea plants. Leafhoppers were collected with a sweep net on the symptomatic citrus and pigeon pea plants (2.5 meters). Five trees from each plant were sampled randomly at Isabela, Adjuntas, San Sebastián and Juana Díaz by net sweeping 10 times per tree. At Juana Díaz and Isabela, due to lack of leafhoppers captured in citrus trees, the systematic sampling was changed. In this regard, six pigeon pea plants were sampled by net swept (5 sweeps per plant). All insects collected were stored at 4 ^oC until their use.

Insects were morphologically identified under the stereoscope. Specimens were compared with reference specimens in collection at the Luis F. Martorell Insectarium maintained at the University of Puerto Rico Alzamora research farm in Mayagüez, PR. A reference collection of leafhoppers recovered from citrus and pigeon pea was created at the Insectarium. Using the taxonomic key of Caldwell and Martorell (1951), the specimen identification and classification was confirmed by Dr. Alejandro E. Segarra, Entomologist and Professor and in the Department of Crops and Agro-Environmental Sciences, College of Agricultural Sciences University of Puerto Rico, Mayagüez. After insect identification, five specimens from each genus were selected for DNA extraction and phytoplasma detection. Insect genomic DNA extraction was carried out for five specimens (whole body) from each genus using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). In this sense, five complete bodies of each insect genus and two ceramic beads were placed in a 1.5 ml microcentrifuge tube. In each tube 180 µl of ATL buffer and 20µl of proteinase K were added. The samples were then mixed by vortexing, and incubated at 56 °C until completely lysed. Vortexing was occasionally performed during incubation. Prior to step two, the tubes were vortexed for 15 sec. Then, 200 µl of AL buffer were added and samples were mixed thoroughly by vortexing. The samples were incubated at 56 °C for 10 min. Next, 200 µl ethanol (96–100%) was added and once again the samples were mixed thoroughly by vortexing. The entire mixture was then transferred placed into a spin column provided with the kit. The tubes were centrifuged at 8,000 rpm for 1 min. The flow-through and collection tube were discarded. The spin columns were placed in a new 2 ml collection tube and 500 µl buffer AW1 was added. The tubes were centrifuged for 1 min at 8,000 rpm and the flow-through and collection tubes were discarded. Once again the spin column were placed in new 2 ml collection tubes, and 500 µl buffer AW2 was added. The tubes were centrifuged for 3 min at 14,000 rpm and the flow-through and collection tube were discarded. Finally, the spin columns were placed in a new 1.5 ml or 2 ml microcentrifuge tube. The DNA was eluted by adding of 200 µl buffer AE in the center of the spin column membrane. The tubes were incubated for 1 min at room temperature (24°C) and centrifuged for 1 min at 8,000 rpm. DNA concentration was measured with a nano-spectrophotometer (Nanopearl®, Westlake Village, CA). The insect DNA samples were stored at -20 ^{o}C until their use (Figure 7). Genomic DNA from each insect genus was used for PCR and nested PCR assays. All insect samples were tested for phytoplasma infection using universal primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) and primers R16F2n/R16R2 (Gundersen and Lee, 1996) and fU5/rU3 (Seemüller *et al.*, 1994) for nested PCR. The thermocycler conditions were as described before. Because control insects (positive for phytoplasma and negative) were not available, the DNA from healthy periwinkle plants maintained in nurseries was used as a negative control.

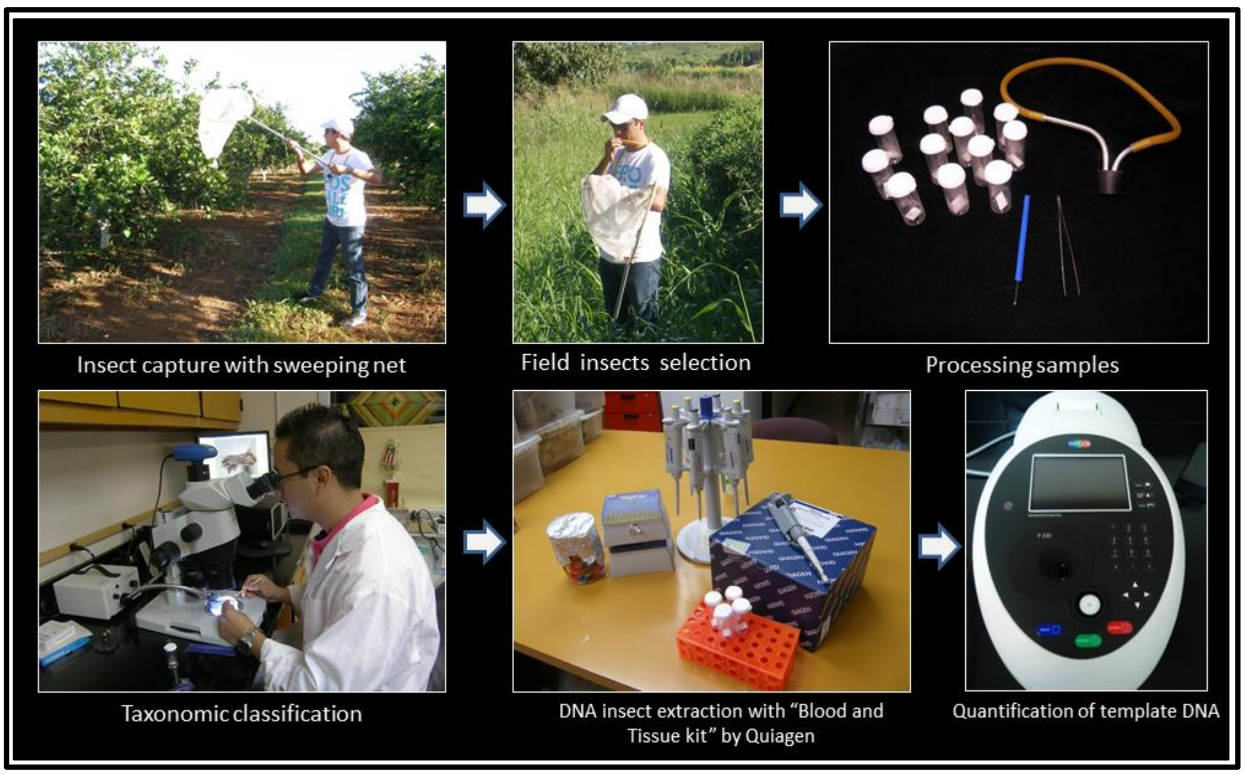

Figure 7. Illustration of insect capture by sweeping net method, insect classification and identification of pigeon pea witches'-broom phytoplasma (PPWB) in nine potential insect vectors in Puerto Rico.

3.7. Phylogeny of 16S rDNA, *rpIV* (*rpl22*) and *rpsC* (*rps3*) genes

Contigs from phytoplasma DNA sequences were assembled and edited using BioEdit (version 7.1.9) (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). Multiple sequence alignment based on the MUSCLE function in Guidance® (http://guidance.tau.ac.il/) was used to align the 16S rDNA and rp genes (Penn *et al.*, 2010) (Appendix 2 and 3). Phylogenetic analysis of nucleotide sequences was conducted employing the maximum likelihood method with the software package MEGA 6 (Molecular Evolutionary Genetics Analysis) (Table 5) (Tamura *et al.*, 2011) (http://www.megasoftware.net/). Clade support was assessed by 2000 bootstrap replications of the 16S rDNA and rp genes. Estimation of nucleotide substitution was made using the Tamura Nei Model. Tree inferences were made using the nearest neighborhood interexchange (NNI) heuristic method. Further, to determine the difference in substitution patterns for a pair of sequences out an analysis of disparity index per site (Tamura *et al.*, 2012) was carried.

Table 5. Representative phytoplasma strains used to construct phylogeny. RFLP classification for 16S rDNA and two ribosomal protein (rp) group affiliations and accession numbers for 16S rDNA and rp genes are included

Phytoplasma strains	RFLP classification		Accession number	
	16Sr group	rp group	16S rDNA gen	rp genes
Alder Yellow	I	I	AY197642	AY197667
Ca. phytoplasma trifolli	I		AY500130	
Oenothera phytoplasma	I	I	M30790	
Aster yellows	I	I	AY265206	
Sweat potato witches'-broom	II		L33770	
Peanut witches'-broom	II		L33765	EF193375
Picris echioides phyllody	II		Y16393	EF193381
Crotalaria phyllody	II			EF186818
Cleome phyllody	II			EF193379
Soybean phyllody	II			EF186816
Lime witches'-broom	II			EF186815

Table 5. (cont.) Representative phytoplasma strains used to construct phylogeny. RFLP classification for 16S rDNA and two ribosomal protein (rp) group affiliations and accession numbers for 16S rDNA and rp genes are included

Phytoplasma strains	RFLP classification		Accession number	
	16Sr group	rp group	16S rDNA gen	rp genes
Italian alfalfa witches'-broom	II		EF193356	EF193380
Milkweed yellows	III	III	AF510724	
Walnut witches'-broom	III	III	AF190227	EF186812
Goldenrod yellows	III	III		EF186810
Coconut lethal yellowing	IV		AF498308	
'*Ca*. phytoplasma fraxini'	IV		JQ868445	EF183493
Lethal yellowing	IV		EF186822	EF186804
'*Ca*. phytoplasma fraxini'	IV			EF183492
Carludovica palmata leaf yellowing	IV			DQ318239
Elm yellow	V	V	AY197655	AY197675
Cherry lethal yellows	V	V	AY197659	AY197679
Peach yellows	V	V	AY197660	AY197680
'*Ca*. phytoplasma ulmi'	V	V		EU116428
Clover proliferation	VI		AF409070	
Potato witches'-broom	VI			AY197683
Illinois elm yellows	VI			EF183490
Ash yellow	VII		AF105316	
HLB-phytoplasma	IX		HQ423159	
Periwinkle virescens	IX		HQ589191	
Colombian periwinkle	IX		EU816776	
Pigeon pea witches'-broom	IX	VI	AF248957	EF193383
Pigeon pea witches'-broom ja	IX	VI	EF186825	EF183496
Caribbean PPWB	IX		U18763	
Pigeon pea witches'-broom fl	IX	VI	EF186826	EF183495
Rynchosia LL	IX	VI	AF361019	EF186799
Pigeon pea witches'-broom pr	IX	VI	EF186824	EF183497
Almond witches'-broom	IX		AF390136	EF186803
'*Ca*. phytoplasma phoenicium'	IX		AF515636	JN712787
'*Ca*. phytoplasma mali'	X		AJ542541	EF193366
'*Ca*. phytoplasma mali'	X		AJ542542	
'*Ca*. phytoplasma prunorum'	X			EF193369
Phormium yellow leaf	XII		U43570	
'*Ca*. phytoplasma australiense'	XII			AY376666
'*Ca*. phytoplasma australiense'	XII			AY303560
Tomato stolbur	XII			EF193364
Strawberry lethal yellowing	XIII		AJ243045	

Table 5. (cont.) Representative phytoplasma strains used to construct phylogeny. RFLP classification for 16Sr and ribosomal protein (rp) group affiliations and accession numbers for 16S rDNA and rp genes are included.

Phytoplasma strains	RFLP classification		Accession number	
	16Sr group	rp group	16S rDNA gen	rp genes
Mexican periwinkle virescence	XIII			EF193365
Phytoplasma STRAWB1	XIII			U96615
'*Ca.* phytoplasma americanum'	XVIII		DQ174121	
'*Ca.* phytoplasma americanum'	XVIII		DQ174120	
American potato purple top	XVIII			EF193362
Acholeplasma palmae	Outgroup	Outgroup	L33734	EF197116
Acholeplasma laidawii	Outgroup	Outgroup	M23932	M74771

3.8. 16S rDNA and *rpIV* (*rpl22*) and *rpsC* (*rps3*) genes RFLP analysis

RFLP analysis of direct PCR products from complete 16S rDNA gene (1.8 kb) and *rpIV* (*rpl22*) and *rpsC* (*rps3*) genes (1.2 kb) were used to detect polymorphisms in phytoplasmas obtained during the survey. Direct PCR products (5 µl to 200 ng/µl) of complete genes mentioned above were digested individually with restriction enzymes *AluI*, *MseI*, *RsaI*, *HinfI* and *HaeIII* (New England Biolabs, Beverly, MA) according to manufacturer's instructions. Restriction products were separated by electrophoresis in a 3% agarose gel in 1X TAE for 1h at 100V. Gel was stained with 3 µl of Gel Red Nucleic Acid Stain (10,000X in water) and visualized with an ultraviolet transilluminator.

3.9. Real time PCR (qPCR) to improve phytoplasma detection using SYBR® Green method

3.9.1. Primer design

Based on the sequences obtained from the amplification of the 16S rDNA gene, specific primers were designed for PPWB phytoplasma detection using qPCR. A consensus

DNA sequence of the expected size of 1.8 kb was created based on multiple sequence alignment obtained from the MUSCLE algorithm using MEGA software. The consensus sequence was placed in the program Primer3 to generate specific primers (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) (Rozen and Skaletsky, 1998). General setting parameters are indicated in the figure below (Figure 8).

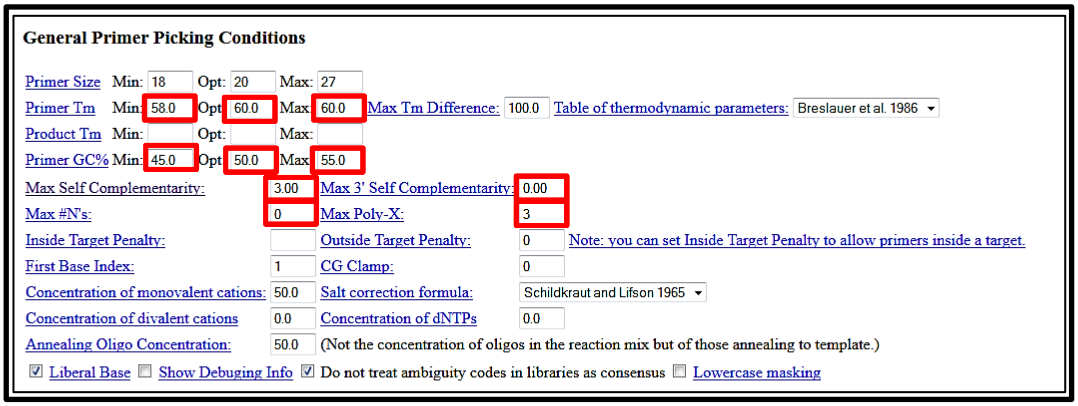

Figure 8. Specific primers for PPWB phytoplasma were generated by Primer3 program to be used in a qPCR assay. General parameters are enclosed in red boxes above.

The Primer3 program generated three different primers (one forward, one reverse and one probe) (Table 6 and Figure 9 and 10). Based on the results of the Primer3 program, a set of forward and reverse primers was selected based on their sensitivity and specificity.

Table 6. Sequence of specific primer pair and probe designed to detect PPWB phytoplasma using a qPCR assay.

Name	Sequences	Start position	Strand	Amplicon	Tm[1]	any[2]	3'[3]
RT-F1	5'TGGACTGAGAGGTCGAACAG	288	forward	93	58.96	3	0
RT-R1	5'CGGTCAGAGTTTCCTCCATT	370	reverse	…	59.14	3	0
RT-P1[4]	5'Fam-CGGCCCAAACTCCTACGGGA-3' Tamra	327	probe	…	68.55	2	0

[1] Tm = Melting temperature of the primer or oligo
[2]Any = Self-complementarity score of the oligo or primer (taken as a measure of its tendency to anneal to itself or form secondary structure)
[3] Primer or oligo 3' self-complementarity (taken as a measure of its tendency to form a primer-dimer with itself)
[4] Primer probe designed for a TaqMan® assay (not evaluated during this study)

The forward and reverse primer set was validated using the program Mfold (http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form) *in silico.* Parameters to analyze the primer's ability to form secondary structures were changed as indicated below figure (Figure 9). In this case, the delta G index (used to describe how 'bad' a duplexed structure derived from hairpin (mFold) and hetero and homo dimers analysis methods may be) was used to determine the necessary energy to avoid any hairpin secondary structure in the primers. After determining primer's delta G, their specificity was evaluated with Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi).

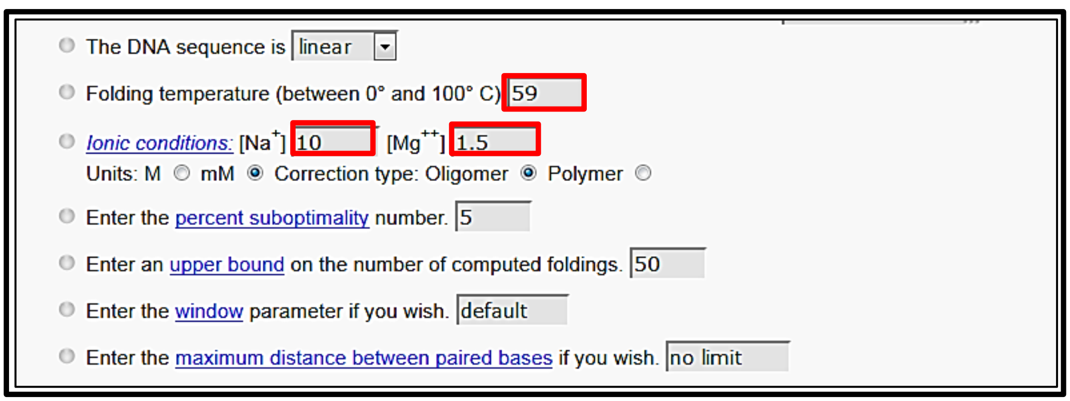

Figure 9. General parameters used with Mfold software program to evaluate primer capability to form secondary structures. The parameters enclosed in red box were changed.

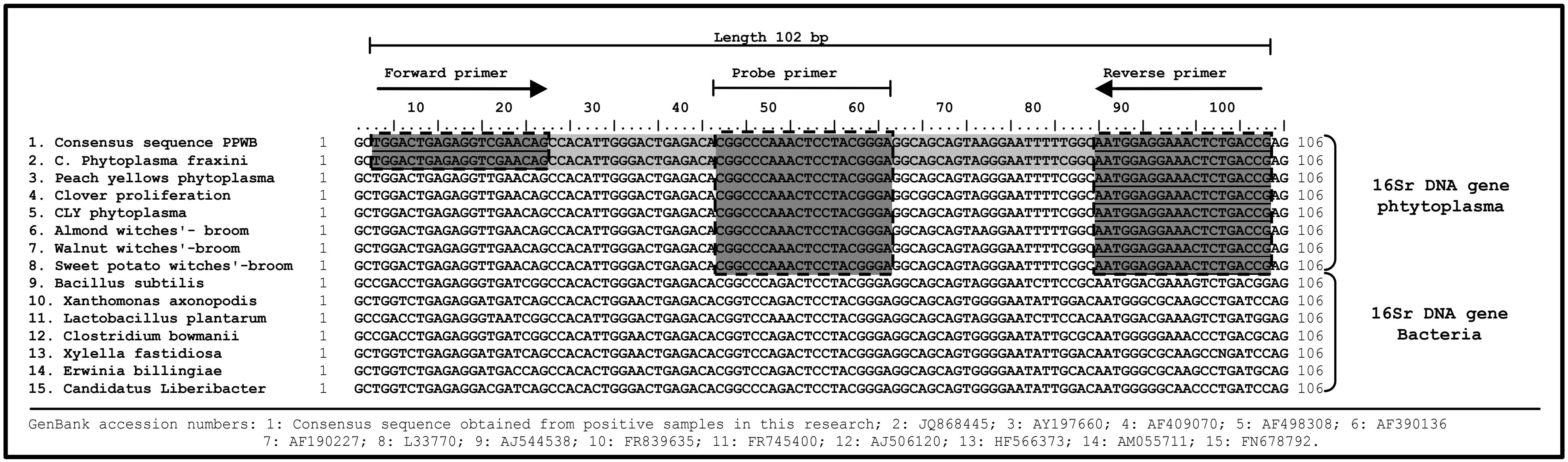

Figure 10. Location and selectivity of forward and reverse primers and probe, generated to amplify a small region (102 bp) of the 16S rDNA gene using a qPCR assay.

3.9.2. Real time PCR protocol

Based on positive samples for PPWB phytoplasma generated in this study a qPCR assay was developed. Positive samples with higher DNA concentrations were tested for PPWB phytoplasma detection using SYBR® the Green method. Three qPCR assays were carried out to improve the phytoplasma detection. To determine the efficiency of primers designed for phytoplasma detection, in the first assay were tested eigth different symptomatic and asymptomatic plant samples (Appendix 4). To confirm the sensibility of primers designed for phytoplasma detection, a second assay was carried out where were tested 25 citrus asymptomatic and symptomatic samples with Citrus greening disease (Appendix 5). Finally, to evaluate the specificity of primers designed for PPWB phytoplasma detection a last assay was carried out with different DNA from 12 phytoplasma subgroups (16SrI-B, 16SrX-A, 16SrV-A, 16SrIII-H, 16SrII-D, 16SrV-C, 16SrII-C, 16SrVI-A, 16SrXII-A, 16SrIX-C, 16SrIII-A, 16SrVII-A) (Appendix 6). For the last assay, DNA from *Pseudomonas saccharophila*, *Sphingomonas phyllosphaerae* and Haloarchaea (extremophile microorganisms living in harsh environments, such as hot springs, salt lakes, soils and oceans) were used as outgroups (Appendix 6). The phytoplasma DNAs were provided by Prof. Assunta Bertaccini (University of Bologna, Plant Pathology, Viale Fanin 42, 40127 – Bologna, Italy). All samples tested in the three qPCR assays were compared with the positive control collected from an infected pigeon pea (*Cajanus cajan*) sample (Pigeon pea (JD) 33) in Juana Díaz, PR. This sample was confirmed positive for PPWB phytoplasma by Dr. Robert E. Davis at the Molecular Plant Pathology Laboratory in USDA–ARS, Beltsville, Maryland, USA. For the three qPCR assays water HPLC grade and DNA from a healthy periwinkle plant were used as a negatives control to detect possible cross contamination.

The qPCR assays were carried out in an Eco™ Real Time PCR System (Illumina®, San Diego, CA) in MicroAmp optical of 48-well plates. Each reaction was performed in a total volume of 20μl, containing 2μl of template DNA (20-50ng/μl), 0.4μl of each primer (200pM), and 12μl of KAPA SYBR® FAST qPCR Kit Master Mix (2X) Universal (KAPA, Biosystems, Boston, MA) and 7.2μl of molecular water (HLPC Sigma-Aldrich, St. Louis, MI). Thermal cycling parameters were: 3 min at 95°C for polymerase activation followed by 40 cycles of denaturation at 95°C for 2 sec and annealing or extension at 60°C for 20 sec. Baseline and fluorescent threshold were set automatically. Melt curve analysis (derivative and component melt calculated the temperature of dissociation of double-stranded DNA during heating, that leading to a rise in the absorbance intensity and hyperchromicity) was performed to identify the occurrence of primer-dimer or missing primer through the melting temperature (Tm). Specificity of the reaction was analyzed by an standard curve obtained from positive sample from Juana Díaz diluted in a dilution series (1:10, 1:100, 1:1000 and 1:10000) to obtain the efficiency (*e*), linear regression and correlation coefficient (R^2) of real-time PCR assay. The assay was calibrate by dilutions series (10^{-1} [9.84ng/μl], 10^{-2}, 10^{-3} and 10^{-4}) of Pigeon pea (JD) 33 sample DNA (123ng/μl).

Data obtained from the amplification of the small region (102bp) of 16S rDNA gene, corresponding to Cq (Cycle quantification), Cq Threshold, Baseline End, Quantity and Tm (Melting Temperature) were calculated by the computer program EcoStudy (Illumina®, San Diego, CA).

4. RESULTS AND DISCUSSION

4.1. Sample collection

From 2012 to 2013, 62 samples were collected from different plant species in eight municipalities of the island of Puerto Rico. Plant species collected included coffee (*Coffea arabica*) (7 samples = 11%); citrus samples (orange [*C. sinensis*], tangerine[*C. reticulata*] and lemon [*C. limon*]) (20 samples = 33%); pigeon pea (*Cajanus cajan*) (15 samples = 24%); periwinkle (*Catharanthus roseus*) (2 samples = 3%); tabebuia (*Tabebuia pallida*) (3 samples = 5%); Spanish lime (*Melicoccus bijugatus*) (5 samples = 8%); Ixora (*Ixora coccinea*) (3 samples = 5%); mango (*Mangifera indica*) (6 samples = 9%) and cactus (*Opuntia* sp.) (1 sample = 2%) (Figure 11).

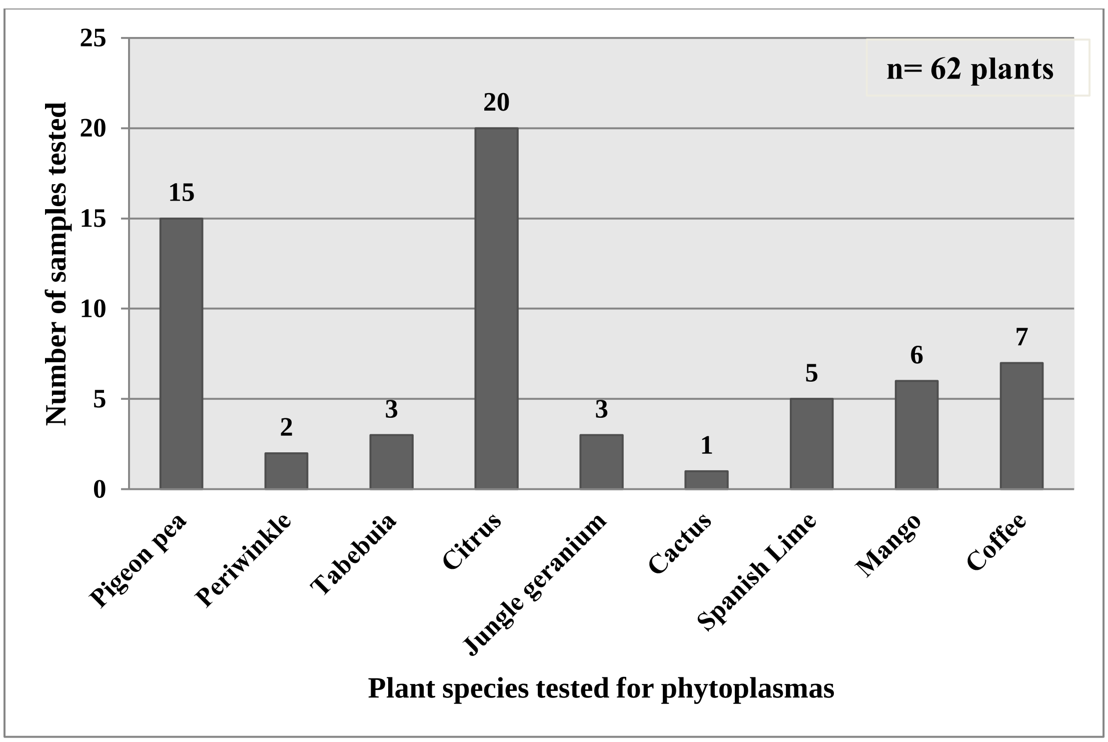

Figure 11. Total number of plant species examined for phytoplasma infection from 2012 to 2014.

4.2. PCR amplification and sequence analysis of 16S rDNA gene

4.2.1. First survey

From August to November 2012, PCR assays were carried out to detect phytoplasma infections using universal primers (P1/P7) and specific primers (fU5/rU3) for nested PCR. No amplifications were obtained from mango, cactus, Ixora and Spanish lime samples (Figure 12). These results suggested that the symptoms observed in the field might be caused by other agents or factors and not by phytoplasmas. In these plants few reports exist on phytoplasmas causing disease. In Egypt, Om-Hashem and El-Deeb (2007) used light and scanning electron microscopy (SEM) to examine cortical cells as well as parenchymatous cells of mango vascular tissues. Based on microscopy exclusively, they established that phytoplasmas were the causal agent of mango malformation in apical tissues. Recent reports identified two new species of *Fusarium subglutinans* as causal agents of mango malformation (Steenkamp *et al.*, 2002). In Yunnan Province, south-western China, Hong *et al.* (2008) reported a phytoplasma belonging to the PPWB phytoplasma group (16SrII) causing witches'-broom in cladodes of cactus (*Opuntia* spp.)

Periwinkle (*Catharanthus roseus*) plants showed typical symptoms associated with phytoplasma infection such as phyllody, big bud, virescent flowers, yellowing and little leaf. Periwinkle samples from different organs such as flowers, petioles and leaf midribs were positive for phytoplasma infections. Amplicons of 1.8 and 0.8kb were obtained using primer sets P1/P7 and fU5/rU3, respectively. DNA sequence analysis of PCR products showed 99% of homology with PPWB phytoplasma belonging to group 16SrIX (GenBank accession: AF248957).

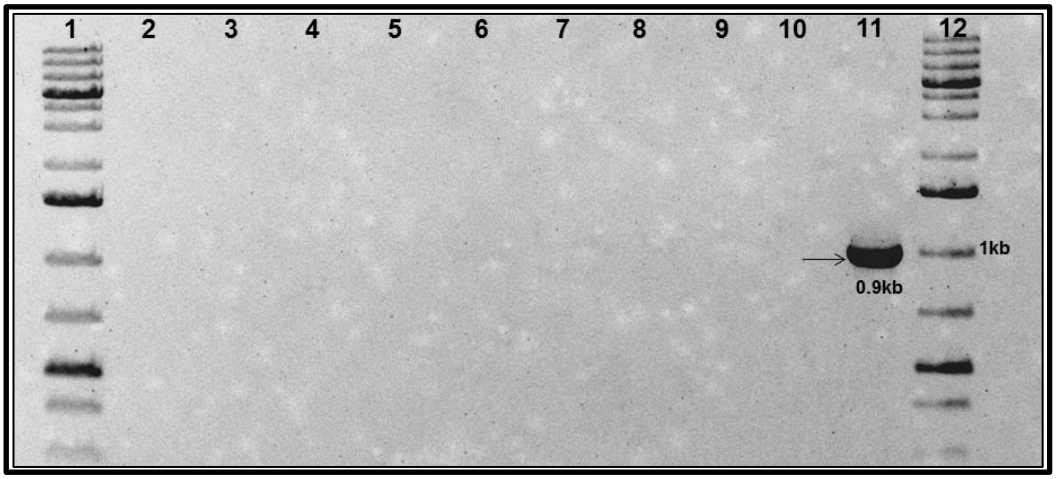

Figure 12. Nested PCR amplicons of the 16S rDNA gene on a 1% agarose gel. The region was amplified with primers fU5/rU3 to detect phytoplasma infections in different plant species. Lane 1 and 12, 1kb DNA ladder (Fermentas); 2: Negative control (water); 3 and 4: Mango (Ma) 3, 4; 5: Cactus (CR) 1; 6 and 7: Ixora (Ma) 1, 2; 8-10: Spanish lime (CR) 1, 2, 3; 11: Periwinkle (Ma). Locations: Ma = Mayagüez and CR = Cabo Rojo.

Periwinkle is a common host plant for phytoplasmas (Mancore *et al.,* 1997). It is easy to infect (mechanically or naturally) with a large number of phytoplasma species, producing very distinctive symptoms such as phyllody and significantly reduced leaf size (little leaf) (Nejat *et al.,* 2012). In Puerto Rico, this plant is commonly used in private gardens as an ornamental plant. We observed natural phytoplasma infections in periwinkle plants cultivated in private gardens in the Mayagüez area. According to our results, a phytoplasma belonging to group 16SrIX have been detected in Brazil in periwinkle collected in the state of Recife (Barros *et al.*, 2002). In tropical areas periwinkle plants may serve as a natural reservoir for this pathogen, spreading the disease to other plant species of economic importance such as citrus trees (Barbosa *et al.*, 2012).

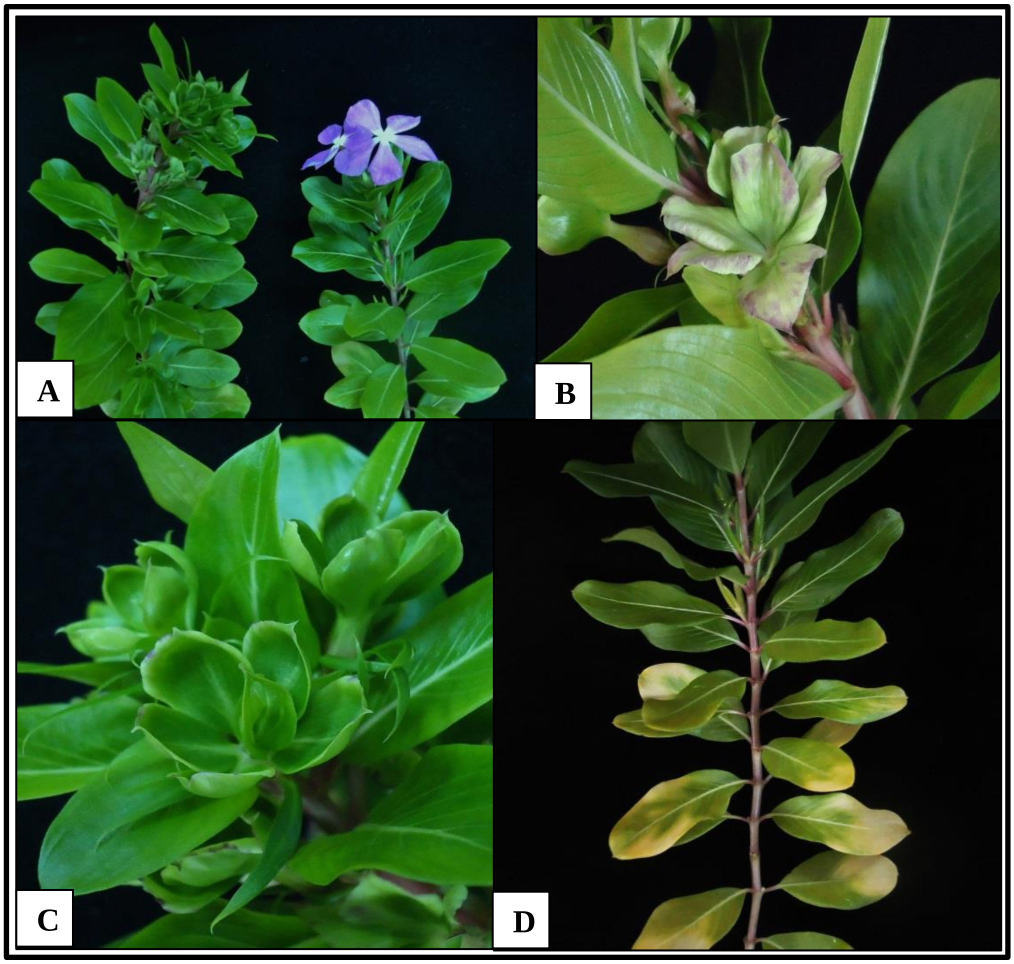

Figure 13. Common symptoms of phytoplasma infection observed in periwinkle (*Catharanthus roseus*) samples collected in Mayagüez, Puerto Rico. **A.** Infected (left) vs healthy (right) plant; **B.** Flower virescence; **C.** Phyllody; and **D.** Leaf mottling.

4.2.2. Second, third, fourth and fifth survey

4.2.2.1. Citrus samples

From January to May 2013, a total of 20 symptomatic citrus samples were collected from the UPR-Agricultural Research Stations located in the townships of Adjuntas, Corozal, Isabela and Juana Díaz. PCR assays using universal primers (P1/P7) and nested PCR using primers fU5/rU3 were carried out for all *Citrus* spp. samples including those from orange, lemon and mandarin. Plant tissues showed symptoms similar to citrus HLB disease. Leaves showing chlorosis (yellowing), blotchy mottling, and upright leaf deformations were tested (Figure 15). Twelve out of 20 samples (60%) were positive for phytoplasma infection producing amplicons of 0.8 kb, suggesting the presence of an unknown phytoplasma within the citrus trees. Only seven produced a large enough sequenced fragement for BLAST comparisons. PCR amplicons from samples collected at Corozal, Isabela, Juana Díaz and San Sebastián using BLAST showed 99% of identity with PPWB phytoplasma (PPWB) (GenBank accession: AF248957) (Figure 14).

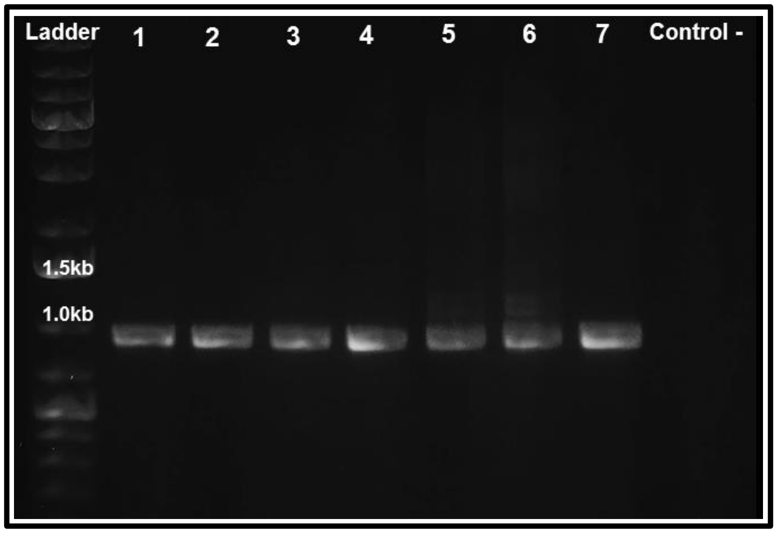

Figure 14. Nested PCR amplicons (0.8 kb) of the 16S rDNA gene on a 1% agarose gel. The region was amplified with primers fU5/rU3 used to detect phytoplasma infections in orange (*C. sinensis*), tangerine(*C. reticulata*) and lemon (*C. limon*). First lane: 1kb DNA ladder (Fermentas); 1-2: Lemon (Co) 11, 12; 3: Orange (Is) 40; 4: Orange (JD) 32; 5: Orange (SS) 28; 6: Orange (LM) 9; 7: Positive control (Periwinkle (Ma) 5); Last lane: Negative control (water). Locations: Co= Corozal; Is= Isabela; JD = Juana Díaz; SS= San Sebastián; LM = Las Marías; and Ma = Mayagüez.

Our findings agree with reports from Brazil where 109 of 117 samples (89%) of symptomatic sweet orange trees examined from 16 municipalities in the state of São Paulo belonged to the 16SrIX PPWB phytoplasma group (Teixeira *et al.*, 2008). Similar results were also reported from *Citrus* spp. (mandarin, sweet orange and pomelo) showing HLB-like symptoms (leaf yellowing and mottling) in six localities of Guangdong Province, China during a survey from October 2006 to 2007 (Chen *et al.,* 2008). Phytoplasma amplification products were detected using specific primers (P1/P7) and nested primers (fU5/rU3) in 70.8% of plants tested. *Chen et al.* (2008) identified a '*Candidatus* Phytoplasma asteris' which had been reported causing onion yellows in Japan, aster yellows 'watercress' in Hawaii and valeriana yellows in Lithuania. Further test confirmed mixed infection with '*Ca.* Liberibacter asiaticus', HLB causal agent. Their results showed that 69 (48.9%) samples were positive for both ‘*Ca*. P. asteris’ and ‘*Ca*. L. asiaticus’, indicating that the symptoms observed could be caused by the synergistic effects of both microorganisms. Both ‘*Ca*. P. asteris’ and ‘*Ca*. L. asiaticus’ have at least one feature in common and that is that they are strictly restricted to the phloem sieve tubes and could have the same mechanism of pathogenicity (Gaurivaud *et al.*, 2000). *Spiroplasma citri*, phytoplasmas and *C.* liberibacter (HLB), which are restricted to phloem sieves tubes, use fructose as primary source of carbohydrates. As a consequence, fructose concentration decreases with an increase of an invertase activity (enzyme that catalyzes the hydrolytic “breakdown” of sucrose), resulting in accumulation of glucose (André *et al.*, 2005). Fructose concentration remains low, invertase activity remains high, but glucose concentration increases, up to 20 times higher than that of healthy leaves. According to these authors, physiologically the accumulation of carbohydrates interferes with photosynthesis in the leaves causing chlorotic and mottling variegations, and a lower photosynthetic activity occurred in infected plants.

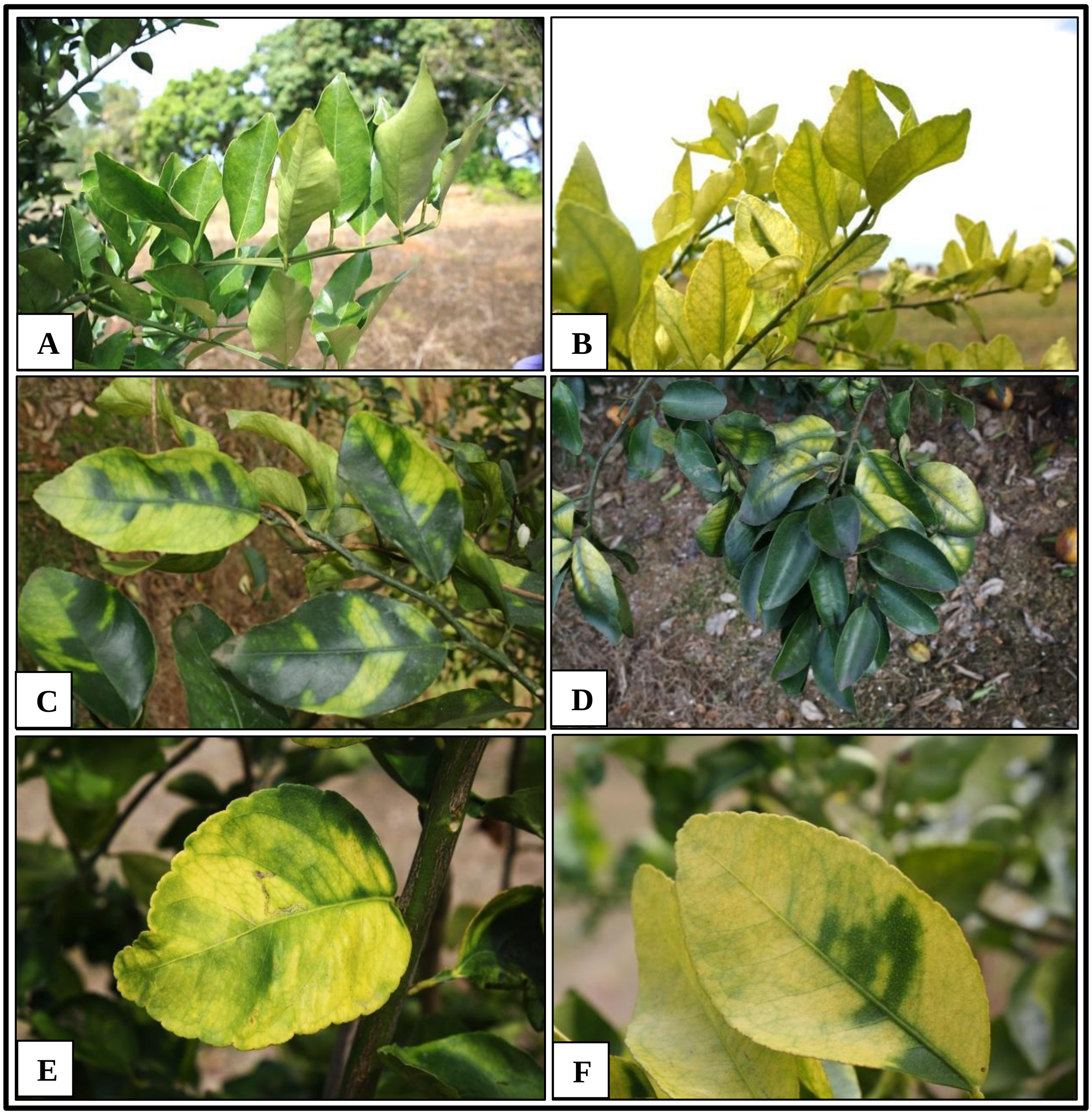

Figure 15. Common symptoms observed in *Citrus* spp. fields, and in samples collected from UPR-Agricultural Research Stations located in the townships of: Corozal, Isabela, San Sebastián, Las Marías and Juana Díaz. **A.** Upright leaves**; B.** Pronounced chlorosis **C.; D.; E.; and F.** Different degrees of mottling of leaves.

To confirm results obtained from positive citrus samples, a collection was conducted in June, 2013 of symptomatic trees corresponding to Lemon (Co) 11, 12; Orange (Is) 40; Orange (JD) 31, 32; Orange (SS) 28 and Orange (LM) 9. PCR assays were carried out with P1/P7 direct primers and fU5/rU3 nested primers. Amplicons from nested PCR with expected size of 0.8 kb were obtained in the following samples: Lemon (Co) 12 and Orange (LM) 9 (Figure 16). Samples corresponding to Lemon (Co) 11, Orange (Is) 40; Orange (JD) 31, 32; and Orange (SS) 28 were negative for phytoplasma infection and did not amplify using nested PCR assays (Figure 16).

Previous citrus samples were collected from February to March, 2013 at UPR-Agriculture Research Station at Isabela; during the survey average temperature and precipitation was 22.5 ^{o}C and 68.83 mm (NOAA, 2014), respectively. The second sampling was conducted in June 2013 with average temperature and precipitation was 27.7 ^{o}C and 127.5 mm (NOAA, 2014), respectively. The first sampling resulted in a 20% of positive samples for phytoplasma infection when tested by PCR. During the second sampling, to confirm results, no amplifications were detected in these samples taken from the same trees. This phenomenon may be explained by changes in plant physiology where phytoplasma concentration fluctuates in leaves (André *et al.,* 2005). Phytoplasma cells might be present in extremely low concentrations and could not be detected by nested PCR. Studies on periwinkle seedlings inoculated with jujube witches’-broom (JWB) phytoplasma via grafting, facilitate the understanding of the migration of JWB phytoplasma within the host tissues (Lee *et al.,* 2012). Results provided evidence that JWB phytoplasma migrate downward to roots along the main stem during the early stages of infection. They observed that the phytoplasma was able to reproduce in the roots and then moved upward, colonizing twigs along the stem until they reached the apex. Research focused on physiological changes occurring in palms infected with Coconut Lethal Yellowing determined that in roots with high phytoplasma concentration, photosynthetic rates and root carbohydrate concentrations decreased (Maus *et al.,* 2003). In contrast, leaf carbohydrate concentrations increased suggesting inhibition of sugar transport in the phloem leading to stress in sink tissues and development of visual symptoms of CLY. To date, knowledge of phytoplasma distribution within plant tissues is not well understood.

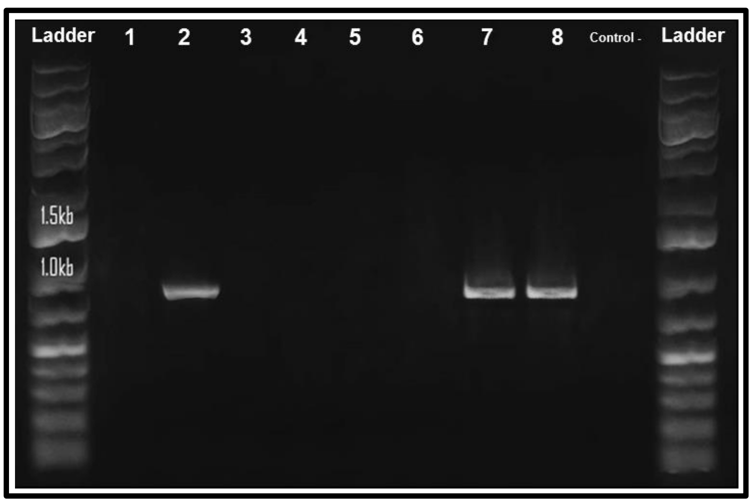

Figure 16. Nested PCR amplicons (0.8 kb) of the 16S rDNA gene on a 1% agarose gel The region was amplified with primers fU5/rU3 to detect phytoplasma infections in in orange (*C. sinensis*), tangerine(*C. reticulata*) and lemon (*C. limon*). First and last lane: 1kb DNA ladder (Fermentas); 1-2: Lemon (Co) 11, 12; 3: Orange (Is) 40; 4, 5: Orange (JD) 31, 32; 6: Orange (SS) 28; 7: Orange (LM) 9; 8: Positive control (Periwinkle (Ma) 5); 9: Negative control (molecular water). Locations: Co= Corozal; Is= Isabela; JD = Juana Díaz; SS= San Sebastián; LM = Las Marías; and Ma = Mayagüez.

4.2.2.2. Citrus samples (*Spiroplasma citri*)

Citrus samples showing HLB associated symptoms such as leaves with yellowing and blotchy mottling, and upright leaves were tested for *Spiroplasma citri* infection. Twenty samples were analyzed using primer set ScR16F/ScR16R1 for direct PCR and ScR16F1A/ScR16R2 primer set for nested PCR. Six out of 20 samples examined from orange and lemon produced amplicons of expected size (1.2 kb) (Figure 17). Using BLAST DNA sequences were identified as *Sphingomonas phyllosphaerae*, a common bacteria occurring in terrestrial and water habitats, plant root systems, clinical specimens, and other sources (GenBank accession: AM989065); *Pseudomonas saccharophila,* another bacterium found in soil, marshes, coastal marine habitats, and plant and animal tissue (GenBank accession: AB819482); *Terriglobus* spp., a widely distributed bacterial genus in nature and abundant in soils (GenBank accession: NR_074294); and four strains of uncultured bacteria (possibly endophytic bacteria). We could not identify spiroplasmas related with symptoms observed in citrus fields. Our results agree with Teixeira *et al.* (2008) in which by using fD1/rP1 primers on symptomatic citrus samples they identified *Sphingomonas phyllosphaerae* and *Pseudomonas saccharophila*. The findings suggested that these bacterial genera are common in the citrus plant environment and the primers are not specific to *Spiroplasma citri* detection. There are two reports describing the occurrence of diseases caused by *Spiroplasma citri* in important crops. Stubborn disease is caused by *S. citri* in citrus (Yokomi *et al.*, 2008). In carrots, *S. citri* causes yellow and bronze foliage discoloration and formation of secondary taproots (Lee *et al.*, 2006).

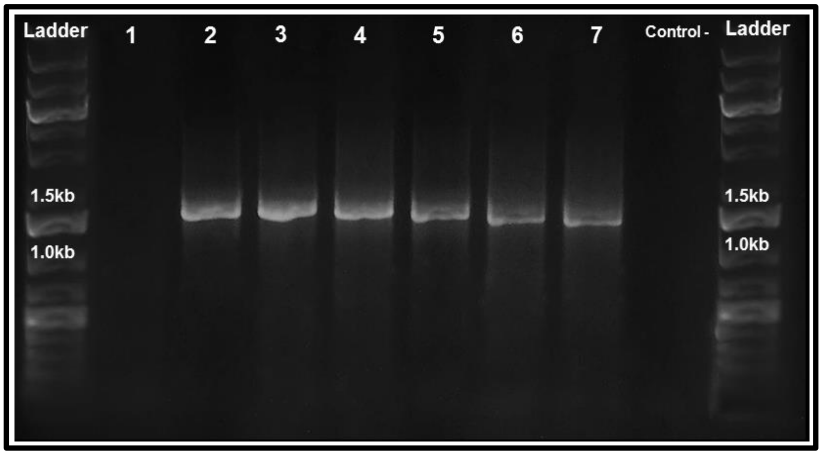

Figure 17. Nested PCR amplicons (1.5 kb) of the 16S rDNA gene on a 1% agarose gel. The region was amplified with primers ScR16F1A/ ScR16R2 specific for *Spiroplasma citri*. First and last lane: 1kb DNA ladder (Fermentas); 1 and 2: Lemon (Co) 16, 17; 3, 4 and 5: Orange (Is) 39, 40, 41; 6 and 7: Orange (JD) 34, 35; 8: Negative control (water). Locations: Co= Corozal; Is= Isabela; and JD = Juana Díaz.

4.2.2.3. Pigeon pea samples

Fifteen pigeon pea samples were collected at UPR Agricultural Research Stations during 2013. Plant exhibited witches'-broom symptoms in which little leaf and proliferating shoots were observed in branches (Figure 19). PCR assays were carried out using direct P1/P7 and nested fU5/rU3 primers. All samples tested from Juana Díaz and Isabela were positive producing amplicons of 1.8 and 0.8 kb, respectively (Figure 18). DNA sequences were identified by BLAST, showing a 99% homology to PPWB phytoplasma belonging to 16SrIX group (GenBank accessions: HQ423159 and AF248957). Specimen of sample Pigeon pea (JD) 33 was confirmed positive for PPWB phytoplasma by the USDA Molecular Plant Pathology Laboratory in Beltsville, Maryland and was used as positive control in subsequent analysis.

Proliferating shoots and witches'-broom were typical symptoms observed in the field in mature pigeon pea plants at the UPR Agricultural Research Station located in Isabela, PR. (Bósques Vega, Researcher, personal communication). In Puerto Rico pigeon pea fields are replanted every eight months in one production cycle, therefore it is rare to observe these symptoms in commercial farms. Our studies confirmed findings by Licha (1979) who observed MLO's using TEM in ultrathin sections of petioles from pigeon pea plants severely affected with witches'-broom disease and bushy canopy. The author concluded that the disease was caused by an unknown MLO. In the same survey the author observed leafhoppers of the genus *Empoasca* spp. on all pigeon pea plants collected. By mechanical transmission trials with *Empoasca* spp. the severity of the disease in pigeon pea plants was determined. Licha (1979) concluded that high populations of *Empoasca* spp. (>10 insects in young plant) cause severe bushy canopy and possibly witches'-broom symptoms, thus confirming *Empoasca* spp. as an insect vector and potential transmitter of PPWB phytoplasma.

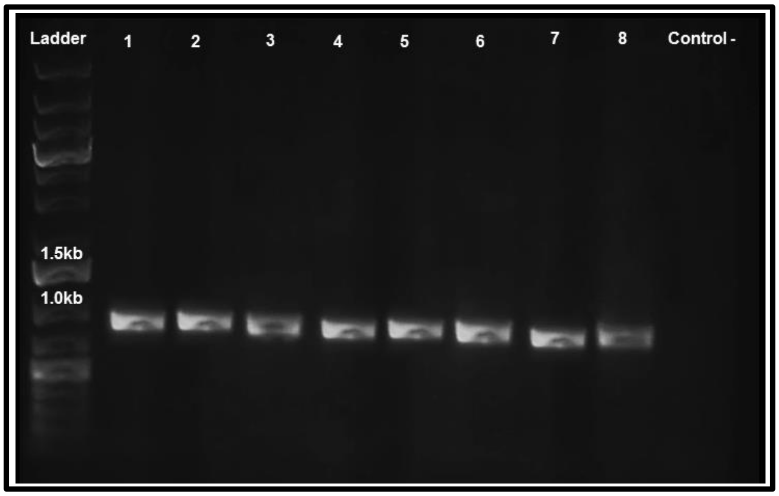

Figure 18. Nested PCR amplicons (0.8 kb) of the 16S rDNA gene on a 1% agarose gel. The region was amplified with primers fU5/rU3 for phytoplasma. First lane: 1kb DNA ladder (Fermentas); 1, 2, 3, 4 and 5: Pigeon pea (Is) 41, 42, 43, 44, 45; 6 and 7: Pigeon pea (JD) 32, 33; 8: Positive control (Periwinkle (Ma) 5); Last lane: Negative control (water). Locations: Is= Isabela; JD = Juana Díaz and Ma = Mayagüez.

In 1983 in Homestead Florida, McCoy *et al.* (1983) reported the presence of a mycoplasma within pigeon pea plants causing witches' broom-like symptoms in branches, in addition to stunting, colorless florets and proliferating shoots. TEM was used to examine ultrathin sections of petioles for phytoplasma detection. Polymorphic structures related to MLO's were observed in plant tissue sections. The authors identified two commons leafhoppers: *Empoasca plebeia* and *Acinopterus* sp. and proposed them as potential insect vectors of the MLO. Unfortunately, no transmission experiments were conducted with the insects mentioned above.

Another common host for PPWB phytoplasma is gliricidia (*Gliricidia sepium*) (Kenyon *et al.,* 1998). In 2008 in Honduras, PCR reactions were carried out using P1/P7 primers for direct PCR and PPf1/Tint primers for nested PCR from tissues collected from young symptomatic trees showing little leaves (Kenyon *et al.,* 1998). Positive samples for phytoplasmas produced amplicons of 1.8 kb. By RLFP patterns and DNA sequencing of the PCR products, the researchers identified a phytoplasma that was very similar to PPWB phytoplasma (16SrIX group).

Recently, PPWB phytoplasma infecting annual phlox (*Phlox drummondii*) was reported at the Indian Agricultural Research Institute Campus, New Delhi (Madhupriya and Khurana, 2013). Symptoms included extensive yellowing and stunting, proliferation of shoots, little leaves and reduced flower size. Using specific primers for phytoplasma detection (P1/P7) and nested PCR primers (R16F2n/R16R2) to amplify 16S rDNA gene an amplicon of 1.2 kb the authors sequenced and performed a BLAST analysis. Results showed 99% identity with phytoplasma members of group 16SrIX (PPWB phytoplasma).

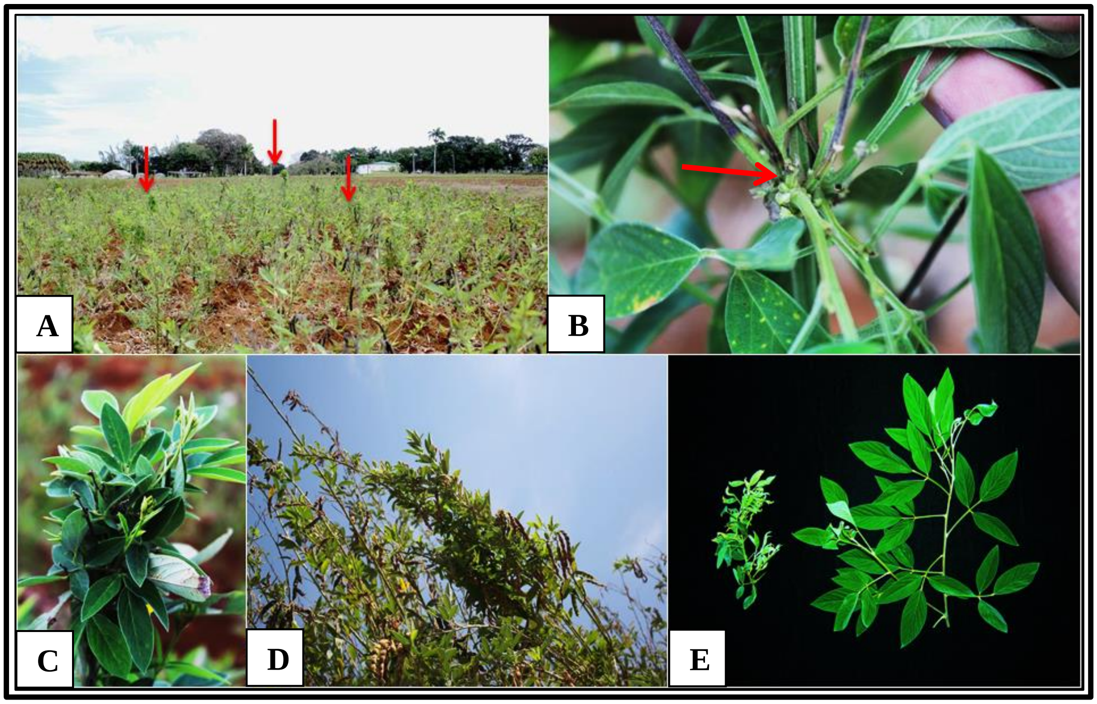

Figure 19. Common symptoms associated with PPWB disease observed in pigeon pea (*Cajanus cajan*) samples collected at the UPR-Agricultural Research Stations located in Isabela (Is) and Juana Díaz (JD), PR. **A.** Field showing apical proliferation (red arrows); **B.** Branch proliferation detail; **C.** Shoot proliferation; **D.** Apical proliferating shoots; **E.** Little leaf (left) and healthy plant (right).

4.2.2.4. Coffee, tabebuia and Ixora samples

Witches'-broom and crispiness (curled leaves and massive vegetative growth that results in the branches) were observed in coffee samples collected during 2013 at the UPR Agricultural Research Station in Adjuntas, PR. (Figure 21). PCR using P1/P7 direct and fU5/rU3 nested primers of DNA extracted from infected coffee petioles produced amplicons of 0.8 kb in five out of seven samples collected (Figure 20). PCR products corresponding to samples Coffee (Ad) 22 and 25 were sequenced and analyzed by BLAST. Samples showed 99% and 98% of identity with PPWB phytoplasma strain PPWBja from Japan (GenBank accession: HQ423159) and *Candidatus* Phytoplasma phoenicium strain

PwK-CP3 (GenBank accession: JN792516), respectively. Regarding the other coffee samples (21, 23 and 24) the DNA concentration of purified PCR products was not high enough for sequencing (concentration from 5 to 7 ng/µl).

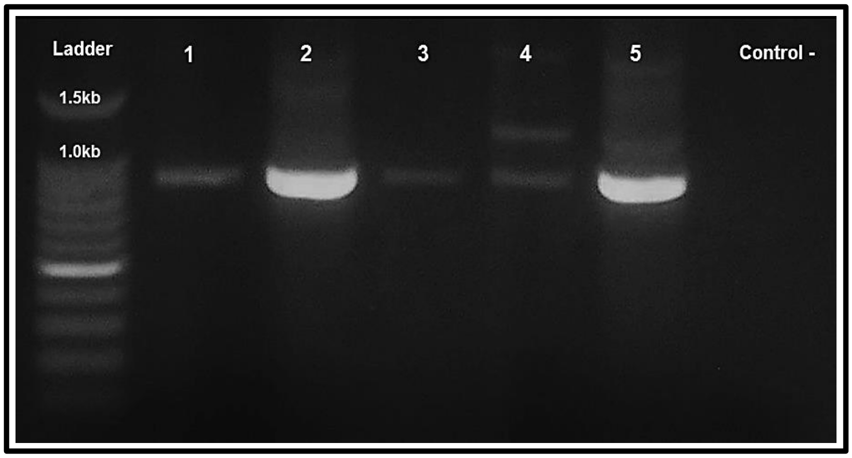

Figure 20. PCR products (0.8 kb) of the 16S rDNA gene generated by direct amplification using the P1/P7 primer combination followed by nested amplification using the fU5/rU3 primer combination. Amplification products were separated 1% agarose gel and visualized with GelRed nucleic acid stain. Lane 1: 1 kb DNA ladder (Promega); lanes 1-5: Coffee (Ad) 21, 22, 23, 24, 25; lane 6: negative water control. Location: Ad = Adjuntas.

In a five-year-old coffee plantation in Bogotá, Colombia, plants showed proliferation, abundant short and narrow leaves, phyllody, floral abortion, monospermic fruit, and dwarfism indicating phytoplasma infections (Galvis *et al.,* 2007). By DNA analysis of a partial sequence of 914 bp of the 16S rDNA obtained using primer pairs P1/P7 followed by fU5/rU3, a new strain member of group 16SrIII (X-disease group) was identified. Galvis et al. (2007) suggested the presence of potential insect vectors (Family: Cicadellidae) that can disseminate the disease to other trees. Our report of the association of crispiness and witches'-broom of coffee with PPWB phytoplasma is a new report and needs further testing for validation.

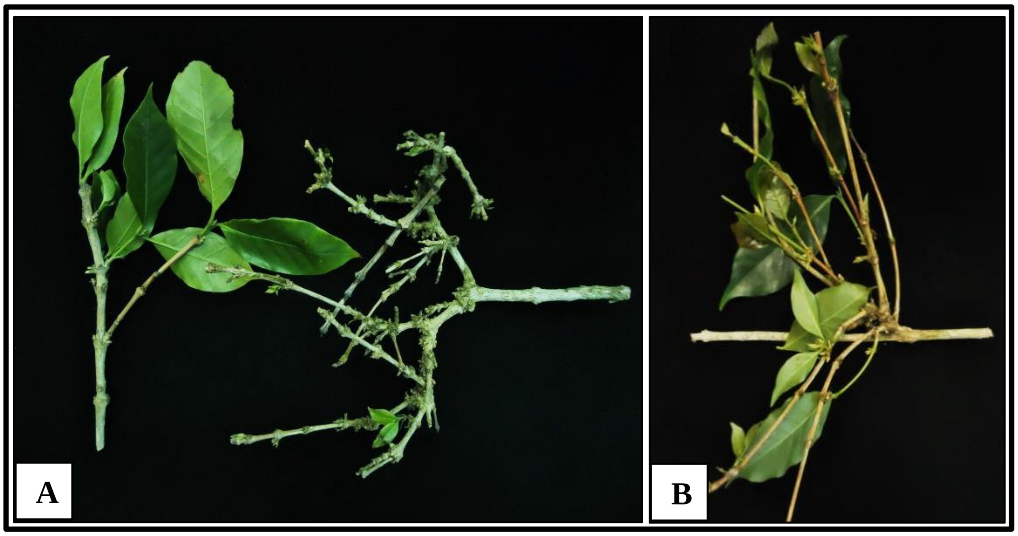

Figure 21. Witches'-broom symptoms observed in the field in coffee samples collected at the UPR-Agricultural Research Stations in Adjuntas, PR. **A.** Coffee (Ad) 22**;** and **B.** Coffee (Ad) 25. Location: Ad= Adjuntas.

Ixora coccinea and *Tabebuia pallida* samples with witches'-broom and proliferating shoot symptoms, respectively were collected on the UPR-Mayagüez campus gardens, Mayagüez, PR. (Figure 23). PCR reactions were carried out with P1/P7 primers in a first amplification and followed by nested PCR with R16F2n/R16R2 primers. Amplicons of 1.2 kb were obtained from two tabebuia samples. PCR products from symptomatic tabebuia samples were sequenced and analyzed by BLAST. DNA sequences had higher homology (99%) with PPWB phytoplasma strain PPWBja (GenBank accessions: EF186825 and AF248957). No amplifications were obtained from three *I. coccinea* samples examined; indicating that proliferation of shoots could be caused by another causal agent.

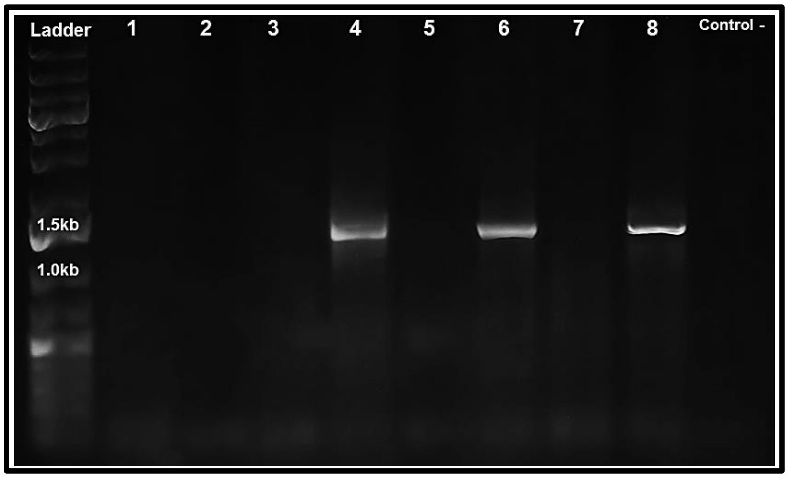

Figure 22. Nested PCR amplicons (1.2 kb) of the 16S rDNA gene in a 1% agarose gel. The region was amplified with primers R16F2n/R16R2 for phytoplasma detection in tabebuia (*Tabebuia pallida*) and ixora samples (*Ixora coccinea*). First lane: 1kb DNA ladder (Fermentas); 1-3: Ixora (Ma) 46, 47, 48; 4-7: Tabebuia (Ma) 2, 3, 4, 5; 8: Positive control (Periwinkle (Ma) 5); Last lane: Negative control (water). Location: Ma = Mayagüez.

Cook (1938) and Ciferri (1949) reported witches'-broom disease in tabebuia (*T. pallida*) occurring in Puerto Rico and Venezuela, respectively. At that time the lack of technology prevented the identification of the microorganisms restricted to the phloem. Based on symptoms, Cook proposed a virus as the causal agent of the disease. More recently in 2007, similar symptoms were observed in *T. pentaphylla* trees in Rio de Janeiro, Brazil (Mafia *et al.,* 2007). By PCR and sequence analyses the phytoplasma was identified with 98% of similarity to *Ca.* Phytoplasma aurantifolia belonging to 16SrII group isolate from lime (*Citrus aurantifolia*) (GenBank accession: U15442). In addition, Mafia *et al.* (2007) examined ultrathin sections of leaf petioles of *T. pentaphylla* with fluorescence and TEM microscopy, observing typical phytoplasma cells of spherical to ovoid shapes with inconsistent sizes.

Results in the present research differed from findings reported by Mafia *et al.*,(2007) from Brazil in which PPWB phytoplasma is reported associated with a disease called witches'-broom of tabebuia caused by *Ca.* Phytoplasma aurantifolia. Future studies are necessary to clarify phytoplasma identity and to study potential insect vectors related to disease dissemination.

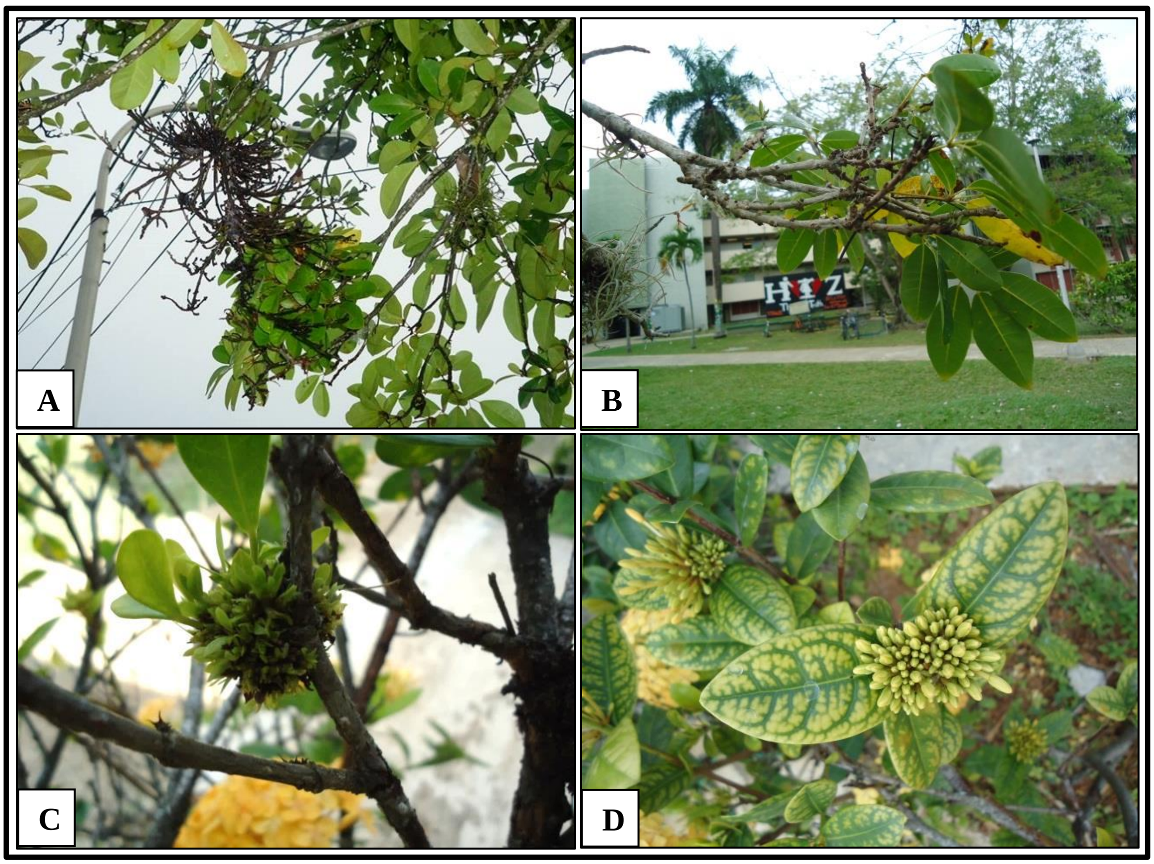

Figure 23. Common symptoms associated with phytoplasma infections observed in *T. pallida* and in *I. coccinea*. **A.** and **B.** Witches'-broom in *Tabebuia pallida* trees; **C.** Shoot proliferation in *I. coccinea* and **D.** Chlorotic variegation in leaves in *I. coccinea.*

4.3. Ribosomal protein (rp) gene amplifications

Ribosomal protein genes: *rpIV* (*rpl22*) and *rpsC* (*rps3*) have proven to be a useful in differentiating and classifying phytoplasmas strains. These genes have help resolve phytoplasmas strains which are biologically and ecologically distinct and that cannot be distinguished by analysis of the 16S rDNA gene alone (Martini *et al.*, 2007). In our studies, a DNA fragment of expected size (1.2 kb) was obtained using primers pair rpL2F3/rp(I)R1 from genomic DNA of positive samples for phytoplasma obtained by 16S rDNA analysis. However, amplification of ribosomal protein (rp) genes in some samples did not work due to low phytoplasma DNA concentration. It is important to point out that fewer copies of these genes are present in the genome compared to 16S rDNA. Samples corresponding to Periwinkle (Ma) 5, Orange (JD) 32, Pigeon pea (Is) 43 and Tabebuia (Ma) 2 produced amplicons of expected size, confirming positive samples for phytoplasma detection (Figure 24). PCR products were sequenced and analyzed by BLAST, sequences showed 99% homology to PPWB phytoplasma from Puerto Rico (strain PPWBpr and GenBank accession No.: EF183497) and to PPWB phytoplasma from Florida (strain PPWBfl and GenBank accession No.: EF183495).

Ribosomal protein gene-based phylogeny allowed the classification and differentiation of various members of the class *Mollicutes*, including 46 phytoplasma strains, representing 12 phytoplasmas of the 16Sr groups (Martini *et al.,* 2007). By analysis of the two rp genes (*rpIV* (*rpl22*) and *rpsC* (*rps3*)), the PPWB phytoplasma group formed a distinct subclade (with a consistent bootstrap of 100). This group included three strains of PPWB phytoplasma from Florida (strain PPWBfl and GenBank accession: EF183495), Puerto Rico (strain PPWBpr and GenBank accession: EF183497), and Jamaica (strain PPWBja and GenBank accession: EF183496); Honduran *Gliricidia* little leaf phytoplasma (GenBank accession: EF186800); *Rhynchosia* little leaf from Florida (GenBank accession: EF186799). The subclade also included another two phytoplasmas from Italy, *Picris echioides* phyllody phytoplasma strain PEY (GenBank accession: EF186802) and *Knautia arvensis* phyllody phytoplasma strain KAP (GenBank accession: EF186801). This clade grouped similar to 16S rDNA gene in which all belonged to group IX, using ribosomal genes these phytoplasmas grouped with subclade VI. Other genes such as *secY* and *tuf* can serve as additional phylogenetic tools for finer classification of groups and subgroups within a given 16Sr phytoplasma group (Martini *et al.*, 2007).

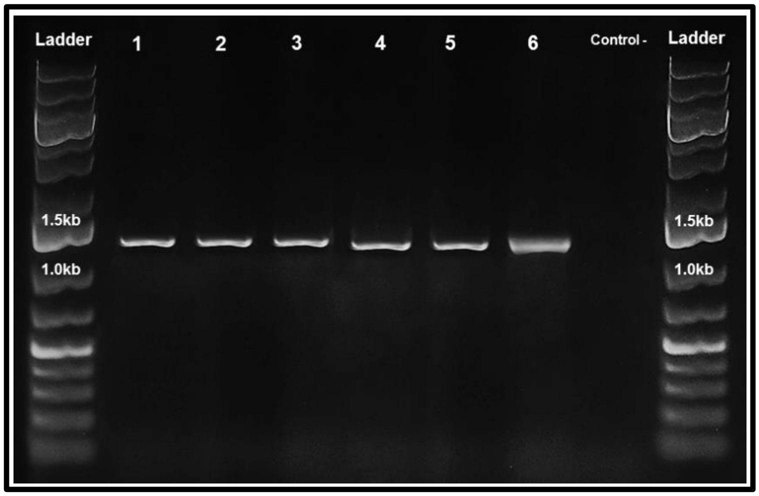

Figure 24. Nested PCR amplicons (1.2 kb) of two ribosomal protein genes *rpIV* (*rpl22*) and *rpsC* (*rps3*) in a 1% agarose gel. The region was amplified with primers rpL2F3/rp(I)R1A for phytoplasma detection. First and last lane: 1kb DNA ladder (Fermentas); 1 and 2: Periwinkle (Ma) 5; 3: Orange (JD) 32; 4: Pigeon pea (Is) 43 and 5: Tabebuia (Ma) 2; 6: Positive control (Pigeon pea (JD) 33); Last lane: Negative control (water). Locations: Is= Isabela; JD = Juana Díaz; and Ma = Mayagüez.

4.4. PPWB phytoplasma insect vectors

Once phytoplasmas were identified in their host plants, populations and abundance of potential insect vectors were studied. Leafhoppers were sweep-collected and tested for phytoplasma infection in symptomatic citrus and pigeon pea trees. Insects were sampled in four sites located at: Isabela, San Sebastián, Adjuntas and Juana Díaz, Puerto Rico. Common insects families collected were Cicadellidae (*Empoasca kraemeri* and *Agallia* sp.), Flatidae (*Melornemis antillarum* and *Flatornemis* sp.), Nogodinidae (*Colpoptera maculifrons*), Cixiidae (*Oliarus complectus* and *Bothriocera* sp.), Derbidae (*Omolicna puertana*) and Psyllidae (*Diaphorina citri*). Insect families Cicadellidae, Flatidae, Nogodinidae and Psyllidae represented 87% of the specimens collected (Figure 25; Table 7). According to Beanland (1998), these insect families can represent the principal mechanism to transmit the phytoplasmas.

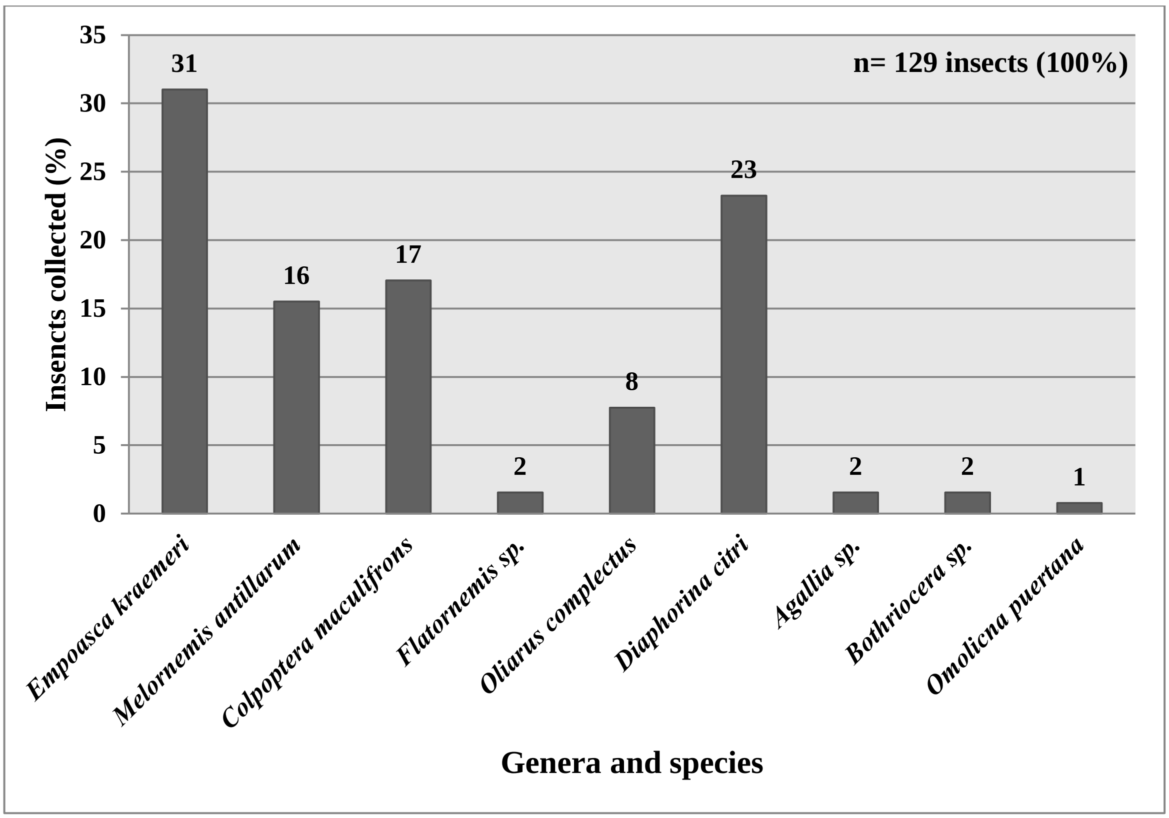

Figure 25. Percentage of insect species collected in the field and tested for phytoplasma infection from 2012 to 2014.

A total of 129 insects were collected belonging to seven families. From these families five specimens were evaluated for phytoplasma infection. Only *Empoasca kraemeri* and *Melornemis antillarum* collected near pigeon pea plants at Juana Díaz and San Sebastián, and *Colpoptera maculifrons* collected near citrus orange at Adjuntas, were positive for phytoplasma infections. All specimens produced amplicons of 0.8kb with primer set fU5/rU3 for nested PCR (Figure 27). No amplifications were obtained with other insects specimens examined. PCR products were sequenced and analyzed by BLAST. All three insect specimens' sequences showed 99% of identity with PPWB phytoplasma belonging to 16SrIX group (GenBank accessions: AF248957 and HQ423159) confirming *Empoasca kraemeri, M. antillarum* and *C. maculifrons* as potential vectors of PPWB phytoplasma. Transmission assays are needed to confirm their role in phytoplasma disease cycles.

Worldwide the role of insect vectors, especially leafhoppers, in phytoplasma dissemination has not been fully studied. Scientific literature described and classified several insect species as vectors of a diverse group of phytoplasmas (Nielson, 1979 and 1985). Insects are able to acquire and transmit equally phytoplasmas from different infected plant species. For example, *Euscelidius variegatus, Macrosteles quadripunctulatus,* and *Euscelis incises* are able to acquire from and transmit chrysanthemum yellows phytoplasma to chrysanthemum plants (Weintraub and Beanland, 2006). Most insect vector species belong to four families of fulgorids: Cixiidae, Delphacidae, Derbidae, and Flatidae. We found representative populations of insects belonging to these families, being *Melornemis* *antillarum* and *Colpoptera maculifrons* new reports for Puerto Rico as potential insect vectors of phytoplasmas, specifically, of PPWB phytoplasma.

In Sao Paulo, Brazil, potential leafhopper vectors of HLB-associated phytoplasma were identified using sticky yellow traps placed at different heights in the citrus tree canopy and by net sweeping of ground vegetation near sweet oranges (Marques *et al.*, 2013). They collected leafhoppers belonging to families Cicadellidae (i.e., *Agallia albidula* Uhler) and Deltocephalinae (i.e., *Scaphytopius* [*Convelinus*] *marginelineatus*) in weeds and the influence of weed species composition on leafhopper abundance in low-lying vegetation. DNA analysis of *S. marginlineatus* collected near the weeds, *Sida rhombifolia* L. and *Althernantera tenella* close to citrus orange plantations were positive for phytoplasmas. Marques *et al.* (2013) detected amplicons of expected size (0.8 kb) which were obtained using specific primer for phytoplasma detection. PCR products were sequenced and analyzed by BLAST indicating that *S. marginlineatus* might be a potential vector of HLB-associated phytoplasma reported by Teixeira *et al.* (2008), which relates to PPWB phytoplasma.

Table 7. Leafhopper genera and species collected by net sweeping near pigeon pea (*Cajanus cajan*) and sweet orange (*Citrus sinensis*) trees at Adjuntas, Isabela, San Sebastián and Juana Díaz, PR.

Binomial name	Family	# collected	% collected	Sampling sites
Empoasca kraemeri	Cicadellidae	40	31	Juana Díaz[1] and San Sebastián[2]
Melornemis antillarum	Flatidae	20	16	Juana Díaz and Isabela[1]
Colpoptera maculifrons	Nogodinidae	22	17	Adjuntas[1]
Flatornemis sp.	Flatidae	2	2	Adjuntas
Oliarus complectus	Cixiidae	10	8	San Sebastián
Diaphorina citri	Psyllidae	30	23	Juana Díaz and Isabela
Agallia sp.	Cicadellidae	2	2	Adjuntas
Bothriocera sp.	Cixiidae	2	2	Adjuntas
Omolicna puertana	Derbidae	1	1	San Sebastián

[1] UPR- Agricultural Research Station
[2] Private Farm located at San Sebastián, P.R.

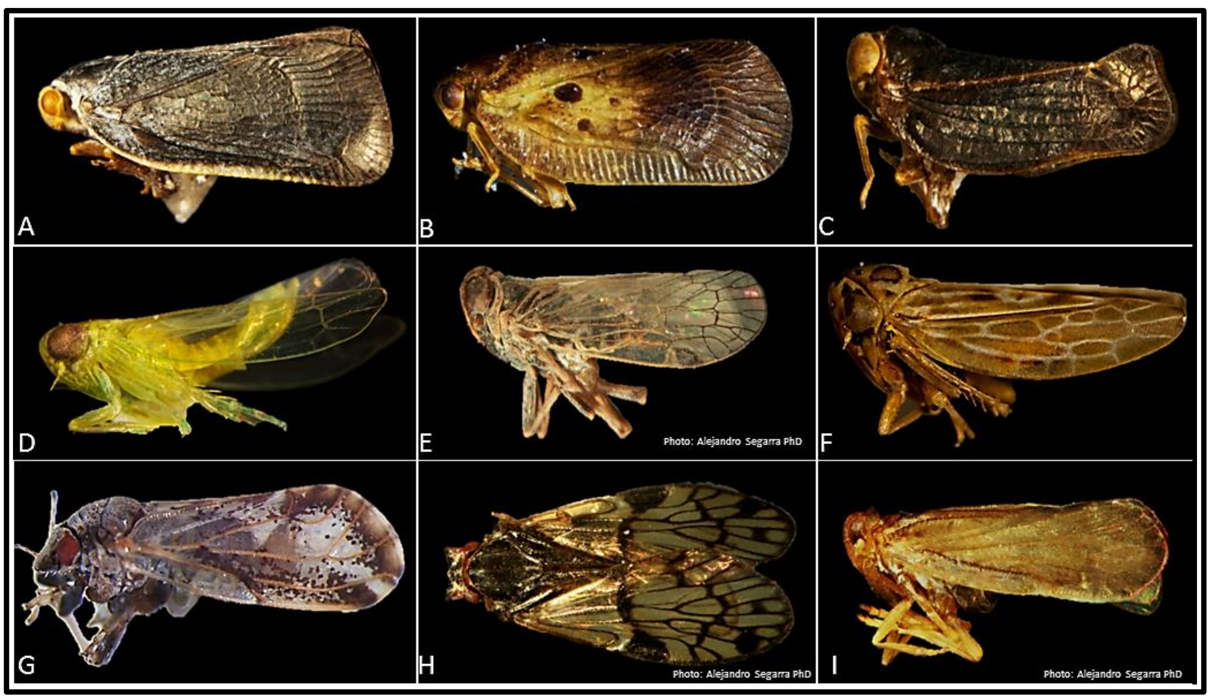

Figure 26. Leafhopper species collected by net sweeping near pigeon pea (*Cajanus cajan*) and sweet orange (*Citrus sinensis*) trees at Adjuntas, Isabela, San Sebastián and Juana Díaz, Puerto Rico from 2012 to 2014. **A.** *Flatornemis* sp. (Fam. Flatidae); **B.** *Melornemis antillarum* (Fam. Flatidae); **C.** *Colpoptera maculifrons* (Fam. Nogodinidae); **D.** *Empoasca kraemeri*. (Fam. Cicadellidae); **E.** *Oliarus complectus* (Fam. Cixiidae), **F.** *Agallia* sp. (Fam. Cicadellidae); **G.** *Diaphorina citri* (Fam. Psyllidae); **H.** *Bothriocera* sp. (Fam. Cixiidae); **I.** *Omolicna puertana* Caldwell (Fam. Derbidae).

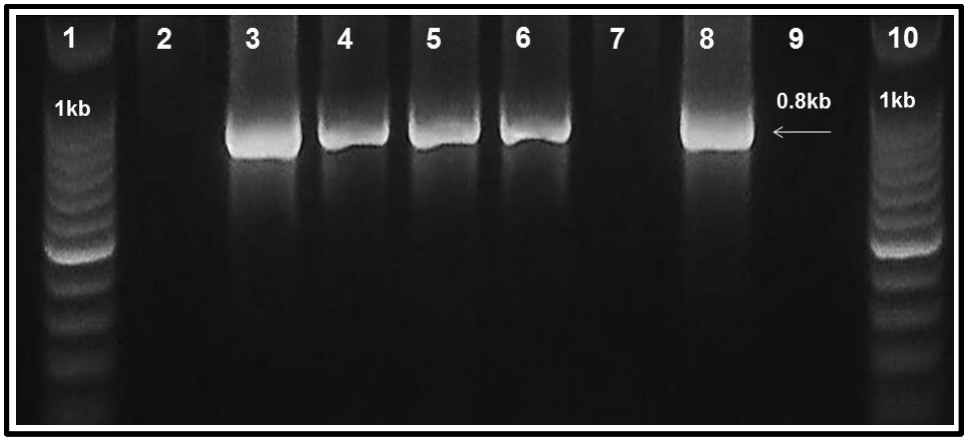

Figure 27. Nested PCR amplicons (0.8 kb) of 16S rDNA gene in a 1% agarose gel. The region was amplified with primers fU5/rU3 for phytoplasma detection. First and last lane: 1kb DNA ladder; 2: *Flatornemis* sp. 3: *Empoasca kraemeri* (Juana Díaz); 4: *Empoasca kraemeri*. (Isabela); 5: *Melornemis antillarum*; 6: *Colpoptera maculifrons*; 7: *Diaphorina citri*; 8: Pigeon pea (JD) 33 Positive control; 9: Negative control (water).

In Puerto Rico Martorell reported two *Scaphytopius* species, *S. fuliginosus* and *S. neoloricatus* on wild beans (*Phaseolus* spp). *Scaphytopius marginlineatus* has not been reported in the island (Martorell, 1976). We focused our insect collections near citrus trees (orange and lemon) and pigeon pea plants. In Brazil, Teixeira *et al.* (2008) collected near weeds close to the citrus plantations. We provided basic information for future studies correlating phytoplasma infection with insect populations on weeds and on citrus trees and pigeon pea plants on the island.

4.5. Phylogeny of 16S rDNA, *rpIV* (*rpl22*) and *rpsC* (*rps3*) genes

Through primer walking a complete contig of 16S rDNA gene was assembled using primers P1/P7, R16F2n/R16R2, fU5/rU3 and fU5/p7. Phylogenetic trees were constructed using complete sequences of 16S rDNA gene from various samples: Orange (JD) 32 and (Is) 40, Pigeon pea (JD) 33, (Is) 43, 44 and 45, *Tabebuia* (Ma) 2 and 3, Periwinkle (Ma) 5, Periwinkle little leaf (Ma) 6, Periwinkle yellowing (Ma) 7, *Empoasca* spp. (Ma), *Melornemis antillarum* (JD) and *Colpoptera maculifrons* (Ad). In addition, thirty-seven representative phytoplasma strains belonging to different groups or clades were obtained from GenBank (Table 5). Phylogenetic tree generated by MEGA showed that all phytoplasma samples detected in periwinkle, citrus, pigeon pea, tabebuia, coffee and in insects grouped closed to the 16SrIX clade defined by Lee *et al.* (2007) (Figure 28). This confirmed that symptoms observed in these plants are associated with the presence of PPWB phytoplasma which appeared to prevail in the island.

We were able to relate symptomatic tabebuia sample with the phytoplasma found in the leafhopper, *Empoasca kraemeri*, because it formed one subclade within the IX group clade. Based on these results, there might be a relationship between *Empoasca* spp. as potential vector of PPWB phytoplasma to tabebuia trees. Most known phytoplasma vectors belong to the Cicadellidae family which includes *Empoasca* spp. Galetto *et al.* (2011), studied the ability of *Empoasca decipiens* Paoli in transmitting chrysanthemum yellows phytoplasma (CYP, "*Ca.* Phytoplasma asteris" classified in group 16SrI-B) and Flavescence dorée phytoplasma (FDP), classified in group 16SrV-C, to *Chrysanthemum carinatum* Schousboe (tricolor daisy) and *Vicia faba* (L.) (broad bean). Results showed that *Empoasca decipiens* had low capability of transmitting CYP, since the microorganism was found in low concentrations in their salivary glands, indicating these organs represent a barrier for phytoplasma colonization.

In addition, a phylogenetic tree was construted with sequences of *rpIV-rpsC* genes from four symptomatic samples: Periwinkle (Ma) 5, citrus Orange (JD) 32, Pigeon pea (Is) 43 and Tabebuia (Ma) 2. All grouped in one clade with phytoplasmas belonging to group VI defined by Lee *et al.* (2007) (Figure 29). These genes are polymorphic compared to 16S rDNA gene and serve as a good phylogenetic parameter and allow to differentiate and classify several microorganisms including phytoplasmas.

Disparity index per site for all sequence pairs obtained from 16S rDNA gene sequences were examined (Table 8). Our results recorded values ranging from 0.00 to 0.02, indicating that the differences in base composition biases based on evolutionary divergence is lower, suggesting that there are no significant evolutionary changes between sequences analyzed (Kumar and Gadagkar, 2001). Similarly, disparity index obtained from sequence analysis of *rpIV* (*rpl22*) and *rpsC* (*rps3*) genes showed that there are no significance differences in base composition bases based on evolutionary divergence (Table 9). Index values ranged from 0.00 to 0.08, suggesting that these genes are more evolutionarily variable than 16S rDNA gene (Tamura *et al*., 2012).

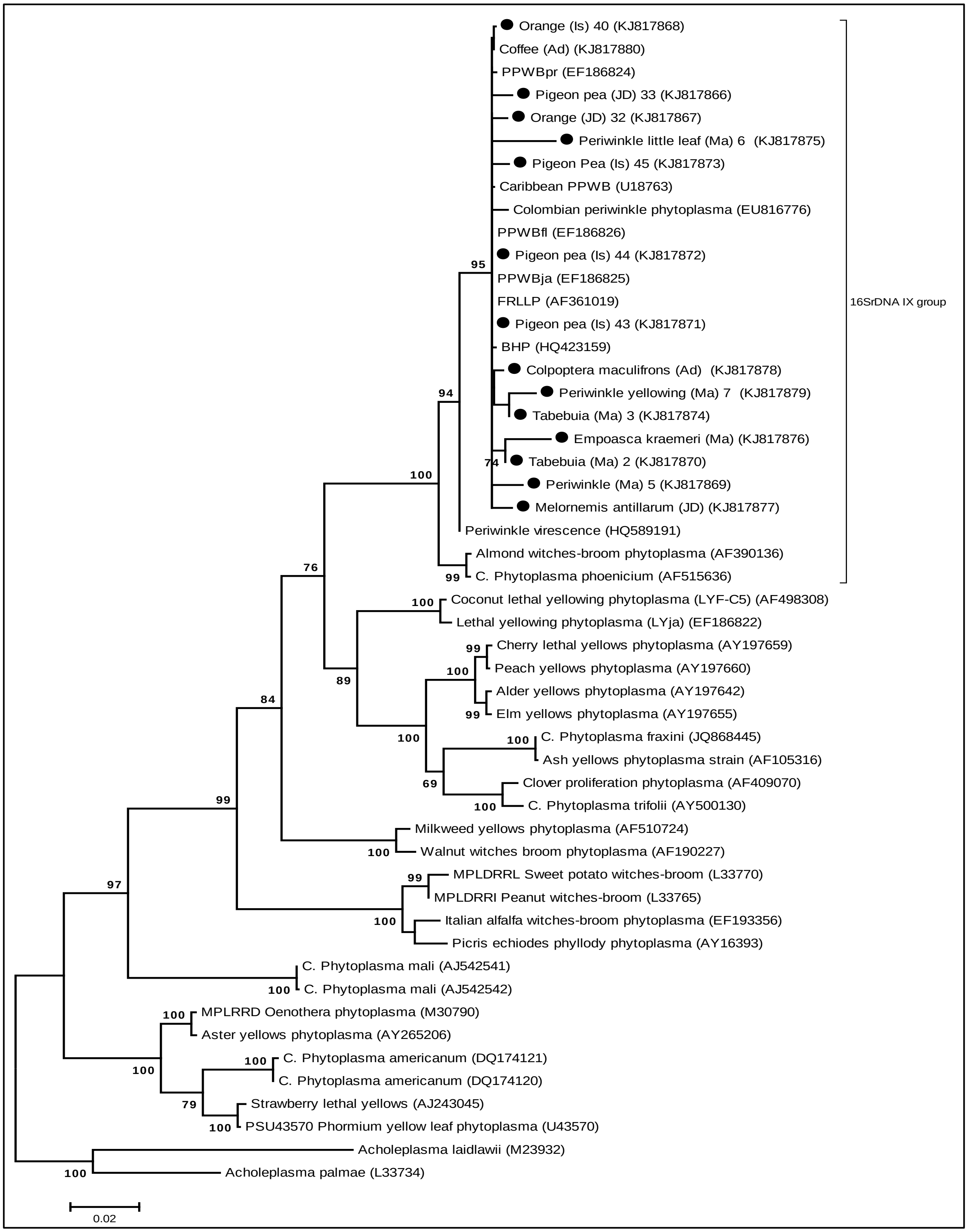

Figure 28. Phylogenetic tree inferred from 16S rDNA sequences using the Maximum Likelihood method, obtained from 51 members of the class *Mollicutes*, including to *Acholeplasma palmae* and *Achoeplasma laidawii* as outgroups. Codon-based MUSCLE alignment function in Guidance was used to align the sequences. Bootstrap values are shown next to the branches. GenBank accession numbers are indicated in parentheses. Black dots indicate PR samples.

Table 8. Estimates of net base composition bias disparity between sequences clustered in 16SrIX Group clade

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
Periwinkle (Ma) 5 (KJ817869)																			
Periwinkle little leaf (Ma) 6 (KJ817875)	0.00[a]																		
Periwinkle yellowing (Ma) 7 (KJ817879)	0.00	0.00																	
Pigeon pea (JD) 33 (KJ817866)	0.00	0.00	0.00																
Pigeon pea (Is) 43 (KJ817870)	0.00	0.00	0.00	0.00															
Pigeon pea (Is) 44 (KJ817871)	0.00	0.00	0.00	0.00	0.00														
Pigeon Pea (Is) 45 (KJ817872)	0.00	0.00	0.01	0.00	0.00	0.00													
Tabebuia (Ma) 2 (KJ817870)	0.00	0.00	0.00	0.00	0.00	0.00	0.00												
Tabebuia (Ma) 3 (KJ817874)	0.00	0.01	0.00	0.01	0.01	0.01	0.02	0.01											
Orange (JD) 32 (KJ817867)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01										
Orange (Is) 40 (KJ817868)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00									
Coffee (Ad) 22 (KJ817880)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00								
***Empoasca kraemeri* (Ma) (KJ817876)**	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00							
***Melornemis antillarum* (JD) (KJ817877)**	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00						
***Colpoptera maculifrons* (Ad) (KJ817878)**	0.00	0.00	0.00	0.01	0.00	0.00	0.02	0.01	0.00	0.00	0.01	0.00	0.01	0.00					
BHP (HQ423159)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.00	0.00				
PPWBpr (EF186824)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.00			
PPWBfl (EF186826)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
PPWBja (EF186825)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
RLLP (AF361019)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

[a]. Values greater than 0 indicate larger differences in base composition biases than expected based on evolutionary divergence between sequences and by chance alone. The analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding.

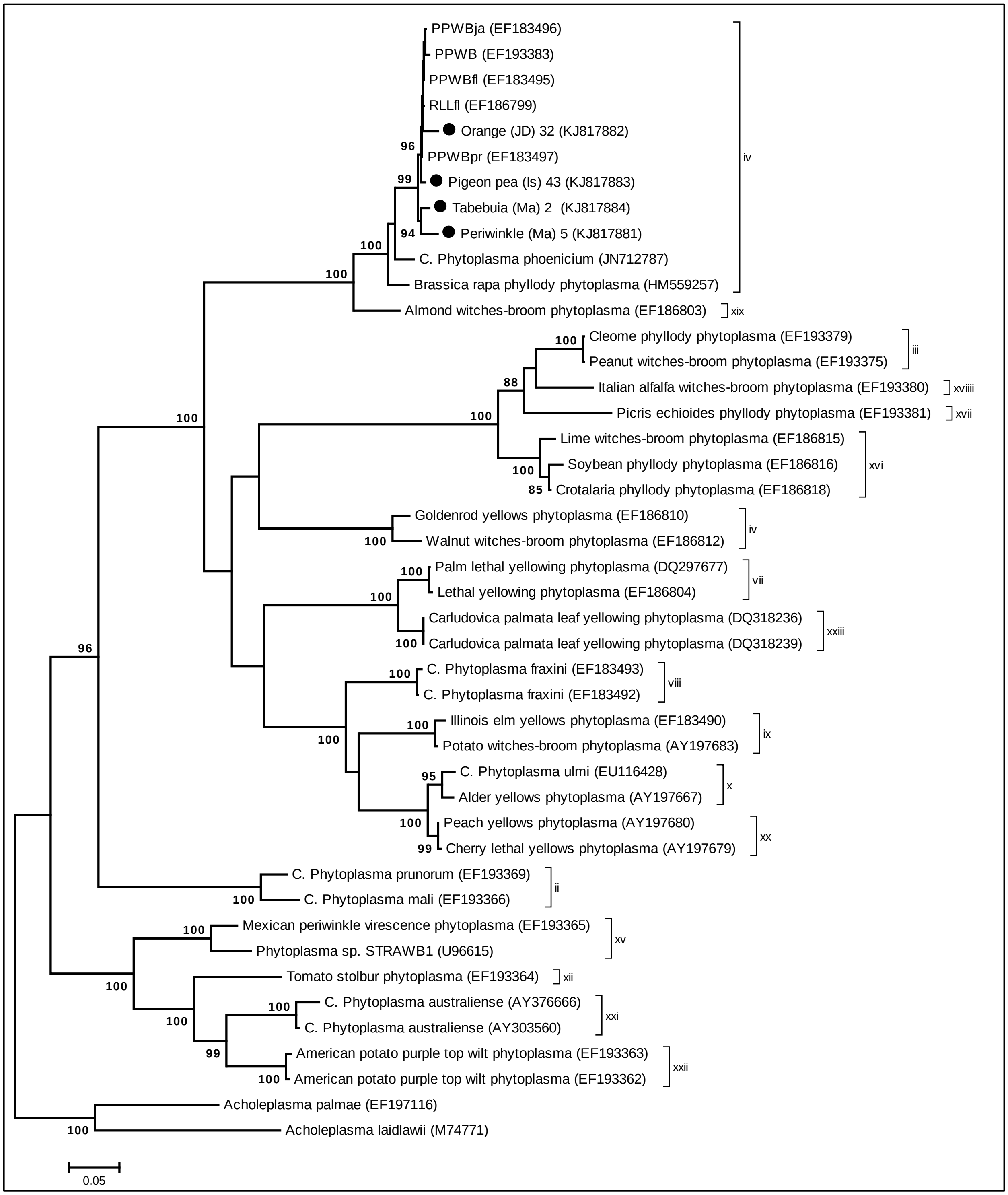

Figure 29. Phylogenetic tree inferred from *rpIV* (*rpl22*) and *rpsC* (*rps3*) sequences using the Maximum Likelihood method, obtained from 51 members of the class *Mollicutes*, including *Acholeplasma palmae* and *Achoeplasma laidawii* as outgroups. Codon-based MUSCLE alignment function in Guidance was used to align the sequences. Bootstrap values are shown next to the branches. GenBank accession numbers are indicated in parentheses. Black dots indicate PR samples.

Table 9. Estimates of net base composition bias disparity between sequences clustered in *rpIV* (*rpl22*) and *rpsC* (*rps3*)-IX Group clade.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Periwinkle (Ma) 5 (KJ817881)									
Orange (JD) 32 (KJ817882)	0.08[a]								
Tabebuia (Ma) 2 (KJ817884)	0.05	0.00							
PPWB (EF193383)	0.00	0.02	0.00						
Pigeon pea (Is) 43 (KJ817883)	0.00	0.03	0.00	0.00					
PPWBja (EF183496)	0.00	0.03	0.01	0.00	0.00				
PPWBpr (EF183497)	0.00	0.03	0.01	0.00	0.00	0.00			
RLLfl (EF186799)	0.00	0.02	0.00	0.00	0.00	0.00	0.00		
PPWBfl (EF183495)	0.00	0.03	0.01	0.00	0.00	0.00	0.00	0.00	
***C.* Phytoplasma phoenicium (JN712787)**	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00

[a]. Disparity Index per site is shown for all sequence pairs. Values greater than 0 indicate larger differences in base composition biases than expected based on evolutionary divergence between sequences and by chance alone. The analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding.

4.6. Restriction maps using restriction endonucleases for the 16S rDNA, *rpIV* (*rpl22*) and *rpsC* (*rps3*) genes

PCR products (DNA concentration 200 ng) of 16S rDNA, *rpIV* (*rpl22*) and *rpsC* (*rps3*) genes obtained using P1/P7 (1.8 kb) and rpF1C/rp(1)-R1A (1.2 kb) primer set, respectively, of samples corresponding to Pigeon pea (JD) 33, (Is) 43, 44 and 45, periwinkle (Ma) 5, periwinkle little leaf (Ma) 6, periwinkle yellowing (Ma) 7 and Orange (JD) 32, were digested with the following endonucleases: *AluI*, *MseI*, *RsaI*, *Hinf1* and *HaeIII*. In general and following restriction, *rpIV* (*rpl22*) and *rpsC* (*rps3*) genes generated variable profiles compared to 16S rDNA gene indicating that rp genes had more polymorphisms than 16S rDNA gene. RFLP patterns obtained were similar to those characteristic of phytoplasma strains belonging to 16SrIX group (Lee *et al.*, 1998a). RFLP profiles confirmed that PPWB phytoplasma is widely disseminated in Puerto Rico, affecting several plant species and periwinkle plants might act as natural reservoir. Periwinkle samples collected and tested showed typical symptoms of phytoplasma infection which included phyllody, big bud, virescence, yellowing and little leaf.

RFLP profiles of 16S rDNA gene from all restriction enzymes examined generated the same restriction pattern revealing no apparent differences between phytoplasma strains except for *AluI* endonuclease (Figure 30A). A different restriction pattern was generated with *AluI* endonuclease in a Pigeon Pea (JD) 33 sample. After digestion with *AluI* this sample showed four restriction sites, generating five restriction fragments, compared with other restriction enzymes digestions. RFLPs of *rpIV* (*rpl22*) and *rpsC* (*rps3*) genes using *AluI* endonuclease generated three different restriction profiles that can be distinguished in agarose gel by electrophoresis (Figure 30A). In the first restriction profile are the samples corresponding to Pigeon Pea (JD) 33, (Is) 44 and 45, Periwinkle little leaf (Ma) 6, Periwinkle yellowing (Ma) 7 and Orange (JD) 32, in which the enzyme found three and four restriction sites generating four or five DNA fragments (Figure 30A). For example, samples corresponding to Pigeon Pea (Is) 43 and Periwinkle (Ma) 5 produced three or four restriction sites, showing different polymorphic profiles compared to the others samples. This indicated possible mutations in this gene detected by *AluI* (Figure 30A).

Endonuclease enzymes *MseI* and *RsaI* digestion detected six different restriction profiles of *rpIV*-*rpsC* genes. Periwinkle yellowing (Ma) 7 and Orange (JD) 32 samples were not polymorphic for these genes, suggesting that they belong to the same 16S rDNA and *rpIV* (*rpl22*) and *rpsC* (*rps3*)-IX groups (Figure 30B and C). Endonuclease enzymes *MseI* and *RsaI* detected several polymorphic sites in ribosomal proteins (*rpIV* (*rpl22*) and *rpsC* (*rps3*)) genes, although bands were faint. These differences in RFLP patterns might indicate that these samples are not phylogenetically related. RFLP with endonuclease enzymes *HinfI* and *HaeIII* allowed us to differentiate two samples from pigeon pea, JD 33 and Is 43, from the other samples (Figure 31A and B). Mutation index might be responsible for generating several restriction patterns in the samples tested. According to Martini *et al.* (2007), the ribosomal protein genes are more variable than 16S rDNA because this gene can accumulate several mutations that indicate adaptability to different hosts. Both genes 16S rDNA and *rpIV* (*rpl22*) and *rpsC* (*rps3*) are involved in the quaternary structure of ribosome and the changes (mutations) can compromise cell life (Case *et al.,* 2007). In certain conditions phytoplasma need to adapt to several hosts or insect carriers. RFLP profiles of *rpIV* (*rpl22*) and *rpsC* (*rps3*) genes in pigeon pea samples showed different restriction patterns when compared with periwinkle and citrus samples digested with the same enzymes (*AluI*, *MseI*, *RsaI*, *Hinf1* and *HaeIII*), indicating polymorphism probably caused by mutations.

Our results indicate that all plant species tested, which exhibited characteristic symptoms of phytoplasma infection, were infected with strains belonging to group IX, one of 28 phytoplasma groups delineated by 16S rDNA and RFLP classification schemes elaborated by Lee *et al.* (1998a) and Wei *et al*. (2007), respectively. Although phytoplasmas detected in these 8 samples exhibited different RFLP patterns, we considered them PPWB phytoplasma, but several strains with DNA sequences heterogeneous for *rpIV* (*rpl22*) and *rpsC* (*rps3*) genes.

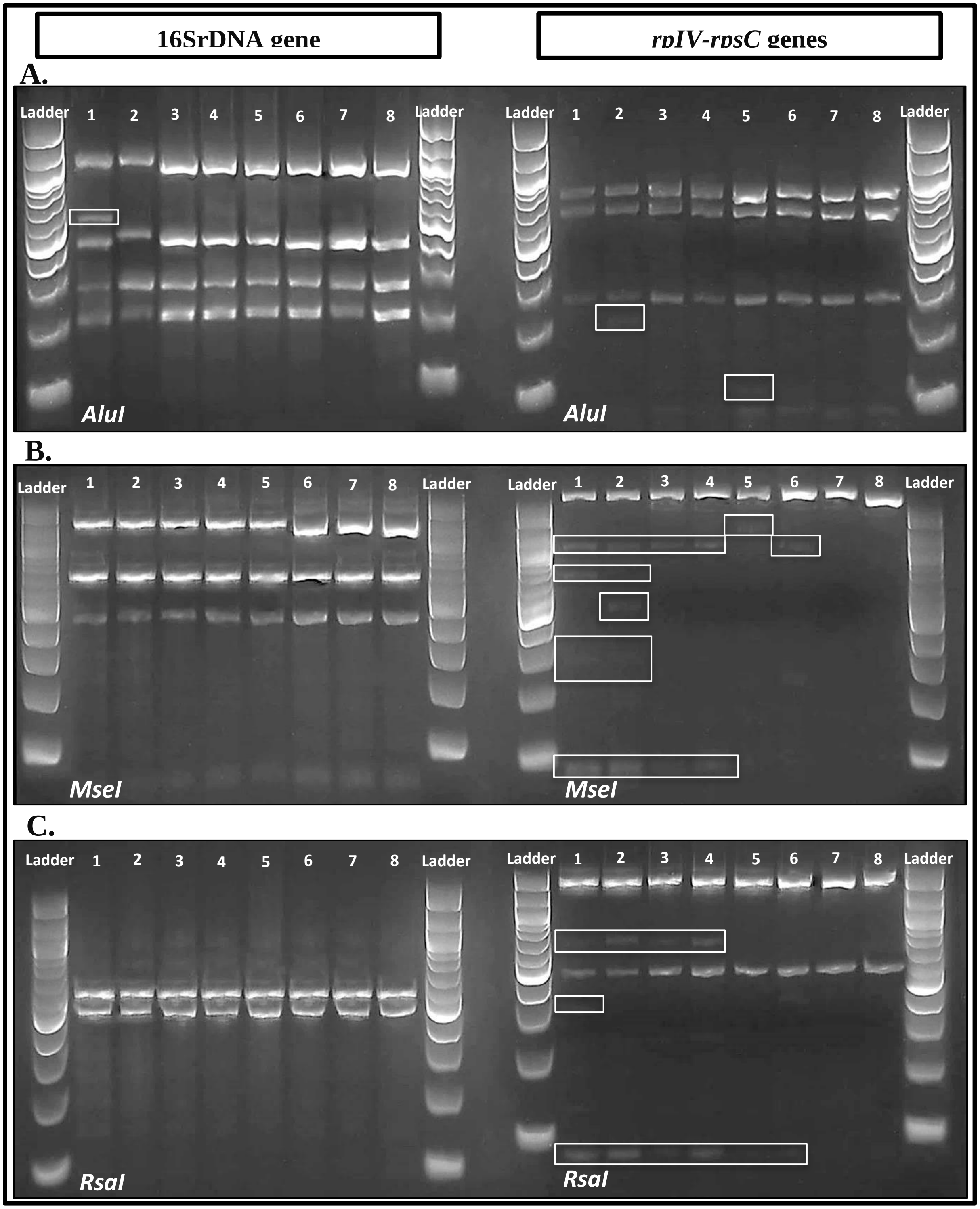

Figure 30. RFLP profiles of 16S rDNA and *rpIV* (*rpl22*) and *rpsC* (*rps3*) genes sequences amplified by PCR with primers P1/P7 and rpL2-F3/rp(1)-R1A from symptomatic plants infected with pigeon pea witches'-broom phytoplasma. Phytoplasma DNA was digested with: **A.** *AluI*; **B.** *MseI*; **C.** *RsaI*. Digested products were separated by electrophoresis in a 3% agarose gel. Template DNA for PCR was derived from plants with: First and last lane: 1 kb DNA Ladder. Samples 1: Pigeon Pea (JD), 33; 2: Pigeon Pea (Is) 43; 3: Pigeon Pea (Is) 44; 4: Pigeon Pea (Is) 45; 5: Periwinkle (Ma) 5; 6: Periwinkle little leaf (Ma) 6; 7: Periwinkle yellowing (Ma) 7 and 8: Orange (JD) 32. Locations: JD= Juana Díaz; Is= Isabela; Ma= Mayagüez. The white boxes show the polymorphism detected by the enzyme indicated.

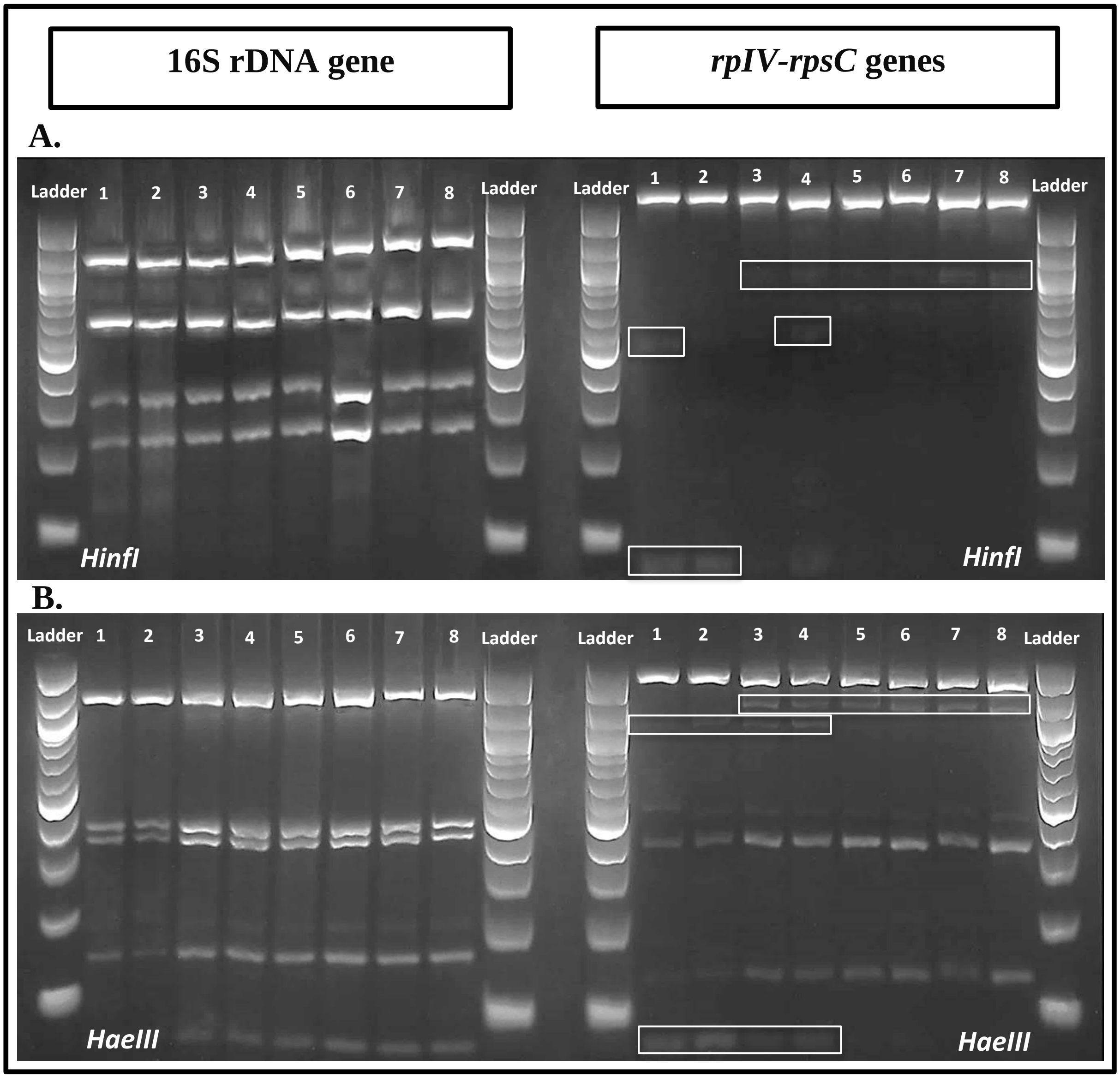

Figure 31. RFLP profiles of 16S rDNA and *rpIV* (*rpl22*) and *rpsC* (*rps3*) gene sequences amplified by PCR with primers P1/P7 and rpL2-F3/rp(1)-R1A from symptomatic plants infected with pigeon pea witches'-broom phytoplasma. Phytoplasma DNA was digested with: **A.** *Hinf1* and **B**. *HaeIII*. Digested products were separated by electrophoresis in a 3% agarose gel. Template DNA for PCR was derived from plants with: First and last lane: 1 kb DNA Ladder. Samples 1: Pigeon Pea (JD), 33; 2: Pigeon Pea (Is) 43; 3: Pigeon Pea (Is) 44; 4: Pigeon Pea (Is) 45; 5: Periwinkle (Ma) 5; 6: Periwinkle little leaf (Ma) 6; 7: Periwinkle yellowing (Ma) 7 and 8: Orange (JD) 32. Locations: JD= Juana Díaz; Is= Isabela; Ma= Mayagüez. The white boxes show the polymorphism detected by the enzyme indicated.

4.7. Real-time PCR protocol using SYBR® Green method

Specific primer pairs were designed to amplify a small region (102 bp) of 16S rDNA gene for rapid and accurate detection and quantification of PPWB phytoplasma with quantitative PCR (qPCR - also known as real-time PCR) using the SYBR® green method. (Figure 31C).

For the first qPCR assay, the positive control (Pigeon pea (JD) 33 sample) and the respective standard solutions produced the same melting temperature (Tm = 82.3 ^{o}C) (Table 10). Two samples from Tabebuia (Ma) 2 and 3, healthy periwinkle and negative control (water) had Tm values of 82 ^{o}C, 81.7 ^{o}C, 75.4 ^{o}C and 75.4 ^{o}C, respectively. The assay was not sensitive enough to detect phytoplasma in extremely low DNA concentrations. The assay was not sensitive enough to detect phytoplasma in extremely low DNA concentrations. In this case, the nested PCR assays were more robust detecting PPWB phytoplasma in both Tabebuia samples (Ma) 2 and 3.

Derivative and component melt graphics (Figure 32A and 32B), showed that samples of Pigeon pea (JD) 33 (positive control), Pigeon pea (Is) 43 and Periwinkle (Ma) 5 recorded a maximum fluorescence and a single derivative and component melt curve at Tm = 82.3 ^{o}C. Assay efficiency was calculated by a linear regression coefficient (R^2), where DNA phytoplasma concentrations from the standard solutions (dilutions series) showed R^2 = 0.988 (Figure 32C). This means that there is a 98.8% relationship between cycle threshold value and increase in fluorescence for each sample and standard solutions. To discriminate false positives, normalized qPCR data is necessary (Tm and Cq Threshold) from positive controls and compare with negative samples and controls (Giulietti *et al.*, 2001; Roberts *et al.,* 2000). Nonspecific amplifications (equivalent to fluorescence level) were observed in curves of negative samples; nonspecific amplification began at 31 cycles (see amplification plot, Figure 32D). The non-specific amplifications might be produced by primer dimers and mispriming. These results can occur in the absence of the phytoplasma (false positives) or with low phytoplasma concentration where qPCR was unable to detect the phytoplasma presence.

PPWB phytoplasma identification is based on nested PCR-RFLP analysis of 16S rDNA amplicons (Martini *et al.,* 2007). qPCR has been used to detect PPWB (group 16SrIX) phytoplasmas in infected periwinkle plants using TaqMan® probes (Crosslin *et al.,* 2006). However these primers were designed based on Columbia Basin potato purple top phytoplasma which belong to a different group (16SrVI) (GenBank accession: AY692280). The primers designed in the currently study are based on DNA sequences obtained from positive samples of PPWB phytoplasma found in this research and use the SYBR Green® assay. Therefore the qPCR assays utilized different detection chemistries. In a second published report, a separate primer pair set designed for qPCR (5′CGTACGCAAGTATGAAACTTAAAGGA/5’TCTTCGAATTAAACAACATGATCA) and reported as a universal primer set for phytoplamsa was used to amplify a region of expected size of 0.7 kb from 16S rDNA gene (Christensen *et al.,* 2004). The author reports a qPCR assay to diagnose numerous phytoplasmas using a TaqMan® probe to detect phytoplasma in *Catharanthus roseus* and *Euphorbia pulcherrima* (Christensen *et al*., 2004). All our samples were negative using this set of primers (Christensen *et al*., 2004). Hren *et al.* (2007) and Baric *et al.* (2004) similarly, reported the use of TaqMan® probes to detect phytoplasma associated with grapevine yellows and apple proliferation, respectively. Galetto *et al.* (2005) and Torres *et al.* (2005) developed a protocols using SYBR Green® assay to diagnose of apple proliferation phytoplasma.

Generally, qPCR is more specific and sensitive than conventional PCR. However this latter technique sometimes can be more useful for identification of the plant pathogen (e.g., small nucleiotide polymorphism). From our experience the success of RT-PCR technique using TaqMan® probes or SYBR® Green, depends on specificity and primer concentration, pathogen DNA concentration, and setup of the cycling program for quantification. High-quality DNA is an important aspect for a successful qPCR assay. Woody plants (such as *Tabebuia* tissue) might have PCR inhibitors (polysaccharides, polyphenols, proteins, and other secondary metabolites) that need to be examined in order to have a successful assay.

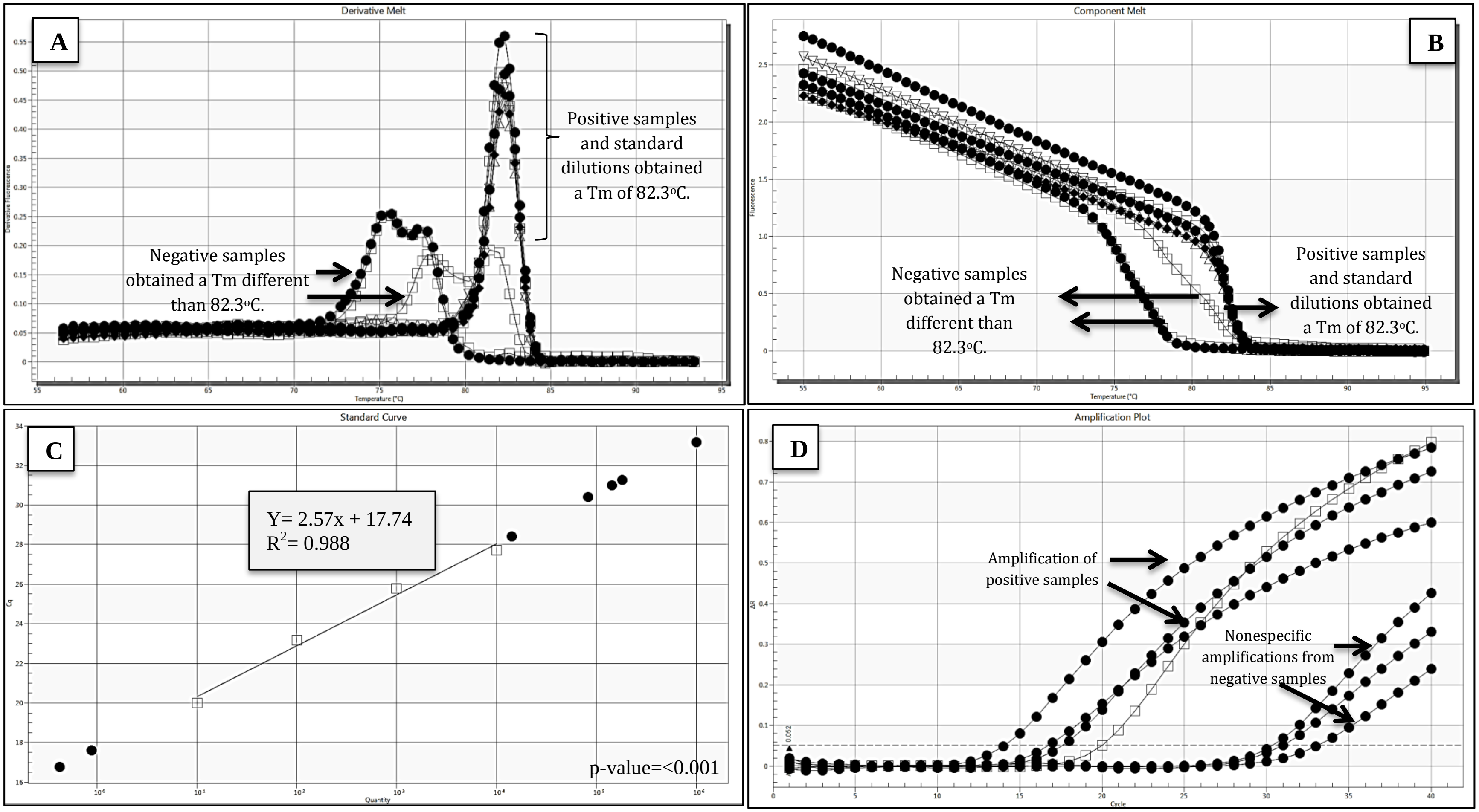

Figure 32. qPCR amplification of DNA from PPWB phytoplasma using SYBR® Green assay. **A.** Derivative and **B.** Component Melt (Melting Temperature 82.3^{o}C) obtained with pigeon pea samples (JD) 33 (Positive control) and (Is) 43, Orange (JD) 32 and Periwinkle (Ma) 5 and standard dilutions from positive control. Negative melt curve were obtained with samples from Tabebuia (Ma 2 and 3), Healthy periwinkle plant and Negative control (molecular water). **C.** Linear regression coefficient (R^2) from dilutions series and samples tested is given in the equation Y= 2.57x + 17.74 and a 98.8 % assay efficiency are shown in graph. **D.** Amplification plot showing specific amplifications from pigeon pea samples (JD) 33 (Positive control) and (Is) 43, Orange (JD) 3 and Periwinkle (Ma) 5; and nonspecific amplifications obtained from samples Tabebuia (Ma) 2 and 3 and Healthy periwinkle plant. Locations: JD= Juana Díaz; Is= Isabela; Ma= Mayagüez.

For the second assay, the symptomatic and asymptomatic *Citrus* spp. samples exhibited citrus greening disease symptoms, but where negative for HLB assays. In this case, the qPCR assay included positive control (Pigeon pea (JD) 33) and dilutions series (10^{-2} to 10^{-4}) obtained from this sample. All citrus samples were negative for phytoplasma and recorded a different melting temperatures (Tm: 74.5 to 79.0^{o}C) when compared to the positive control (Tm = 82.3^{o}C) (Table 11). Similarly, melt and component curves differed in fluorescence levels compared to that obtained with positive sample and the standard solutions (Figure 33A and B).

qPCR assay efficiency was calculated by a linear regression coefficient (R^2), using DNA phytoplasma concentrations from the standard solutions (dilutions series) showed R^2 = 0.303 (Figure 33C). This number means that there is a 30.3% of relationship between the cycle threshold value and the increase of fluorescence in each sample and standard solutions. Although R^2 was not high, Tm achieved by dilutions series and positive control were 82.3^{o}C, confirming that the amplicons corresponded to pigeon pea phytoplasma.

The amplification plot recorded nonspecific amplifications in a group set of samples, showing differences with the curve obtained from positive control (Figure 33D).

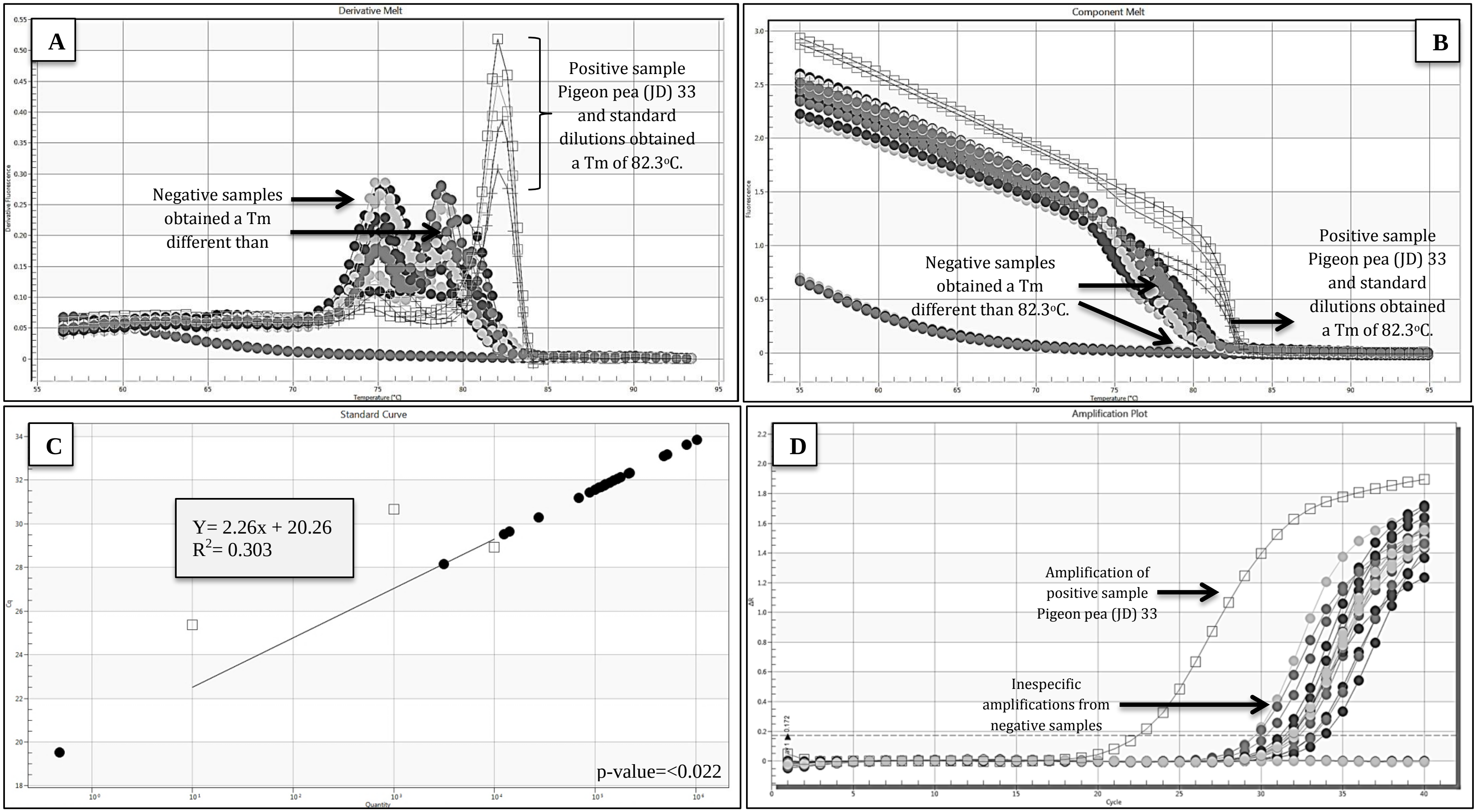

Figure 33. qPCR amplification of DNA from Pigeon pea witches'-broom phytoplasma using SYBR Green assay®. **A.** and **B.** Derivative and Component Melt (Melting Temperature 82.3^{o}C) obtained with pigeon pea samples (JD) 33 (Positive control) and standard dilutions from positive control. Negative melt curve were obtained with the samples from 25 symptomatic and asymptomatic citrus samples **C.** Linear regression coefficient (R^2) from dilutions series and samples tested is given in the equation Y= 2.26x + 20.26 and a 30.3% assay efficiency are shown in graph. **D.** Amplification plot showing specific amplifications from pigeon pea samples (JD) 33 (Positive control) and nonspecific amplifications obtained from 25 citrus samples. Location: JD= Juana Díaz.

Finally, in the third assay the Tm calculated for the phytoplasma samples corresponding to American Aster Yellows (16SrI-B) (74.5 °C), Apple proliferation (16SrX-A) (80.8 °C), Ash yellow (16SrVII-A) (83.2 °C), Cactus phytoplasma (16SrI-B) (81.4 °C) and Peach X disease (16SrIII-A) (82.9 °C), were different to Tm calculated for the positive sample (Pigeon pea (JD) 33) (82.3 °C) (Figure 34 and 35). In this case, the Figure 34 shows eigth nucleotide changes in the amplicon sequences from the samples above mentioned, compared with the amplicon obtained from the positive sample Pigeon pea (JD) 33.

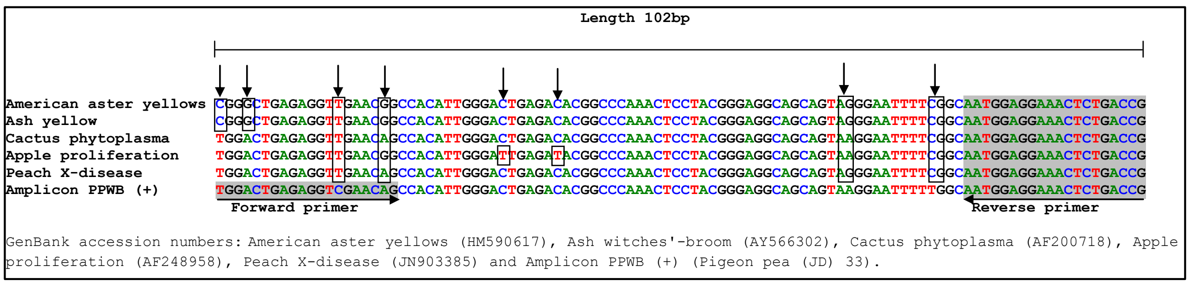

Figure 34. Location of the polymorphisms in the amplicon sequence obtained from the phytoplasma subgroups: American Aster Yellows (16SrI-B), Apple proliferation (16SrX-A), Ash yellow (16SrVII-A), Cactus phytoplasma (16SrI-B) and Peach X disease (16SrIII-A). The black arrow shows the nucleotide changes.

Furthermore, the phytoplasma samples corresponding to Elm yellows (16SrV-A), Poinsettia branching factor (16SrIII-H), Tomato big bud (16SrII-D), Alder yellows (16SrV-C), Faba bean phyllody (16SrII-C), Beet leafhopper transmitted (16SrVI-A), Stolbur (16SrXII-A) and *Pichris echioides* yellows (16SrIX-C), recorded the same Tm compared with the positive sample (Pigeon pea (JD) 33) (82.3°C). Negative controls (water) and outgroups (*Pseudomonas saccharophila*, *Sphingomonas phyllosphaerae* and Haloarchaea), obtained a Tm of 79. 3 °C, 79 °C, 76 °C and 75.7 °C, respectively (Figure 35).

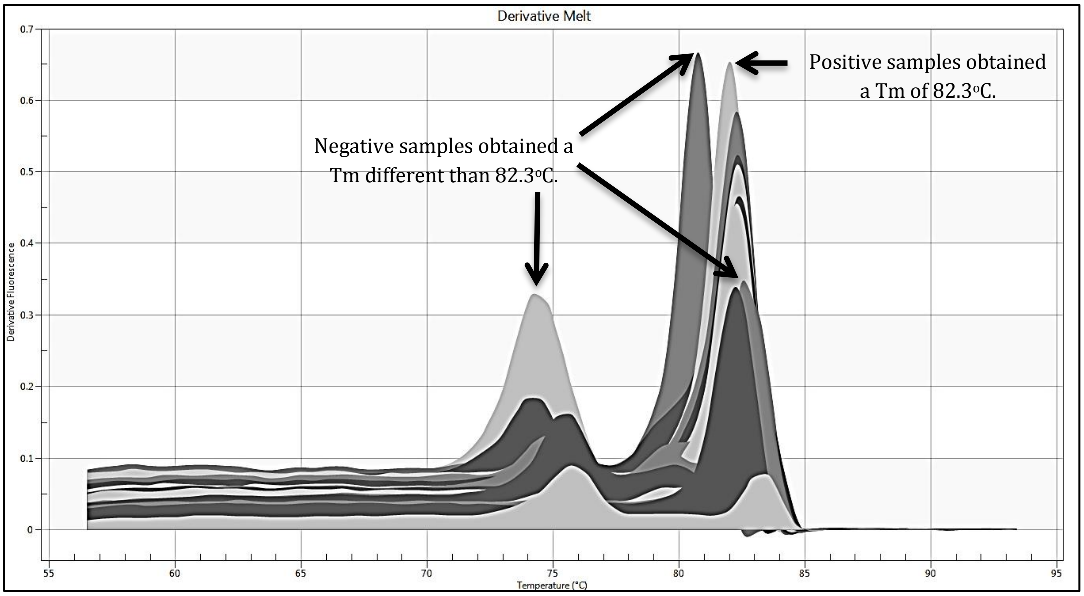

Figure 35. qPCR amplification of DNA from phytoplasma DNA using SYBR Green assay®. Derivative Melt (Melting temperature 82.3^{o}C) obtained with the positive control (Pigeon pea (JD) 33), Elm yellows (16SrV-A), Poinsettia branching factor (16SrIII-H), Tomato big bud (16SrII-D), Alder yellows (16SrV-C), Faba bean phyllody (16SrII-C), Beet leafhopper transmitted (16SrVI-A), Stolbur (16SrXII-A) and *Pichris echioides* yellows (16SrIX-C). The others phytoplasma subgroups and three outgroups (bacteria and arquaea) recorded different Tm (from 75.7 to 83.2 ^{o}C).

Thus, the primers designed for phytoplasma detection can differentiate the phytoplasma groups and subgroups 16SrI-B, 16SrX-A, 16SrVII-A, 16SrIII-A, from the phytoplasma subgroups 16SrV-A, 16SrIII-H, 16SrII-D, 16SrV-C, 16SrII-C, 16SrVI-A, 16SrXII-A and 16SrIX-C. In the same sense, the primers designed for phytoplasma detection identified the phytoplasma DNA from bacteria and archaea DNA (Figure 35).

5. CONCLUSIONS

- Sixty two samples tested positive for phytoplasma infection. Phytoplasma DNA sequences of the 16S rDNA gene from infected samples of Orange (JD) 32 and Is 40, Pigeon pea (JD) 33; (Is) 43, 44 and 45, Tabebuia (Ma) 2 and 3, Periwinkle (Ma) 5, Periwinkle little leaf (Ma) 6, Periwinkle yellowing (Ma) 7 were amplified using end point PCR. DNA sequences were found homologous (99% identity) to PPWB phytoplasma. These findings were confirmed by amplification of two ribosomal genes *rpIV* (*rpl22*) and *rpsC* (*rps3*) in samples corresponding to Periwinkle (Ma) 5, Orange (JD) 32), Pigeon pea (Is) 43 and Tabebuia (Ma) 2.

- *Empoasca kraemeri* (40 individuals), *Melornemis antillarum* (20 individuals) and *Colpoptera maculifrons* (22 individuals) were sweep-collected from field grown pigeon peas and citrus trees as well as from weedy borders around plots. Five insects from each genus tested positive for PPWB phytoplasma by end point PCR and by DNA analysis of 16S rDNA gene. These insects may act as potential PPWB phytoplasma vectors.

- RFLP patterns of samples of 16S rDNA gene from pigeon pea (JD) 33), (Is) 43, 44, and 45, Periwinkle (Ma) 5, Periwinkle little leaf (Ma) 6, Periwinkle yellowing (Ma) 7 and Orange (JD) 32 were found to be similar to patters for strains belonging to group 16SrIX. This confirmed that this phytoplasma is widely disseminated in five plant species such as pigeon pea, periwinkle, tabebuia, citrus and coffee in Puerto Rico.

- Specific primers were designed for phytoplasma using qPCR (SYBR® Green method) improving detection of early and low level infections of phytoplasma. Melting Temperature (Tm) was determined to be 82.3 ^{o}C using samples corresponding to Pigeon pea (Is) 33 (positive control), Pigeon pea (JD) 43, Orange (JD) 32, Periwinkle (Ma) 5 and phytoplasma groups and subgroups 16SrV-A, 16SrIII-H, 16SrII-D, 16SrV-C, 16SrII-C, 16SrVI-A, 16SrXII-A and 16SrIX-C. A different Tm was obtained with samples from Tabebuia (Ma) 2 and 3, 25 *Citrus* spp. samples, and phytoplasma subgroups 16SrI-B, 16SrX-A, 16SrVII-A, 16SrIII-A.

6. RECOMMENDATIONS

- Expand the molecular characterization of PPWB phytoplasma found in this study using sequence data and RFLP for other genes such as *tuf*, *secY and gro*EL.

- Complete infection cycles to determine the capability of insect vectors identified in this study to transmit PPWB phytoplasma to plants of economic importance such as pigeon pea, citrus, coffee and Tabebuia.

- Validate probe designed for qPCR assays using TaqMan® assay method in order to increase assay specificity for PPWB phytoplasma detection.

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Appendix 1

Record of samples collected in the field

Table 12. Plant samples collected in Puerto Rico during survey for phytoplasma infection.

Sample #	Sample name	Place	Plant species	Sampling	Prescence of PPWBP
1	Cactus 1	Cabo Rojo	Cactus	1	No
2	Mango 3	Mayagüez	Mango	1	No
3	Mango 4	Mayagüez	Mango	1	No
4	Mango 5	Mayagüez	Mango	1	No
5	Mango 6	Mayagüez	Mango	1	No
6	Mango 7	Mayagüez	Mango	1	No
7	Mango 8	Mayagüez	Mango	1	No
8	Ixora 1	Mayagüez	Ixora	1	No
9	Ixora 2	Mayagüez	Ixora	1	No
10	Ixora 3	Mayagüez	Ixora	1	No
11	Sapanish lime 1	Cabo Rojo	Sapanish lime	1	No
12	Sapanish lime 2	Cabo Rojo	Sapanish lime	1	No
13	Sapanish lime 3	Cabo Rojo	Sapanish lime	1	No
14	Sapanish lime 4	Cabo Rojo	Sapanish lime	1	No
15	Sapanish lime 5	Cabo Rojo	Sapanish lime	1	No
16	Periwinkle 5	Mayagüez	Periwinkle	1	**Yes**
17	Periwinkle 6	Mayagüez	Periwinkle	1	**Yes**
18	Tabebuia 2	Mayagüez	Tabebuia	1	**Yes**
19	Tabebuia 3	Mayagüez	Tabebuia	1	No
20	Tabebuia 4	Mayagüez	Tabebuia	1	**Yes**
21	Lemon 11	Corozal	Lemon	2	**Yes**
22	Lemon 12	Corozal	Lemon	2	**Yes**
23	Lemon 13	Corozal	Lemon	2	**Yes**
24	Lemon 14	Corozal	Coffee	2	**Yes**
25	Coffee 21	Adjuntas	Coffee	3	No
26	Coffee 22	Adjuntas	Coffee	3	**Yes**
27	Coffee 23	Adjuntas	Coffee	3	No
28	Coffee 24	Adjuntas	Coffee	3	No
29	Coffee 25	Adjuntas	Coffee	3	**Yes**
30	Coffee 26	Adjuntas	Coffee	3	**Yes**
31	Coffee 27	Adjuntas	Coffee	3	**Yes**
32	Orange 26	San Sebastián	Orange	4	No
33	Orange 27	San Sebastián	Orange	4	No
34	Orange 28	San Sebastián	Orange	4	**Yes**
35	Lemon 29	San Sebastián	Lemon	4	No
36	Lemon 30	Juana Díaz	Lemon	4	No
37	Lemon 31	Juana Díaz	Lemon	4	No
38	Orange 32	Juana Díaz	Orange	4	**Yes**

39	Orange 7	Las Marías	Orange	4	**Yes**
40	Orange 8	Las Marías	Orange	4	**Yes**
41	Lemon 33	Isabela	Lemon	4	No
42	Lemon 34	Isabela	Lemon	4	**Yes**
43	Orange 35	Isabela	Orange	4	No
44	Tangerine 36	Isabela	Tangerine	4	No
45	Orange 37	Isabela	Orange	4	**Yes**
46	Orange 38	Isabela	Orange	4	**Yes**
47	Orange 40	Isabela	Orange	4	**Yes**
48	Pigeon pea 32	Juana Díaz	Pigeon Pea	5	**Yes**
49	Pigeon pea 33	Juana Díaz	Pigeon Pea	5	**Yes**
50	Pigeon pea 34	Juana Díaz	Pigeon Pea	5	**Yes**
51	Pigeon pea 35	Juana Díaz	Pigeon Pea	5	**Yes**
52	Pigeon pea 35	Juana Díaz	Pigeon Pea	5	**Yes**
53	Pigeon pea 36	Isabela	Pigeon Pea	5	No
54	Pigeon pea 37	Isabela	Pigeon Pea	5	No
55	Pigeon pea 38	Isabela	Pigeon Pea	5	No
56	Pigeon pea 39	Isabela	Pigeon Pea	5	No
57	Pigeon pea 40	Isabela	Pigeon Pea	5	**Yes**
58	Pigeon pea 41	Isabela	Pigeon Pea	5	**Yes**
59	Pigeon pea 42	Isabela	Pigeon Pea	5	**Yes**
60	Pigeon pea 43	Isabela	Pigeon Pea	5	**Yes**
61	Pigeon pea 44	Isabela	Pigeon Pea	5	**Yes**
62	Pigeon pea 45	Isabela	Pigeon Pea	5	**Yes**

Appendix 2

Multiple sequence alignment of 16S rDNA gene for 14 positive samples of PPWB phytoplasma using GUIDANCE server (Guide-tree based alignment confidence) generated through MUSCLE algorithm.

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                                          10        20        30        40        50        60        70
                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)          1   ---------------------------------------------------------------------- 1
Melornemis antillarum (JD)      1   ---------------------------------------------------------------------- 1
Colpoptera maculifrons (Ad)     1   AAGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGCGCCTAA-TACATGCAAGTCGAACGGGAACCT 69
Periwinkle little leaf (Ma) 6   1   AAGAGTTTGATCCTGGCTCAGGATTAACGCTGGCGGCGCGCCTAA-TACATGCAAGTCGAACGGAAACCT 69
Periwinkle yellowing (Ma) 7     1   AAGAGTTTGATCCTGGCTCAGGATTAACGCTGGCGGCGCGCCTAA-TACATGCAAGTCGAACGGAAACCT 69
Periwinkle (Ma) 5               1   AAGAGTTTGATCCTGGCTCAGGATTAACGCTGGCGGCGCGCCTAT-AACATGCAAGTCGAACGGAAACCT 69
Pigeon pea (JD) 33              1   AAGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGCGCCTA--TAC-TGCAAGTCGAACGGAAACCT 67
Pigeon Pea (Is) 45              1   AAGAGTTTGATCCTGGCTCAGGATTAACGCTGGCGGCGCGCCTAA-TACATGCAAGTCGAACGGAAACCT 69
Tabebuia (Ma) 2                 1   AAGAGTTTGATCCTGGCTCAGGATTAACGCTGGCGGCGCGCCTAA---CATGCAAGTCGAACGGAAACCT 67
Tabebuia (Ma) 3                 1   AAGAGTTTGATCCTGGCTCAGGATTAACGCTGGCGGCGCGCCTAA-TACATGCAAGTCGAACGGAAACCT 69
Orange (Is) 40                  1   AAGAGTTTGATCCTGGCTCAGGATTAACGCTGGCGGCGCGCCTAA-TACATGCAAGTCGAACGGAAACCT 69
Orange (JD) 32                  1   AAGAGTTTGATCCTGGCTCAGGATTAACGCTGGCGGCGCGCCTAA-TACATGCAAGTCGAACGGAAACCT 69
Pigeon pea (Is) 43              1   AAGAGTTTGATCCTGGCTCAGGATTAACGCTGGCGGCGCGCCTAA-TACATGCAAGTCGAACGGAAACCT 69
Pigeon pea (Is) 44              1   AAGAGTTTGATCCTGGCTCAGGATTAACGCTGGCGGCGCGCCTA--TACATGCAAGTCGAACGGAAACCT 68
PPWBja (EF186825)               1   AAGAGTTTGATCCTGGCTCAGGATTAACGCTGGCGGCGCGCCTAA-TACATGCAAGTCGAACGGAAACCT 69
PPWBpr (EF186824)               1   AAGAGTTTGATCCTGGCTCAGGATTAACGCTGGCGGCGCGCCTAA-TACATGCAAGTCGAACGGAAACCT 69
PPWBfl (EF186826)               1   AAGAGTTTGATCCTGGCTCAGGATTAACGCTGGCGGCGCGCCTAATCACATGCAAGTCGAACGGAAACCT 70

                                                80        90       100       110       120       130       140
                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)          1   ---------------------------------------------------------------------- 1
Melornemis antillarum (JD)      1   ---------------------------------------------------------------------- 1
Colpoptera maculifrons (Ad)     70  TAGGGTTTTAGTGGCGAACGGGTGCGTAACACGTAAGCAACCTGCCTTTAAGACGTGGATAACAATTGGA 139
Periwinkle little leaf (Ma) 6   70  TAGGGTTTTAGTGGCGAACGGGTGAGTAACACGTAAGCAACCTGCCTTTAAGACGTGGATAACAATTGGA 139
Periwinkle yellowing (Ma) 7     70  TAGGGTTTTAGTGGCGAACGGGTGAGTAACACGTAAGCAACCTGCCTTTAAGACGTGGATAACAATTGGA 139
Periwinkle (Ma) 5               70  TAGGGTTTTAGTGGCGAACGGGTGAGTAACACGTAAGCAACCTGCCTTTAAGACGTGGATAACAATTGGA 139
Pigeon pea (JD) 33              68  TAGGGTTTTAGTGGCGAACGGGTGAGTAACACGTAAGCAACCTGCCTTTAAGACGTGGATAACAATTGGA 137
Pigeon pea (Is) 45              70  TAGGGTTTTAGTGGCGAACGGGTGAGTAACACGTAAGCAACCTGCCTTTAAGACGTGGATAACAATTGGA 139
Tabebuia (Ma) 2                 68  TAGGGTTTTAGTGGCGAACGGGTGAGTAACACGTCAGTTACCTGCCTTTAAGACGTGGATAACAATTGGA 137
Tabebuia (Ma) 3                 70  TAGGGTTTTAGTGGCGAACGGGTGAGTAACACGTAAGCAACCTGCCTTTAAGACGTGGATAACAATTGGA 139
Orange (Is) 40                  70  TAGGGTTTTAGTGGCGAACGGGTGAGTAACACGTAAGCAACCTGCCTTTAAGACGTGGATAACAATTGGA 139
Orange (JD) 32                  70  TAGGGTTTTAGTGGCGAACGGGTGAGTAACACGTAAGCAACCTGCCTTTAAGACGTGGATAACAATTGGA 139
Pigeon pea (Is) 43              70  TAGGGTTTTAGTGGCGAACGGGTGAGTAACACGTAAGCAACCTGCCTTTAAGACGTGGATAACAATTGGA 139
Pigeon pea (Is) 44              69  TAGGGTTTTAGTGGCGAACGGGTGAGTAACACGTAAGCAACCTGCCTTTAAGACGTGGATAACAATTGGA 138
PPWBja (EF186825)               70  TAGGGTTTTAGTGGCGAACGGGTGAGTAACACGTAAGCAACCTGCCTTTAAGACGTGGATAACAATTGGA 139
PPWBpr (EF186824)               70  TAGGGTTTTAGTGGCGAACGGGTGAGTAACACGTAAGCAACCTGCCTTTAAGACGTGGATAACAATTGGA 139
PPWBfl (EF186826)               71  TAGGGTTTTAGTGGCGAACGGGTGAGTAACACGTAAGCAACCTGCCTTTAAGACGTGGATAACAATTGGA 140

                                               150       160       170       180       190       200       210
                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)          1   ---------------------------------------------------------------------- 1
Melornemis antillarum (JD)      1   ---------------------------------------------------------------------- 1
Colpoptera maculifrons (Ad)     140 AACAGTTGCTAAGACAGGATAGGAAATTAAGAGGCATCTTTTAATTTTTAAAAGACCTTTTTCGAAAGGT 209
Periwinkle little leaf (Ma) 6   140 AACAGTTGCTAAGACAGGATAGGAAATTAAGAGGCATCTTTTAATTTTTAAAAGACCTTTTTCGAAAGGT 209
Periwinkle yellowing (Ma) 7     140 AACAGTTGCTAAGACAGGATAGGAAATTAAGAGGCATCTTTTAATTTTTAAAAGACCTTTTTCGAAAGGT 209
Periwinkle (Ma) 5               140 AACAGTTGCTAAGACAGGATAGGAAATTAAGAGGCATCTTTTAATTTTTAAAAGACCTTTTTCGAAAGGT 209
Pigeon pea (JD) 33              138 AACAGTTGCTAAGACAGGATAGGAAATTAAGAGGCATCTTTTAATTTTTAAAAGACCTTTTTCGAAAGGT 207
Pigeon pea (Is) 45              140 AACAGTTGCTAAGACAGGATAGGAAATTAAGAGGCATCTTTTAATTTTTAAAAGACCTTTTTCGAAAGGT 209
Tabebuia (Ma) 2                 138 AACAGTTGCTAAGACAGGATAGGAAATTAAGAGGCATCTTTTAATTTTTAAAAGACCTTTTTCGAAAGGT 207
Tabebuia (Ma) 3                 140 AACAGTTGCTAAGACAGGATAGGAAATTAAGAGGCATCTTTTAATTTTTAAAAGACCTTTTTCGAAAGGT 209
Orange (Is) 40                  140 AACAGTTGCTAAGACAGGATAGGAAATTAAGAGGCATCTTTTAATTTTTAAAAGACCTTTTTCGAAAGGT 209
Orange (JD) 32                  140 AACAGTTGCTAAGACAGGATAGGAAATTAAGAGGCATCTTTTAATTTTTAAAAGACCTTTTTCGAAAGGT 209
Pigeon pea (Is) 43              140 AACAGTTGCTAAGACAGGATAGGAAATTAAGAGGCATCTTTTAATTTTTAAAAGACCTTTTTCGAAAGGT 209
Pigeon pea (Is) 44              139 AACAGTTGCTAAGACAGGATAGGAAATTAAGAGGCATCTTTTAATTTTTAAAAGACCTTTTTCGAAAGGT 208
PPWBja (EF186825)               140 AACAGTTGCTAAGACAGGATAGGAAATTAAGAGGCATCTTTTAATTTTTAAAAGACCTTTTTCGAAAGGT 209
PPWBpr (EF186824)               140 AACAGTTGCTAAGACAGGATAGGAAATTAAGAGGCATCTTTTAATTTTTAAAAGACCTTTTTCGAAAGGT 209
PPWBfl (EF186826)               141 AACAGTTGCTAAGACAGGATAGGAAATTAAGAGGCATCTTTTAATTTTTAAAAGACCTTTTTCGAAAGGT 210

                                               220       230       240       250       260       270       280
                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)          1   ---------------------------------------------------------------------- 1
Melornemis antillarum (JD)      1   ---------------------------------------------------------------------- 1
Colpoptera maculifrons (Ad)     210 ATACTTAAAGAGGGGCTTGCGCCACATTAGTTAGTTGGTAAGGTAAAAGCTTACCAAGGCGATGATGTGT 279
Periwinkle little leaf (Ma) 6   210 ATACTTAAAGAGGGGCTTGCGCCACATTAGTTAGTTGGTAAGGTAAAAGCTTACCAAGACGATGATGTGT 279
Periwinkle yellowing (Ma) 7     210 ATACTTAAAGAGGGGCTTGCGCCACATTAGTTAGTTGGTAAGGTAAAAGCTTACCAAGACGATGATGTGT 279
Periwinkle (Ma) 5               210 ATACTTAAAGAGGGGCTTGCGCCACATTAGTTAGTTGGTAAGGTAAAAGCTTACCAAGACGATGATGTGT 279
Pigeon pea (JD) 33              208 ATACTTAAAGAGGGGCTTGCGCCACATTAGTTAGTTGGTAAGGTAAAAGCTTACCAAGACGATGATGTGT 277
Pigeon pea (Is) 45              210 ATACTTAAAGAGGGGCTTGCGCCACATTAGTTAGTTGGTAAGGTAAAAGCTTACCAAGACGATGATGTGT 279
Tabebuia (Ma) 2                 208 ATACTTAAAGAGGGGCTTGCGCCACATTAGTTAGTTGGTAAGGTAAAAGCTTACCAAGACGATGATGTGT 277
Tabebuia (Ma) 3                 210 ATACTTAAAGAGGGGCTTGCGCCCCATTAGTTAGTTGGTAAGGTAAAAGCTTACCAAGGCGGTGATGTGT 279
Orange (Is) 40                  210 ATACTTAAAGAGGGGCTTGCGCCACATTAGTTAGTTGGTAAGGTAAAAGCTTACCAAGACGATGATGTGT 279
Orange (JD) 32                  210 ATACTTAAAGAGGGGCTTGCGCCACATTAGTTAGTTGGTAAGGTAAAAGCTTACCAAGACGATGATGTGT 279
Pigeon pea (Is) 43              210 ATACTTAAAGAGGGGCTTGCGCCACATTAGTTAGTTGGTAAGGTAAAAGCTTACCAAGACGATGATGTGT 279
Pigeon pea (Is) 44              209 ATACTTAAAGAGGGGCTTGCGCCACATTAGTTAGTTGGTAAGGTAAAAGCTTACCAAGACGATGATGTGT 278
PPWBja (EF186825)               210 ATACTTAAAGAGGGGCTTGCGCCACATTAGTTAGTTGGTAAGGTAAAAGCTTACCAAGACGATGATGTGT 279
PPWBpr (EF186824)               210 ATACTTAAAGAGGGGCTTGCGCCACATTAGTTAGTTGGTAAGGTAAAAGCTTACCAAGACGATGATGTGT 279
PPWBfl (EF186826)               211 ATACTTAAAGAGGGGCTTGCGCCACATTAGTTAGTTGGTAAGGTAAAAGCTTACCAAGACGATGATGTGT 280
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                                             290       300       310       320       330       340       350
                                      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)          1     ---------------------------------------------------------------------- 1
Melornemis antillarum (JD)      1     ---------------------------------------------------------------------- 1
Colpoptera maculifrons (Ad)     280   AGCTGGACTGAGAGGTCGAACAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAG 349
Periwinkle little leaf (Ma) 6   280   AGCTGGACTGAGAGGTCGAACAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAG 349
Periwinkle yellowing (Ma) 7     280   AGCTGGACTGAGAGGTCGAACAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAG 349
Periwinkle (Ma) 5               280   AGCTGGACTGAGAGGTCGAACAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAG 349
Pigeon pea (JD) 33              278   AGCTGGACTGAGAGGTCGAACAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAG 347
Pigeon pea (Is) 45              280   AGCTGGACTGAGAGGTCGAACAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAG 349
Tabebuia (Ma) 2                 278   AGCTGGACTGAGAGGTCGAACAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAG 347
Tabebuia (Ma) 3                 280   AGCTGGACTGAGAGGTCGAACAGCCACATTGGGACTGGGACACGGCCCAAACTCCTACGGGAGGCAGCAG 349
Orange (Is) 40                  280   AGCTGGACTGAGAGGTCGAACAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAG 349
Orange (JD) 32                  280   AGCTGGACTGAGAGGTCGAACAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAG 349
Pigeon pea (Is) 43              280   AGCTGGACTGAGAGGTCGAACAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAG 349
Pigeon pea (Is) 44              279   AGCTGGACTGAGAGGTCGAACAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAG 348
PPWBja (EF186825)               280   AGCTGGACTGAGAGGTCGAACAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAG 349
PPWBpr (EF186824)               280   AGCTGGACTGAGAGGTCGAACAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAG 349
PPWBfl (EF186826)               281   AGCTGGACTGAGAGGTCGAACAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAG 350

                                             360       370       380       390       400       410       420
                                      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)          1     ---------------ATTGGAGAAAACTCTGACCGAGCGA----CGCCGCGTGAACAATGAAGTACTTC- 50
Melornemis antillarum (JD)      1     ---------------AAGGGGAGGAACTCTGACCGAGCGA----CGCCGCGTGAACAATGAAGTACTTC- 50
Colpoptera maculifrons (Ad)     350   TAAGGAATTTTTGGCATTGGAGGAAACTCTGACCGAGCGA----CGCCGCGTGAACAATGAAGTACTTC- 414
Periwinkle little leaf (Ma) 6   350   TAAGTTATGTATGGCAATAATGAATACTCTCACCGTGCGAGTGTCCTCTCGTGAACAATGAAGTACTTC- 418
Periwinkle yellowing (Ma) 7     350   TAAGTTATGTATGGCAATGGAGGAAACTCTGACGGAGCCA----CGCCGCGTAAACAAGGAAGTACTTC- 414
Periwinkle (Ma) 5               350   TAAGGAATTTTTGGCAATGGAGGAAACTCTGACCGAGCGA----CGCCGCGTGAACAATGAAGTACTTC- 414
Pigeon pea (JD) 33              348   TAAGGAATTTTTGGCAATGGAGGAAACTCTGACCGAGGGA----CGCCGCGTGAAAAATGAAGTACTTCG 413
Pigeon pea (Is) 45              350   TAAGGAATTTTTGGCAATGGAGGAAACTCTGACCGGGCAA----CACCGCGAGAACAATGAAGTACTTCG 415
Tabebuia (Ma) 2                 348   TAAGGAATTTTTGGCAATGGAGGAAACTCTGACCGAGCGA----CGCCGCGTGAACAATGAAGTACTTC- 412
Tabebuia (Ma) 3                 350   TAAGGAATTTTTGGCAATGGAGGAAACTCTGACCGAGCGA----CGCCGCGTGAACAATGAAGTACTTC- 414
Orange (Is) 40                  350   TAAGGAATTTTTGGCAATGGAGGAAACTCTGACCGAGCGA----CGCCGCGTGAACAATGAAGTACTTC- 414
Orange (JD) 32                  350   TAAGGAATTTTTGGCAATGGAGGAAACTCTGACCGAGCGA----CGCCGCGTGAACAATGAAGTACTTC- 414
Pigeon pea (Is) 43              350   TAAGGAATTTTTGGCAATGGAGGAAACTCTGACCGAGCGA----CGCCGCGTGAACAATGAAGTACTTC- 414
Pigeon pea (Is) 44              349   TAAGGAATTTTTGGCAATGGAGGAAACTCTGACCGAGCGA----CGCCGCGTGAACAATGAAGTACTTC- 413
PPWBja (EF186825)               350   TAAGGAATTTTTGGCAATGGAGGAAACTCTGACCGAGCGA----CGCCGCGTGAACAATGAAGTACTTC- 414
PPWBpr (EF186824)               350   TAAGGAATTTTTGGCAATGGAGGAAACTCTGACCGAGCGA----CGCCGCGTGAACAATGAAGTACTTC- 414
PPWBfl (EF186826)               351   TAAGGAATTTTTGGCAATGGAGGAAACTCTGACCGAGCGA----CGCCGCGTGAACAATGAAGTACTTC- 415

                                             430       440       450       460       470       480       490
                                      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)          51    GGTATGTAAAGTTCTTT--TATTGAAAAAGAAAAAATAGTGGAAAAACTATCTTGACATTATTCAATGAA 118
Melornemis antillarum (JD)      51    GGTATGTAAAGTTCTTT--TATTGAAAAAGAAAAAATAGTGGAAAAACTATCTTGACATTATTCAATGAA 118
Colpoptera maculifrons (Ad)     415   GGTATGTAAAGTTCTTT--TATTGAAAAAGAAAAAATAGTGGAAAAACTATCTTGACATTATTCAATGAA 482
Periwinkle little leaf (Ma) 6   419   GGTATGTAAAGTTATATAATATTGAAAAAGAAAAAATAGTGGTAAAATTATCTTGACATAATTCAATGAC 488
Periwinkle yellowing (Ma) 7     415   GGTATGTAAAGTTCTTT--TATTGAAAAAGAAAAAATAGTGGAAAAACTATCTTGACATTATTCAATGAA 482
Periwinkle (Ma) 5               415   GGTATGTAAAGTTCTTT--TATTGAAAAAGAAAAAATAGTGGAAAAACTATCTTGACATTATTCAATGAA 482
Pigeon pea (JD) 33              414   GGTATGTAAAGTTCTTT--TATTGAAAAAGAAAAAATAGTGGAAAAACTATCTTGACATTATTCAATGAA 481
Pigeon pea (Is) 45              416   GGTATGTAAAGTTCTTT--TATTGAAAAAGAAAAAATAGTGGAAAAACTATCTTGACATTATTCAATGAA 483
Tabebuia (Ma) 2                 413   GGTATGTAAAGTTCTTT--TATTGAAAAAGAAAAAATAGTGGAAAAACTATCTTGACATTATTCAATGAA 480
Tabebuia (Ma) 3                 415   GGTATGTAAAGTTCTTT--TATTGAAAAAGAAAAAATAGTGGAAAAACTATCTTGACATTATTCAATGAA 482
Orange (Is) 40                  415   GGTATGTAAAGTTCTTT--TATTGAAAAAGAAAAAATAGTGGAAAAACTATCTTGACATTATTCAATGAA 482
Orange (JD) 32                  415   GGTATGTAAAGTTCTTT--TATTGAAAAAGAAAAAATAGTGGAAAAACTATCTTGACATTATTCAATGAA 482
Pigeon pea (Is) 43              415   GGTATGTAAAGTTCTTT--TATTGAAAAAGAAAAAATAGTGGAAAAACTATCTTGACATTATTCAATGAA 482
Pigeon pea (Is) 44              414   GGTATGTAAAGTTCTTT--TATTGAAAAAGAAAAAATAGTGGAAAAACTATCTTGACATTATTCAATGAA 481
PPWBja (EF186825)               415   GGTATGTAAAGTTCTTT--TATTGAAAAAGAAAAAATAGTGGAAAAACTATCTTGACATTATTCAATGAA 482
PPWBpr (EF186824)               415   GGTATGTAAAGTTCTTT--TATTGAAAAAGAAAAAATAGTGGAAAAACTATCTTGACATTATTCAATGAA 482
PPWBfl (EF186826)               416   GGTATGTAAAGTTCTTT--TATTGAAAAAGAAAAAATAGTGGAAAAACTATCTTGACATTATTCAATGAA 483

                                             500       510       520       530       540       550       560
                                      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)          119   TAAGCCCCGGCTAATTATGTGCCA-GC-AGCC---GCGGTAATACATAAGGGGCGAGCGTTATCCGGAAT 183
Melornemis antillarum (JD)      119   TAAGCCCCGGCTAATTATGTGCCA-GC-AGCC---GCGGTAATACATAAGGGGCGAGCGTTATCCGGAAT 183
Colpoptera maculifrons (Ad)     483   TAAGCCCCGGCTAATTATGTGCCA-GC-AGCC---GCGGTAATACATAAGGGGCGAGCGTTATCCGGAAT 547
Periwinkle little leaf (Ma) 6   489   TAAGCCCCAGCTAATTATGTGCCACGCGAGCC---GCGGTAAAACGTGAGGGGCGAGCGTTATCCGGAAT 555
Periwinkle yellowing (Ma) 7     483   TAAGCCCCGGCTAATTATGTGCCA-GC-AGCC---GCGGTAATACATAAGGGGCGAGCGTTATCCGGAAT 547
Periwinkle (Ma) 5               483   TAAGCCCCGGCTAATTATGTGCCA-GC-AGCC---GCGGTAATACATAAGGGGCGAGCGTTATCCGGAAT 547
Pigeon pea (JD) 33              482   TAAGCCCCGGCTAATTATGTGCCA-GC-AGCC---GCGGTAATACATAAGGGGCGAGCGTTATCCGGAAT 546
Pigeon pea (Is) 45              484   TAAGCCCCGGCTAATTATGTGCCA-GC-AGCC---GCGGTAATACATAAGGGGCGAGCGTTATCCGGAAT 548
Tabebuia (Ma) 2                 481   TAAGCCCCGGCTAATTATGTGCCA-GC-AGCC---GCGGTAATACATAAGGGGCGAGCGTTATCCGGAAT 545
Tabebuia (Ma) 3                 483   TAAGCCCCGGCTAATTATGTGCCA-GC-AGCC---GCGGTAATACATAAGGGGCGAGCGTTATCCGGAAT 547
Orange (Is) 40                  483   TAAGCCCCGGCTAATTATGTGCCA-GC-AGCCGCGGCGGTAATACATAAGGGGCGAGCGTTATCCGGAAT 550
Orange (JD) 32                  483   TAAGCCCCGGCTAATTATGTGCCA-GC-AGCC---GCGGTAATACATAAGGGGCGAGCGTTATCCGGAAT 547
Pigeon pea (Is) 43              483   TAAGCCCCGGCTAATTATGTGCCA-GC-AGCC---GCGGTAATACATAAGGGGCGAGCGTTATCCGGAAT 547
Pigeon pea (Is) 44              482   TAAGCCCCGGCTAATTATGTGCCA-GC-AGCC---GCGGTAATACATAAGGGGCGAGCGTTATCCGGAAT 546
PPWBja (EF186825)               483   TAAGCCCCGGCTAATTATGTGCCA-GC-AGCC---GCGGTAATACATAAGGGGCGAGCGTTATCCGGAAT 547
PPWBpr (EF186824)               483   TAAGCCCCGGCTAATTATGTGCCA-GC-AGCC---GCGGTAATACATAAGGGGCGAGCGTTATCCGGAAT 547
PPWBfl (EF186826)               484   TAAGCCCCGGCTAATTATGTGCCA-GC-AGCC---GCGGTAATACATAAGGGGCGAGCGTTATCCGGAAT 548
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                                                  570       580       590       600       610       620       630
                                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)               184  TATTGGGCGTAAAGGGT-GCGTAGGCGG-TTTGATAAGTCTATAGT-TTAAATGCAGTGCCT-AACGCTG 249
Melornemis antillarum (JD)           184  TATTGGGCGTAAAGGGT-GCGTAGGCGG-TTTGATAAGTCTATAGT-TTAAATGCAGTGCTT-AACGCTG 249
Colpoptera maculifrons (Ad)          548  TATTGGGCGTAAAGGGT-GCGTAGGCGG-TTTGATAAGTCTATAGT-TTAAATGCAGTGCTT-AACGCTG 613
Periwinkle little leaf (Ma) 6        556  TGTTGGGCGTAAAGCGT-GCGTAGGCGG-TTTGATAAGTCTATAGT-CTAAATGCAGTGCTT-AACGCTG 621
Periwinkle yellowing (Ma) 7          548  TATTGGGCGTAAAGGGT-GCGTAGGCGG-TTTGATAAGTCTATAGT-TTAAATGCAGTGCTT-AACGCTG 613
Periwinkle (Ma) 5                    548  TATTGGGCGTAAAGGGT-GCGTAGGCGG-TTTGATAAGTCTATAGT-TTAAATGCAGTGCTT-AACGCTG 613
Pigeon pea (JD) 33                   547  TATTGGGCGTAAAGGGT-GCGTAGGCGG-TTTGATAAGTCTATAGT-TTAAATGCAGTGCTT-AACGCTG 612
Pigeon pea (Is) 45                   549  TATTGGGCGTAAAGGGT-GCGTAGGCGG-TTTGATAAGTCTATAGT-TTAAATGCAGTGCTT-AACGCTG 614
Tabebuia (Ma) 2                      546  TATTGGGCGTAAAGGGT-GCGTAGGCGG-TTTGATAAGTCTATAGT-TTAAATGCAGTGCTT-AACGCTG 611
Tabebuia (Ma) 3                      548  TATTGGGCGTAAAGGGT-GCGTAGGCGG-TTTGATAAGTCTATAGT-TTAAATGCAGTGCTT-AACGCTG 613
Orange (Is) 40                       551  TATTGGGCGTAAAGGGTGGCGTAGGCGGCTTTGATAAGTCTATAGTATTAAATGCAGTGCTTAAACGCTG 620
Orange (JD) 32                       548  TATTGGGCGTAAAGGGT-GCGTAGGCGG-TTTGATAAGTCTATAGT-TTAAATGCAGTGCTT-AACGCTG 613
Pigeon pea (Is) 43                   548  TATTGGGCGTAAAGGGT-GCGTAGGCGG-TTTGATAAGTCTATAGT-TTAAATGCAGTGCTT-AACGCTG 613
Pigeon pea (Is) 44                   547  TATTGGGCGTAAAGGGT-GCGTAGGCGG-TTTGATAAGTCTATAGT-TTAAATGCAGTGCTT-AACGCTG 612
PPWBja (EF186825)                    548  TATTGGGCGTAAAGGGT-GCGTAGGCGG-TTTGATAAGTCTATAGT-TTAAATGCAGTGCTT-AACGCTG 613
PPWBpr (EF186824)                    548  TATTGGGCGTAAAGGGT-GCGTAGGCGG-TTTGATAAGTCTATAGT-TTAAATGCAGTGCTT-AACGCTG 613
PPWBfl (EF186826)                    549  TATTGGGCGTAAAGGGT-GCGTAGGCGG-TTTGATAAGTCTATAGT-TTAAATGCAGTGCTT-AACGCTG 614

                                                  640       650       660       670       680       690       700
                                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)               250  TAGCGCTATAGAAACTGTCTGACTAGAGTTAGATAGAGGCAAGCGGAATTCCATGTGTAGCGGTAAAATG 319
Melornemis antillarum (JD)           250  TAGCGCTATAGAAACTGTCTGACTAGAGTTAGATAGAGGCAAGCGGAATTCCATGTGTAGCGGTAAAATG 319
Colpoptera maculifrons (Ad)          614  TAGCGCTATAGAAACTGTCTGACTAGAGTTAGATAGAGGCAAGCGGAATTCCATGTGTAGCGGTAAAATG 683
Periwinkle little leaf (Ma) 6        622  TAGCGCTATAGAAACTGTCTGACTAGAGTTAGATAGAGGCAAGCGGAATTCCATGTGTAGCGGTAAAATG 691
Periwinkle yellowing (Ma) 7          614  TAGCGCTATAGAAACTGTCTGACTAGAGTTAGATAGAGGCAAGCGGAATTCCATGTGTAGCGGTAAAATG 683
Periwinkle (Ma) 5                    614  TAGCGCTATAGAAACTGTCTGACTAGAGTTAGATAGAGGCAAGCGGAATTCCATGTGTAGCGGTAAAATG 683
Pigeon pea (JD) 33                   613  TAGCGCTATAGAAACTGTCTGACTAGAGTTAGATAGAGGCAAGCGGAATTCCATGTGTAGCGGTAAAATG 682
Pigeon pea (Is) 45                   615  TAGCGCTATAGAAACTGTCTGACTAGAGTTAGATAGAGGCAAGCGGAATTCCATGTGTAGCGGTAAAATG 684
Tabebuia (Ma) 2                      612  TAGCGCTATAGAAACTGTCTGACTAGAGTTAGATAGAGGCAAGCGGAATTCCATGTGTAGCGGTAAAATG 681
Tabebuia (Ma) 3                      614  TAGCGCTATAGAAACTGTCTGACTAGAGTTAGATAGAGGCAAGCGGAATTCCATGTGTAGCGGTAAAATG 683
Orange (Is) 40                       621  TAGCGCTATAAAAACTGTCTGACTAGAGTTAGATAGAGGCAAGCGGAATTCCATGTGTAGCGGTAAAATG 690
Orange (JD) 32                       614  TAGCGCTATAGAAACTGTCTGACTAGAGTTAGATAGAGGCAAGCGGAATTCCATGTGTAGCGGTAAAATG 683
Pigeon pea (Is) 43                   614  TAGCGCTATAGAAACTGTCTGACTAGAGTTAGATAGAGGCAAGCGGAATTCCATGTGTAGCGGTAAAATG 683
Pigeon pea (Is) 44                   613  TAGCGCTATAGAAACTGTCTGACTAGAGTTAGATAGAGGCAAGCGGAATTCCATGTGTAGCGGTAAAATG 682
PPWBja (EF186825)                    614  TAGCGCTATAGAAACTGTCTGACTAGAGTTAGATAGAGGCAAGCGGAATTCCATGTGTAGCGGTAAAATG 683
PPWBpr (EF186824)                    614  TAGCGCTATAGAAACTGTCTGACTAGAGTTAGATAGAGGCAAGCGGAACTCCACGTGTAGCGGTAAAATG 683
PPWBfl (EF186826)                    615  TAGCGCTATAGAAACTGTCTGACTAGAGTTAGATAGAGGCAAGCGGAATTCCATGTGTAGCGGTAAAATG 684

                                                  710       720       730       740       750       760       770
                                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)               320  CGTAAATATATGGAGGAACACCAGAGGCGTAGGCGGCTTGCGGGGTCTTAACTGACGCTGAGGCACGAAA 389
Melornemis antillarum (JD)           320  CGTAAATATATGGAGGAACACCAGAGGCGTAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAA 389
Colpoptera maculifrons (Ad)          684  CGTAAATATATGGAGGAACACCAGAGGCGTAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAA 753
Periwinkle little leaf (Ma) 6        692  CGTAAATATATGGAGGAACACCAGAGGCGTAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAA 761
Periwinkle yellowing (Ma) 7          684  CGTAAATATATGGAGGAACACCAGAGGCGTAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAA 753
Periwinkle (Ma) 5                    684  CGTAAATATATGGAGGAACACCAGAGGCGTAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAA 753
Pigeon pea (JD) 33                   683  CGTAAATATATGGAGGAACACCAGAGGCGTAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAA 752
Pigeon pea (Is) 45                   685  CGTAAATATATGGAGGAACACCAGAGGCGTAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAA 754
Tabebuia (Ma) 2                      682  CGTAAATATATGGAGGAACACCAGAGGCGTAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAA 751
Tabebuia (Ma) 3                      684  CGTAAATATATGGAGGAACACCAGAGGCGTAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAA 753
Orange (Is) 40                       691  CGTAAATATATGGAGGAACACCAGAGGCGTAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAA 760
Orange (JD) 32                       684  CGTAAATATATGGAGGAACACCAGAGGCGTAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAA 753
Pigeon pea (Is) 43                   684  CGTAAATATATGGAGGAACACCAGAGGCGTAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAA 753
Pigeon pea (Is) 44                   683  CGTAAATATATGGAGGAACACCAGAGGCGTAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAA 752
PPWBja (EF186825)                    684  CGTAAATATATGGAGGAACACCAGAGGCGTAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAA 753
PPWBpr (EF186824)                    684  CGTAAATATATGGAGGAACACCAGAGGCGTAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAA 753
PPWBfl (EF186826)                    685  CGTAAATATATGGAGGAACACCAGAGGCGTAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAA 754

                                                  780       790       800       810       820       830       840
                                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)               390  GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAAGTGTCGGGT 459
Melornemis antillarum (JD)           390  GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAAGTGTCGGGT 459
Colpoptera maculifrons (Ad)          754  GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAAGTGTCGGGT 823
Periwinkle little leaf (Ma) 6        762  GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAAGTGTCGGGT 831
Periwinkle yellowing (Ma) 7          754  GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAAGTGTCGGGT 823
Periwinkle (Ma) 5                    754  GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAAGTGTCGGGT 823
Pigeon pea (JD) 33                   753  GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAAGTGTCGGGT 822
Pigeon pea (Is) 45                   755  GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAAGTGTCGGGT 824
Tabebuia (Ma) 2                      752  GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAAGTGTCGGGT 821
Tabebuia (Ma) 3                      754  GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAAGTGTCGGGT 823
Orange (Is) 40                       761  GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAAGTGTCGGGT 830
Orange (JD) 32                       754  GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAAGTGTCGGGT 823
Pigeon pea (Is) 43                   754  GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAAGTGTCGGGT 823
Pigeon pea_(Is)_44                   753  GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAAGTGTCGGGT 822
PPWBja (EF186825)                    754  GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAAGTGTCGGGT 823
PPWBpr (EF186824)                    754  GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAAGTGTCGGGT 823
PPWBfl (EF186826)                    755  GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAAGTGTCGGGT 824
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                                             850        860        870        880        890        900        910
                                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)              460 TTCGACTCGGTACTGAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTA 529
Melornemis antillarum (JD)          460 TTCGACTCGGTACTGAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTA 529
Colpoptera maculifrons (Ad)         824 TTCGACTCGGTACTGAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTA 893
Periwinkle little leaf (Ma) 6       832 TTCGACTCGGTACTGAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTA 901
Periwinkle yellowing (Ma) 7         824 TTCGACTCGGTACTGAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTA 893
Periwinkle (Ma) 5                   824 TTCGACTCGGTACTGAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTA 893
Pigeon pea (JD) 33                  823 TTCGACTCGGTACTGAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTA 892
Pigeon pea (Is) 45                  825 TTCGACTCGGTACTGAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTA 894
Tabebuia (Ma) 2                     822 TTCGACTCGGTACTGAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTA 891
Tabebuia (Ma) 3                     824 TTCGACTCGGTACTGAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTA 893
Orange (Is) 40                      831 TTCGACTCGGTACTGAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTA 900
Orange (JD) 32                      824 TTCGACTCGGTACTGAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTA 893
Pigeon pea (Is) 43                  824 TTCGACTCGGTACTGAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTA 893
Pigeon pea (Is) 44                  823 TTCGACTCGGTACTGAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTA 892
PPWBja (EF186825)                   824 TTCGACTCGGTACTGAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTA 893
PPWBpr (EF186824)                   824 TTCGACTCGGTACTGAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTA 893
PPWBfl (EF186826)                   825 TTCGACTCGGTACTGAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTA 894

                                             920        930        940        950        960        970        980
                                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)              530 AAGGAATTGACGGGACCCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGATACACGAAAAACCTTA 599
Melornemis antillarum (JD)          530 AAGGAATTGACGGGACCCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGATACACGAAAAACCTTA 599
Colpoptera maculifrons (Ad)         894 AAGGAATTGACGGGACCCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGATACACGAAAAACCTTA 963
Periwinkle little leaf (Ma) 6       902 AAGGAATTGACGGGACCCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGATACACGAAAAACCTTA 971
Periwinkle yellowing (Ma) 7         894 AAGGAATTGACGGGACCCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGATACACGAAAAACCTTA 963
Periwinkle (Ma) 5                   894 AAGGAATTGACGGGACCCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGATACACGAAAAACCTTA 963
Pigeon pea (JD) 33                  893 AAGGAATTGACGGGACCCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGATACACGAAAAACCTTA 962
Pigeon pea (Is) 45                  895 AAGGAATTGACGGGACCCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGATACACGAAAAACCTTA 964
Tabebuia (Ma) 2                     892 AAGGAATTGACGGGACCCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGATACACGAAAAACCTTA 961
Tabebuia (Ma) 3                     894 AAGGAATTGACGGGACCCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGATACACGAAAAACCTTA 963
Orange (Is) 40                      901 AAGGAATTGACGGGACCCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGATACACGAAAAACCTTA 970
Orange (JD) 32                      894 AAGGAATTGACGGGACCCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGATACACGAAAAACCTTA 963
Pigeon pea (Is) 43                  894 AAGGAATTGACGGGACCCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGATACACGAAAAACCTTA 963
Pigeon pea (Is) 44                  893 AAGGAATTGACGGGACCCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGATACACGAAAAACCTTA 962
PPWBja (EF186825)                   894 AAGGAATTGACGGGACCCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGATACACGAAAAACCTTA 963
PPWBpr (EF186824)                   894 AAGGAATTGACGGGACCCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGATACACGAAAAACCTTA 963
PPWBfl (EF186826)                   895 AAGGAATTGACGGGACCCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGATACACGAAAAACCTTA 964

                                             990       1000       1010       1020       1030       1040       1050
                                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)              600 CCAGGTCTTGACATAATTTTGCGACATTATAGAAATATA-ATGAAGGTTATCAGAATTACAGGTGGTGCA 668
Melornemis antillarum (JD)          600 CCAGGTCTTGACATAATTTTGCGACATTATAGAAATATA-ATGAAGGTTATCAGAATTACAGGTGGTGCA 668
Colpoptera maculifrons (Ad)         964 CCAGGTCTTGACATAATTTTGCGACATTATAGAAATATA-ATGAAGGTTATCAGAATTACAGGTGGTGCA 1032
Periwinkle little leaf (Ma) 6       972 CCAGGTCTTGACATAATTTTGCGACATTATAGAAATATA-ATGAAGGTTATCAGAATTACAGGTGGTGCA 1040
Periwinkle yellowing (Ma) 7         964 CCAGGTCTTGACATAATTTTGCGACATTATAGAAATATA-ATGAAGGTTATCAGAATTACAGGTGGTGCA 1032
Periwinkle (Ma) 5                   964 CCAGGTCTTGACATAATTTTGCGACATTATAGAAATATA-ATGAAGGTTATCAGAATTACAGGTGGTGCA 1032
Pigeon pea (JD) 33                  963 CCAGGTCTTGACATAATTTTGCGACATTATAGAAATATA-ATGAAGGTTATCAGAATTACAGGTGGTGCA 1031
Pigeon pea (Is) 45                  965 CCAGGTCTTGACATAATTTTGCGACATTATAGAAATATA-ATGAAGGTTATCAGAATTACAGGTGGTGCA 1033
Tabebuia (Ma) 2                     962 CCAGGTCTTGACATAATTTTGCGACATTATAGAAATATA-ATGAAGGTTATCAGAATTACAGGTGGTGCA 1030
Tabebuia (Ma) 3                     964 CCAGGTCTTGACATAATTTTGCGACATTATAGAAATATA-ATGAAGGTTATCAGAATTACAGGTGGTGCA 1032
Orange (Is) 40                      971 CCAGGTCTTGACATAATTTTGCGACATTATAGAAATATACATGAAGGTTATCAGAATTACAGGTGGTGCA 1040
Orange (JD) 32                      964 CCAGGTCTTGACATAATTTTGCGACATTATAGAAATATA-ATGAAGGTTATCAGAATTACAGGTGGTGCA 1032
Pigeon pea (Is) 43                  964 CCAGGTCTTGACATAATTTTGCGACATTATAGAAATATA-ATGAAGGTTATCAGAATTACAGGTGGTGCA 1032
Pigeon pea (Is) 44                  963 CCAGGTCTTGACATAATTTTGCGACATTATAGAAATATA-ATGAAGGTTATCAGAATTACAGGTGGTGCA 1031
PPWBja (EF186825)                   964 CCAGGTCTTGACATAATTTTGCGACATTATAGAAATATA-ATGAAGGTTATCAGAATTACAGGTGGTGCA 1032
PPWBpr (EF186824)                   964 CCAGGTCTTGACATAATTTTGCGACATTATAGAAATATA-ATGAAGGTTATCAGAATTACAGGTGGTGCA 1032
PPWBfl (EF186826)                   965 CCAGGTCTTGACATAATTTTGCGACATTATAGAAATATA-ATGAAGGTTATCAGAATTACAGGTGGTGCA 1033

                                            1060       1070       1080       1090       1100       1110       1120
                                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)              669 TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTAGGTTAAGTCCT-AAAACGAGCGCAACCCTTGCCG-TTA 736
Melornemis antillarum (JD)          669 TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTAGGTTAAGTCCT-AAAACGAGCGCAACCCTTGTCG-TTA 736
Colpoptera maculifrons (Ad)        1033 TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTAGGTTAAGTCCT-AAAACGAGCGCAACCCTTGTCG-TTA 1100
Periwinkle little leaf (Ma) 6      1041 TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTAGGTTAAGTCCT-AAAACGAGCGCAACCCTTGTCG-TTA 1108
Periwinkle yellowing (Ma) 7        1033 TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTAGGTTAAGTCCT-AAAACGAGCGCAACCCTTGTCG-TTA 1100
Periwinkle (Ma) 5                  1033 TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTAGGTTAAGTCCT-AAAACGAGCGCAACCCTTGTCGTTTA 1101
Pigeon pea (JD) 33                 1032 TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTAGGTTAAGTCCT-AAAACGAGCGCAACCCTTGTCG-TTA 1099
Pigeon pea (Is) 45                 1034 TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTAGGTTAAGTCCT-AAAACGAGCGCAACCCTTGTCG-TTA 1101
Tabebuia (Ma) 2                    1031 TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTAGGTTAAGTCCT-AAAACGAGCGCAACCCTTGTCG-TTA 1098
Tabebuia (Ma) 3                    1033 TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTAGGTTAAGTCCT-AAAACGAGCGCAACCCTTGTCG-TTA 1100
Orange (Is) 40                     1041 TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTAGGTTAAGTCCT-AAAACGAGCGCAACCCTTGTCG-TTA 1108
Orange (JD) 32                     1033 TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTAGGTTAAGTCCT-AAAACGAGCGCAACCCTTGTCG-TTA 1100
Pigeon pea (Is) 43                 1033 TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTAGGTTAAGTCCT-AAAACGAGCGCAACCCTTGTCG-TTA 1100
Pigeon pea (Is) 44                 1032 TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTAGGTTAAGTCCT-AAAACGAGCGCAACCCTTGTCG-TTA 1099
PPWBja (EF186825)                  1033 TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTAGGTTAAGTCCT-AAAACGAGCGCAACCCTTGTCG-TTA 1100
PPWBpr (EF186824)                  1033 TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTAGGTTAAGTCCT-AAAACGAGCGCAACCCTTGTCG-TTA 1100
PPWBfl (EF186826)                  1034 TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTAGGTTAAGTCCTAAAAACGAGCGCAACCCTTGTCG-TTA 1102
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                                              1130      1140      1150      1160      1170      1180      1190
                                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)             737  GTTGCG-ACCACGTAATGGT--GAGCAC-TTTAGC-GAGACTGCCAATG-AAAAATTGG-AGGAAGGTGA 799
Melornemis antillarum (JD)         737  GTTGCG-ACCACGTAATGGT--GAGCAC-TTTAGC-GAGACTGCCAATG-AAAAATTGG-AGGAAGGTGA 799
Colpoptera maculifrons (Ad)        1101 GTTGCG-ACCACGTAATGGT--GAGCAC-TTTAGC-GAGACTGCCAATG-AAAAATTGG-AGGAAGGTGA 1163
Periwinkle little leaf (Ma) 6      1109 GTTGCG-ACCACGTAATGGT--GAGCAC-TTTAGC-GAGACTGCCAATG-AAAAATTGG-AGGAAGGTGA 1171
Periwinkle yellowing (Ma) 7        1101 GTTGCG-ACCACGTAATGGT--GAGCAC-TTTAGC-GAGACTGCCAATG-AAAAATTGG-AGGAAGGTGA 1163
Periwinkle (Ma) 5                  1102 GTTGCGTACCACGTAATGGT--GAGCACATTTAGCTGAGACTGCCAATGCAAAAATTTGGAGGAAGGTGA 1169
Pigeon pea (JD) 33                 1100 GTTGCG-ACCACGTAATGGT--GAGCAC-TTTAGC-GAGACTGCCAATG-AAAAATTGGAAGGAAGGTGA 1163
Pigeon pea (Is) 45                 1102 GTTGCG-ACCACGTAATGGT--GAGCAC-TTTAGC-GAGACTGCCAATG-AAAAATTGG-AGGAAGGTGA 1164
Tabebuia (Ma) 2                    1099 GTTGCG-ACCACGTAATGGT--GAGCAC-TTTAGC-GAGACTGCCAATG-AAAAATTGG-AGGAAGGTGA 1161
Tabebuia (Ma) 3                    1101 GTTGCG-ACCACGTAATGGT--GAGCAC-TTTAGC-GAGACTGCCAATG-AAAAATTGG-AGGAAGGTGA 1163
Orange (Is) 40                     1109 GTTGCG-ACCACGTAATGGTTCGAGCAC-TTTAGC-GAGACTGCCAATG-AAAAATTGG-AGGAAGGTGA 1173
Orange (JD) 32                     1101 GTTGCG-ACCACGTAATGGT--GAGCAC-TTTAGC-GAGACTGCCAATG-AAAAATTGG-AGGAAGGTGA 1163
Pigeon pea (Is) 43                 1101 GTTGCG-ACCACGTAATGGT--GAGCAC-TTTAGC-GAGACTGCCAATG-AAAAATTGG-AGGAAGGTGA 1163
Pigeon pea (Is) 44                 1100 GTTGCG-ACCACGTAATGGT--GAGCAC-TTTAGC-GAGACTGCCAATG-AAAAATTGG-AGGAAGGTGA 1162
PPWBja (EF186825)                  1101 GTTGCG-ACCACGTAATGGT--GAGCAC-TTTAGC-GAGACTGCCAATG-AAAAATTGG-AGGAAGGTGA 1163
PPWBpr (EF186824)                  1101 GTTGCG-ACCACGTAATGGT--GAGCAC-TTTAGC-GAGACTGCCAATG-AAAAATTGG-AGGAAGGTGA 1163
PPWBfl (EF186826)                  1103 GTTGCG-ACCACGTAATGGT--GAGCAC-TTTAGC-GAGACTGCCAATG-AAAAATTGG-AGGAAGGTGA 1165

                                              1200      1210      1220      1230      1240      1250      1260
                                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)             800  GGATTACATCAAATCATCATGCCCCTTATGATCTGGGCTA-CAAACGTGATACAATGGC-TGTTACAA-A 866
Melornemis antillarum (JD)         800  GGATTACGTCAAATCATCATGCCCCTTATGATCTGGGCTA-CAAACGTGATACAATGGC-TGTTACAA-A 866
Colpoptera maculifrons (Ad)        1164 GGATTACGTCAAATCATCATGCCCCTTATGATCTGGGCTA-CAAACGTGATACAATGGC-TGTTACAA-A 1230
Periwinkle little leaf (Ma) 6      1172 GGATTACGTCAAATCATCATGCCCCTTATGATCTGGGCTA-CAAACGTGATACAATGGC-TGTTACAA-A 1238
Periwinkle yellowing (Ma) 7        1164 GGATTACGTCAAATCATCATGCCCCTTATGGTCTGGGCTA-CAAACGTGATACAATGGC-TGTTACAA-A 1230
Periwinkle (Ma) 5                  1170 GGATTACGTCAAATCATCATGCCCCTTATGATCTGGGCTATCAAACGTGATACAATGGC-TGTTACAA-A 1237
Pigeon pea (JD) 33                 1164 GGATTACGTCAAATCATCATGCCCCTTATGATCTGGGCTA-CAAACGTGATACAATGGC-TGTTACAA-A 1230
Pigeon pea (Is) 45                 1165 GGATTACGTCAAATCATCATGCCCCTTATGATCTGGGCTA-CAAACGTGATACAATGGC-TGTTACAA-A 1231
Tabebuia (Ma) 2                    1162 GGATTACGTCAAATCATCATGCCCCTTATGATCTGGGCTA-CAAACGTGATACAATGGCTTGTTACAAGA 1230
Tabebuia (Ma) 3                    1164 GGATTACGTCAAATCATCATGCCCCTTATGATCTGGGCTA-CAAACGTGATACAATGGC-TGTTACAA-A 1230
Orange (Is) 40                     1174 GGATTACGTCAAATCATCATGCCCCTTATGATCTGGGCTA-CAAACGTGATACAATGGC-TGTTACAA-A 1240
Orange (JD) 32                     1164 GGATTACGTCAAATCATCATGCCCCTTATGATCTGGGCTA-CAAACGTGATACAATGGC-TGTTACAA-A 1230
Pigeon pea (Is) 43                 1164 GGATTACGTCAAATCATCATGCCCCTTATGATCTGGGCTA-CAAACGTGATACAATGGC-TGTTACAA-A 1230
Pigeon pea (Is) 44                 1163 GGATTACGTCAAATCATCATGCCCCTTATGATCTGGGCTA-CAAACGTGATACAATGGC-TGTTACAA-A 1229
PPWBja (EF186825)                  1164 GGATTACGTCAAATCATCATGCCCCTTATGATCTGGGCTA-CAAACGTGATACAATGGC-TGTTACAA-A 1230
PPWBpr (EF186824)                  1164 GGATTACGTCAAATCATCATGCCCCTTATGATCTGGGCTA-CAAACGTGATACAATGGC-TGTTACAA-A 1230
PPWBfl (EF186826)                  1166 GGATTACGTCAAATCATCATGCCCCTTATGATCTGGGCTA-CAAACGTGATACAATGGC-TGTTACAA-A 1232

                                              1270      1280      1290      1300      1310      1320      1330
                                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)             867  GAGTAGCTGTAACGTGAGTTTATGGCCAATCTCAAAAAAACAGTCTCAGTTCGGATTGAAGTCTGCAACT 936
Melornemis antillarum (JD)         867  GAGTAGCTGTAACGTGAGTTTATGGCCAATCTCAAAAAAACAGTCTCAGTTCGGATTGAAGTCTGCAACT 936
Colpoptera maculifrons (Ad)        1231 GAGTAGCTGTAACGTGAGTTTATGGCCAATCTCAAAAAAACAGTCTCAGTTCGGATTGAAGTCTGCAACT 1300
Periwinkle little leaf (Ma) 6      1239 GAGTAGCTGTAACGTGAGTTTATGGCCAATCTCAAAAAAACAGTCTCAGTTCGGATTGAAGTCTGCAACT 1308
Periwinkle yellowing (Ma) 7        1231 GAGTAGCTGTAACGTGAGTTTATGGCCAATCTCAAAAAAACAGTCTCAGTTCGGATTGAAGTCTGCAACT 1300
Periwinkle (Ma) 5                  1238 GAGAGGCTGTAACGGGAGTTTATGGCCAATCTCAAAAAAACAGTCTTAGTTCGAATTGAAGTCTGCAACT 1307
Pigeon pea (JD) 33                 1231 GAGTAGCTGTAACGTGAGTTTATGGCCAATCTCAAAAAAACAGTCTCAGTTCGGATTGAAGTCTGCAACT 1300
Pigeon pea (Is) 45                 1232 GAGTAGCTGTAACGTGAGTTTATGGCCAATCTCAAAAAAACAGTCTCAGTTCGGATTGAAGTCTGCAACT 1301
Tabebuia (Ma) 2                    1231 AAGTAGCTGTAACGTGAGTTTATGGCCAATCTCAAAAAAACAGTCTCAGTTCGGATTGAAGTCTGCAACT 1300
Tabebuia (Ma) 3                    1231 GAGTAGCTGTAACGTGAGTTTATGGCCAATCTCAAAAAAACAGTCTCAGTTCGGATTGAAGTCTGCAACT 1300
Orange (Is) 40                     1241 GAGTAGCTGTAACGTGAGTTTATGGCCAATCTCAAAAAAACAGTCTCAGTTCGGATTGAAGTCTGCAACT 1310
Orange (JD) 32                     1231 GAGTAGCTGTAACGTGAGTTTATGGCCAATCTCAAAAAAACAGTCTCAGTTCGGATTGAAGTCTGCAACT 1300
Pigeon pea (Is) 43                 1231 GAGTAGCTGTAACGTGAGTTTATGGCCAATCTCAAAAAAACAGTCTCAGTTCGGATTGAAGTCTGCAACT 1300
Pigeon pea (Is) 44                 1230 GAGTAGCTGTAACGTGAGTTTATGGCCAATCTCAAAAAAACAGTCTCAGTTCGGATTGAAGTCTGCAACT 1299
PPWBja (EF186825)                  1231 GAGTAGCTGTAACGTGAGTTTATGGCCAATCTCAAAAAAACAGTCTCAGTTCGGATTGAAGTCTGCAACT 1300
PPWBpr (EF186824)                  1231 GAGTAGCTGTAACGTGAGTTTATGGCCAATCTCAAAAAAACAGTCTCAGTTCGGATTGAAGTCTGCAACT 1300
PPWBfl (EF186826)                  1233 GAGTAGCTGTAACGTGAGTTTATGGCCAATCTCAAAAAAACAGTCTCAGTTCGGATTGAAGTCTGCAACT 1302

                                              1340      1350      1360      1370      1380      1390      1400
                                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)             937  CGACTTCATGAAGTTGG-AATCGCTAGTAATCGCGAATCAGCATGTCGC--GGTGAATACGTTCTCGGGG 1003
Melornemis antillarum (JD)         937  CGACTTCATGAAGTTGG-AATCGCTAGTAATCGCGAATCAGCATGTCGC--GGTGAATACGTTCTCGGGG 1003
Colpoptera maculifrons (Ad)        1301 CGACTTCATGAAGTTGG-AATCGCTAGTAATCGCGAATCAGCATGTCGC--GGTGAATACGTTCTCGGGG 1367
Periwinkle little leaf (Ma) 6      1309 CGACTTCATGAAGTTGG-AATCGCTAGTAATCGCGAATCAGCATGTCGC--GGTGAATACGTTCTCGGGG 1375
Periwinkle yellowing (Ma) 7        1301 CGACTTCATGAAGTTGG-AATCGCTAGTAATCGCGAATCAGCATGTCGC--GGTGAATACGTTCTCGGGG 1367
Periwinkle (Ma) 5                  1308 CGACTTCATGAAGTTGG-AATCGCTAGTAATCGCGAATCAGCATGGCGC--GGTGAATACGTTCCGGGGG 1374
Pigeon pea (JD) 33                 1301 CGACTTCATGAAGTTGGAAATCGCTAGTAATCGCGAATCAGCATGTCGCCGGGTGAAAACGTTCTCGGGG 1370
Pigeon pea (Is) 45                 1302 CGACTTCATGAAGTTGG-AATCGCTAGTAATCGCGAATCAGCATGTCGC--GGTGAATACGTTCTCGGGG 1368
Tabebuia (Ma) 2                    1301 CGACTTCATGAAGTTGG-AATCGCTAGTAATCGCGAATCAGCATGTCGC--GGTGAATACGTTCTCGGGG 1367
Tabebuia (Ma) 3                    1301 CGACTTCATGAAGTTGG-AATCGCTAGTAATCGCGAATCAGCATGTCGC--GGTGAATACGTTCTCGGGG 1367
Orange (Is) 40                     1311 CGACTTCATGAAGTTGG-AATCGCTAGTAATCGCGAATCAGCATGTCGC--GGTGAATACGTTCTCGGGG 1377
Orange (JD) 32                     1301 CGACTTCATGAAGTTGG-AATCGCTAGTAATCGCGAATCAGCATGTCGC--GGTGAATACGTTCTCGGGG 1367
Pigeon pea (Is) 43                 1301 CGACTTCATGAAGTTGG-AATCGCTAGTAATCGCGAATCAGCATGTCGC--GGTGAATACGTTCTCGGGG 1367
Pigeon pea (Is) 44                 1300 CGACTTCATGAAGTTGG-AATCGCTAGTAATCGCGAATCAGCATGTCGC--GGTGAATACGTTCTCGGGG 1366
PPWBja (EF186825)                  1301 CGACTTCATGAAGTTGG-AATCGCTAGTAATCGCGAATCAGCATGTCGC--GGTGAATACGTTCTCGGGG 1367
PPWBpr (EF186824)                  1301 CGACTTCATGAAGTTGG-AATCGCTAGTAATCGCGAATCAGCATGTCGC--GGTGAATACGTTCTCGGGG 1367
PPWBfl (EF186826)                  1303 CGACTTCATGAAGTTGG-AATCGCTAGTAATCGCGAATCAGCATGTCGC--GGTGAATACGTTCTCGGGG 1369
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                                                1410      1420      1430      1440      1450      1460      1470
                                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)             1004 TTTGTACACACC-GCCCGTCAAACCAC------------------------------------------- 1029
Melornemis antillarum (JD)         1004 TTTGTACACACC-GCCCGTCAAACCAC------------------------------------------- 1029
Colpoptera maculifrons (Ad)        1368 TTTGTACACACC-GCCCGTCAAACCACGAAAGTTGATAATA-CCCGAAAGCGGTCGCCTAACTTCGTTAG 1435
Periwinkle little leaf (Ma) 6      1376 TTTGTACACACC-GCCCGTCAAACCACGAAAGTTGATAATA-CCCGAAAGCGGTCGCCTAACTTCGTTAG 1443
Periwinkle yellowing (Ma) 7        1368 TTTGTACACACC-GCCCGTCAAACCACGAAAGTTGATAATA-CCCGAAAGCGGTCGCCTAACTTCGTTAG 1435
Periwinkle (Ma) 5                  1375 TTTGTACCCACCGGCCCGTCAAACCACGAAAGTTGATAATA-CCCGAAAGTGGTCGCCTAACTTCGTTAG 1443
Pigeon pea (JD) 33                 1371 TTTGTACACACC-GCCCGTCAAACCACGAAAGTTGATAATACCCCGAAAGCGGTCGCCTAACTTCGTTAG 1439
Pigeon pea (Is) 45                 1369 TTTGTACACACC-GCCCGTCAAACCACGAAAGTTGATAATA-CCCGAAAGCGGTCGCCTAACTTCGTTAG 1436
Tabebuia (Ma) 2                    1368 TTTGTACACACC-GCCCGTCAAACCACGAAAGTT------------------------------------ 1400
Tabebuia (Ma) 3                    1368 TTTGTACACACC-GCCCGTCAAACCACGAAAGTTGATAATA-CCCGAAAGCGGTCGCCTAACTTCGTTAG 1435
Orange (Is) 40                     1378 TTTGTACACACC-GCCCGTCAAACCACGAAAGTTGATAATA-CCCGAAAGCGGTCGCCTAACTTCGTTAG 1445
Orange (JD) 32                     1368 TTTGTACACACC-GCCCGTCAAACCACGAAAGTTGATAATA-CCCGAAAGCGGTCGCCTAACTTCGTTAG 1435
Pigeon pea (Is) 43                 1368 TTTGTACACACC-GCCCGTCAAACCACGAAAGTTGATAATA-CCCGAAAGCGGTCGCCTAACTTCGTTAG 1435
Pigeon pea (Is) 44                 1367 TTTGTACACACC-GCCCGTCAAACCACGAAAGTTGATAATA-CCCGAAAGCGGTCGCCTAACTTCGTTAG 1434
PPWBja (EF186825)                  1368 TTTGTACACACC-GCCCGTCAAACCACGAAAGTTGATAATA-CCCGAAAGCGGTCGCCTAACTTCGTTAG 1435
PPWBpr (EF186824)                  1368 TTTGTACACACC-GCCCGTCAAACCACGAAAGTTGATAATA-CCCGAAAGCGGTCGCCTAACTTCGTTAG 1435
PPWBfl (EF186826)                  1370 TTTGTACACACC-GCCCGTCAAACCACGAAAGTTGATAATA-CCCGAAAGCGGTCGCCTAACTTCGTTAG 1437

                                                1480      1490      1500      1510      1520      1530      1540
                                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)             1029 ---------------------------------------------------------------------- 1029
Melornemis antillarum (JD)         1029 ---------------------------------------------------------------------- 1029
Colpoptera maculifrons (Ad)        1436 AAGAGGGAGCCGTCTAAGGTAGGATCGATG-ATTGGGGTTAAGTCGTAACAAGGTATCCCTACCGG-AAG 1503
Periwinkle little leaf (Ma) 6      1444 AAGAGGGAGCCGTCTAAGGTAGGATCGATG-ATTGGGGTTAAGTCGTAACAAGGTATCCCTACCGG-AAG 1511
Periwinkle yellowing (Ma) 7        1436 AAGAGGGAGCCGTCTAAGGTAGGATCGATG-ATTGGGGTTAAGTCGTAACAAGGTATCCCTACCGG-AAG 1503
Periwinkle (Ma) 5                  1444 AAGAGGGAGCCGTCTAAGGTAGGATCGATG-ATTGGGGTTAAGTCGTAACAAGGTATCCCTACCGG-AAG 1511
Pigeon pea (JD) 33                 1440 AAGAGGGAGCCGTCTAAGGTAGGATCGATGAATTGGGGTTAAGTCGTAACAAGGTATCCCTACCGGAAAG 1509
Pigeon pea (Is) 45                 1437 AAAAGGGAGCCGTCTAAGGTAGGATCGATG-ATTGGGGTTAAGTCGTAACAAGGTATCCCTACCGG-AAG 1504
Tabebuia (Ma) 2                    1400 --------------------------------------TTAA---------------------------- 1404
Tabebuia (Ma) 3                    1436 AAGAGGGAGCCGTCTAAGGTAGGATCGATG-ATTGGGGTTAAGTCGTAACAAGGTATCCCTACCGG-AAG 1503
Orange (Is) 40                     1446 AAGAGGGAGCCGTCTAAGGTAGGATCGATG-ATTGGGGTTAAGTCGTAACAAGGTATCCCTACCGG-AAG 1513
Orange (JD) 32                     1436 AAGAGGGAGCCGTCTAAGGTAGGATCGATG-ATTGGGGTTAAGTCGTAACAAGGTATCCCTACCGG-AAG 1503
Pigeon pea (Is) 43                 1436 AAGAGGGAGCCGTCTAAGGTAGGATCGATG-ATTGGGGTTAAGTCGTAACAAGGTATCCCTACCGG-AAG 1503
Pigeon pea (Is) 44                 1435 AAGAGGGAGCCGTCTAAGGTAGGATCGATG-ATTGGGGTTAAGTCGTAACAAGGTATCCCTACCGG-AAG 1502
PPWBja (EF186825)                  1436 AAGAGGGAGCCGTCTAAGGTAGGATCGATG-ATTGGGGTTAAGTCGTAACAAGGTATCCCTACCGG-AAG 1503
PPWBpr (EF186824)                  1436 AAGAGGGAGCCGTCTAAGGTAGGATCGATG-ATTGGGGTTAAGTCGTAACAAGGTATCCCTACCGG-AAG 1503
PPWBfl (EF186826)                  1438 AAGAGGGAGCCGTCTAAGGTAGGATCGATG-ATTGGGGTTAAGTCGTAACAAGGTATCCCTACCGG-AAG 1505

                                                1550      1560      1570      1580      1590      1600      1610
                                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)             1029 ---------------------------------------------------------------------- 1029
Melornemis antillarum (JD)         1029 ---------------------------------------------------------------------- 1029
Colpoptera maculifrons (Ad)        1504 GTGGGGATGGATCACCTCCTTTCTAAGGAAATGTAGA--------------------------------- 1540
Periwinkle little leaf (Ma) 6      1512 GTGGGGATGGATCACCTCCTTCTTAAGGAAATTTCCAATCATCATCTTCAGTTTTGAAAGACTTAGTTAA 1581
Periwinkle yellowing (Ma) 7        1504 GTGGGGATGGATCACCTCCTTCTTAAGGAAATTTCC---------------------------------- 1539
Periwinkle (Ma) 5                  1512 GTGGGGATGGATCACCTCCTTTCTAAGGAAAT-------------------------------------- 1543
Pigeon_pea (JD) 33                 1510 GTGGGGATGGATCACCTCCTTTCTAAGGAAATGTAAAATCATCATCTTCAGTTTTGAAA-ACTTAGTTAA 1578
Pigeon_Pea (Is) 45                 1505 GTGGGGATGGATCACCTCCTTTCTAAGGAAATGTAGAATCATCATCTTCAGTTTTGAAAGACTTAGTTAA 1574
Tabebuia (Ma) 2                    1404 ---------------------------------------------------------------------- 1404
Tabebuia (Ma) 3                    1504 GTGGGGATGGATCACCTCCTTTCTAAGGAAATTTCCCATCATCATCTTCAGTTTTGAAAGACTTAGTTAA 1573
Orange (Is) 40                     1514 GTGGGGATGGATCACCTCCTTTCTAAGGAAATGTAGAATCATCATCTTCAGTTTTGAAAGACTTAGTTAA 1583
Orange (JD) 32                     1504 GTGGGGATGGATCACCTCCTTTCTAAGGAAATGTAGAATCATCATCTTCAGTTTTGAAAGACTTAATTAA 1573
Pigeon pea (Is) 43                 1504 GTGGGGATGGATCACCTCCTTTCTAAGGAAATGTAGAATCATCATCTTCAGTTTTGAAAGACTTAGTTAA 1573
Pigeon pea_(Is)_44                 1503 GTGGGGATGGATCACCTCCTTTCTAAGGAAATGTAGAATCATCATCTTCAGTTTTGAAAGACTTAGTTAA 1572
PPWBja (EF186825)                  1504 GTGGGGATGGATCACCTCCTTTCT---------------------------------------------- 1527
PPWBpr (EF186824)                  1504 GTGGGGATGGATCACCTCCTTTCT---------------------------------------------- 1527
PPWBfl (EF186826)                  1506 GTGGGGATGGATCACCTCCTTTCT---------------------------------------------- 1529

                                                1620      1630      1640      1650      1660      1670      1680
                                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)             1029 ---------------------------------------------------------------------- 1029
Melornemis antillarum (JD)         1029 ---------------------------------------------------------------------- 1029
Colpoptera maculifrons (Ad)        1540 ---------------------------------------------------------------------- 1540
Periwinkle little leaf (Ma) 6      1582 GTTTTTCTCATTTATTTTGTTTTTTTGATATCCTGGGCCTATAGCTCAGTTGGTTAGAGCACACGCCTGA 1651
Periwinkle yellowing (Ma) 7        1539 ---------------------------------------------------------------------- 1539
Periwinkle (Ma) 5                  1543 ---------------------------------------------------------------------- 1543
Pigeon pea (JD) 33                 1579 GTTTTTCTCATTTATTTTATTTTTTTGA-ATCCTGGGCCTATAGCTCAGTTGGTTAGAGCACACGCCTGA 1647
Pigeon pea (Is) 45                 1575 GTTTTCCCATTTTATTTTATTTTTTTGATATCCTGGGCCTATAGCTCAGTTGGTTAGAGCACACGCCTGA 1644
Tabebuia (Ma) 2                    1404 ---------------------------------------------------------------------- 1404
Tabebuia (Ma) 3                    1574 GTTTTTCTCATTTATTTTGTTTTTTTGATATCCTGGGCCTATAGCTCAGTTGGTTAGAGCACACGCCTGA 1643
Orange (Is) 40                     1584 GTTTTTCTCATTTATTTTGTTTTTTTGATATCCTGGGCCTATAGCTCAGTTGGTTAGAGCACACGCCTGA 1653
Orange (JD) 32                     1574 ATTTTTCTCATTTATTTTATTTTTTTGATATCCTGGGCCTATAGCTCAGTTGGTTAAAGCACACCCCTGA 1643
Pigeon pea (Is) 43                 1574 GTTTTTCTCATTTATTTTATTTTTTTGATATCCTGGGCCTATAGCTCAGTTGGTTAGAGCACACGCCTGA 1643
Pigeon pea (Is) 44                 1573 GTTTTT---------------------------------------------------------------- 1578
PPWBja (EF186825)                  1527 ---------------------------------------------------------------------- 1527
PPWBpr (EF186824)                  1527 ---------------------------------------------------------------------- 1527
PPWBfl (EF186826)                  1529 ---------------------------------------------------------------------- 1529
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                                          1690      1700      1710      1720      1730      1740      1750
                                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)            1029 ---------------------------------------------------------------------- 1029
Melornemis antillarum (JD)        1029 ---------------------------------------------------------------------- 1029
Colpoptera maculifrons (Ad)       1540 ---------------------------------------------------------------------- 1540
Periwinkle little leaf (Ma) 6     1652 TAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCCACCAAACGTTTTTAT-AAAAAACGTGTTATCAA 1720
Periwinkle yellowing (Ma) 7       1539 ---------------------------------------------------------------------- 1539
Periwinkle (Ma) 5                 1543 ---------------------------------------------------------------------- 1543
Pigeon pea (JD) 33                1648 TAAGCGTGAGGTCGGTGGTTGAATTCCATTTAGGCCC-CCAAACGTTTTTA--AAAAAAAGTGTTAT--A 1712
Pigeon pea(Is) 45                 1645 TAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCCACCAAACGTTTTTATAAAAAAACGTGTTATCAA 1714
Tabebuia (Ma) 2                   1404 ---------------------------------------------------------------------- 1404
Tabebuia (Ma) 3                   1644 TAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCCACCAAACGTTTTTAT-AAAAAACGTGTTATCAA 1712
Orange (Is) 40                    1654 TAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCCACCAAACGTTTTTAT-AAAAAACGTGTTATCAA 1722
Orange (JD) 32                    1644 TAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCCACCAAACGTTTTTTT-AAAAAACGTGGTTTCAA 1712
Pigeon pea (Is) 43                1644 TAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCCACCAAACGTTTTTAT-AAAAAACGTGTTATCAA 1712
Pigeon pea (Is) 44                1578 ---------------------------------------------------------------------- 1578
PPWBja (EF186825)                 1527 ---------------------------------------------------------------------- 1527
PPWBpr (EF186824)                 1527 ---------------------------------------------------------------------- 1527
PPWBfl (EF186826)                 1529 ---------------------------------------------------------------------- 1529

                                           1760      1770      1780      1790      1800      1810      1820
                                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Empoasca kraemeri (Ma)            1029 ---------------------------------------------------------------------- 1029
Melornemis antillarum (JD)        1029 ---------------------------------------------------------------------- 1029
Colpoptera maculifrons (Ad)       1540 ---------------------------------------------------------------------- 1540
Periwinkle little leaf (Ma) 6     1721 AAGAAAGTTCTTTGAAAAGTAGATAAACAAGAAAATAATATCCGTTTTTAAAGGAAGTAAGGGCGTACAG 1790
Periwinkle yellowing (Ma) 7       1539 ---------------------------------------------------------------------- 1539
Periwinkle (Ma) 5                 1543 ---------------------------------------------------------------------- 1543
Pigeon pea (JD) 33                1713 AAGAAAGTTC--TGAAAAGTAGATAAACAAGAAAATAATAGCCATTTTTAAAGGAAGTAAGGGCGTACAG 1780
Pigeon pea (Is) 45                1715 AAGAAAGTTCTTTGAAAAGTAGATAAACAAGAAAATAATATCCGTTTTTAAAGGAAGTAAGGGCGTACAG 1784
Tabebuia (Ma) 2                   1404 ---------------------------------------------------------------------- 1404
Tabebuia (Ma) 3                   1713 AAGAAAGTTCTTTGAAAAGTAGATAAACAAGAAAATAATATCCGTTTTTAAAGGAAGTAAGGGCGTACAG 1782
Orange (Is) 40                    1723 AAGAAAGTTCTTTGAAAAGTAGATAAACAAGAAAATAATATCCGTTTTTAAAGGAAGTAAGGGCGTACAG 1792
Orange (JD) 32                    1713 AAGAAAGTTTTTTGAAAAGTAGATAAACAAGAAAATAATATCCGTTTTTAAAGGAAGTAAGGGCGTACAG 1782
Pigeon pea (Is) 43                1713 AAGAAAGTTCTTTGAAAAGTAGATAAACAAGAAAATAATATCCG-TTTTAAAGGAAGTAAGGGCGTACAG 1781
Pigeon pea (Is) 44                1578 ---------------------------------------------------------------------- 1578
PPWBja (EF186825)                 1527 ---------------------------------------------------------------------- 1527
PPWBpr (EF186824)                 1527 ---------------------------------------------------------------------- 1527
PPWBfl (EF186826)                 1529 ---------------------------------------------------------------------- 1529

                                           1830      1840      1850
                                  ....|....|....|....|....|....|....
Empoasca kraemeri (Ma)            1029 ---------------------------------- 1029
Melornemis antillarum (JD)        1029 ---------------------------------- 1029
Colpoptera maculifrons (Ad)       1540 ---------------------------------- 1540
Periwinkle little leaf (Ma) 6     1791 TGGATGCCTTGGCACTAAGAGCCGATGAAGGACG 1824
Periwinkle yellowing (Ma) 7       1539 ---------------------------------- 1539
Periwinkle (Ma) 5                 1543 ---------------------------------- 1543
Pigeon pea (JD) 33                1781 TGGATGCC-------------------------- 1788
Pigeon pea (Is) 45                1785 TGGATGCCTTGGCACTAAGAGCCGATGAAGGACG 1818
Tabebuia (Ma) 2                   1404 ---------------------------------- 1404
Tabebuia (Ma) 3                   1783 TGGATGCCTTGGCACTAAGAGCCGATGAAGGACG 1816
Orange (Is) 40                    1793 TGGATGCCTTGGCACTAAGAGCCGATGAAGGACG 1826
Orange (JD) 32                    1783 TGGATGCCTTGGCACTAAGAGCCGATGAAGGACG 1816
Pigeon pea (Is) 43                1782 TGGATGCCTT------------------------ 1791
Pigeon pea (Is) 44                1578 ---------------------------------- 1578
PPWBja (EF186825)                 1527 ---------------------------------- 1527
PPWBpr (EF186824)                 1527 ---------------------------------- 1527
PPWBfl (EF186826)                 1529 ---------------------------------- 1529
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Appendix 3

Multiple sequence alignment of *rplV* (*rpl22*) and *rpsC* (*rps3*) genes for 14 positive samples of PPWB phytoplasma using GUIDANCE server (Guide-tree based alignment confidence) generated through MUSCLE algorithm.

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                               10        20        30        40        50        60        70
                       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Periwinkle (Ma) 5    1    ATGGTTGGACATAAATTAGGTGAATTTTCGCCAACAAGAAAATTTCACGGACATACTAAAGATAGTAAAA 70
Orange (JD) 32       1    ATGGTTGGACATAAATTAGGTGAATTTTCGCCAACAAGAAAATTTCACGGACATACTAAAGATAGTAAAA 70
Tabebuia (Ma) 2      1    ATGGTTGGGCATAAATTAGGTGAATTTTCGCCACCAAGAAAATTTCCCGGCCATACTAAAGATAGTAAAA 70
Pigeon pea (Is) 43   1    ATGGTTGGACATAAATTAGGTGAATTTTCGCCAACAAGAAAATTTCACGGACATACTAAAGATAGTAAAA 70
PPWBpr (EF183497)    1    ATGGTTGGGCATAAATTAGGTGAATTTTCGCCAACAAGAAAATTTCACGGACATACTAAAGATAGTAAAA 70
PPWBja (EF183496)    1    ATGGTGGGACACAAGTTAGGTGAATTTTCGCCAACAAGAAAATTTCACGGACATACTAAAGATAGTAAAA 70

                               80        90       100       110       120       130       140
                       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Periwinkle (Ma) 5    71   AAAATATTAAAAAATAAAAAATTTGAGAAGGTATTGACATGAATGTAAAAGCAATTGCTAAACAAATGCC 140
Orange (JD) 32       71   AAAATATTAAAAAATAAAAAATTTGAGAAGGTATTGACATGAATGTAAAAGCAATTGCTAAACAAATGCC 140
Tabebuia (Ma) 2      71   AAAATTTTAAAAAAT-AAAAATTTGGGAAGGTATTGCCATGAATGTAAAAGCAATTGCTAAACAAATGCC 139
Pigeon pea (Is) 43   71   AAAATATTAAAAAATAAAAAATTTGAGAAGGTATTGACATGAATGTAAAAGCAATTGCTAAACAAATGCC 140
PPWBpr (EF183497)    71   AAAATATTAAAAAATAAAAAATTTGAGAAGGTATTGACATGAATGTAAAAGCAATTGCTAAACAAATGCC 140
PPWBja (EF183496)    71   AAAATATTAAAAAATAAAAAATTTGAGAAGGTATTGACATGAATGTAAAAGCAATTGCTAAACAAATGCC 140

                              150       160       170       180       190       200       210
                       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Periwinkle (Ma) 5    141  TATTACTCCACGTAAAACACGTTTAGTTGCAGATTTAATTCGGGGGAAAAATATTAAAGAAGCACAAGCT 210
Orange (JD) 32       141  TATTACTCCACGTAAAACACGTTTAGTTGCAGATTTAATTCGGGGGAAAAATATTAAAGAAGCACAAGCT 210
Tabebuia (Ma) 2      140  TATTACTCCACGTAAAACACGTTTAGTTGCAGATTTAATTCGGGGGAAAAATATTAAAGAAGCACAAGCT 209
Pigeon pea (Is) 43   141  TATTACTCCACGTAAAACACGTTTAGTTGCAGATTTAATTCGGGGGAAAAATATTAAAGAAGCCCAAGCT 210
PPWBpr (EF183497)    141  TATTACTCCACGTAAAACACGTTTAGTTGCAGATTTAATTCGGGGGAAAAATATTAAAGAAGCACAAGCT 210
PPWBja (EF183496)    141  TATTACTCCACGTAAAACACGTTTAGTTGCAGATTTAATTCGGGGGAAAAATATTAAAGAAGCACAAGCT 210

                              220       230       240       250       260       270       280
                       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Periwinkle (Ma) 5    211  ATTTTAATGTTTACGCCCAAATCAGCTTCTCCTATTGTTTTAAAACTTTTAAAAAGTGCAATCGCAAACG 280
Orange (JD) 32       211  ATTTTAATGTTTACACCCAAATCAGCTTCTCCTATTGTTTTAAAACTTTTAAAAAGTGCAATCGCAAACG 280
Tabebuia (Ma) 2      210  ATTTTAATGTTTACGCCCAAATCAGCTTCTCCTATTGTTTTAAAACTTTTAAAAAGTGCAATCGCAAACG 279
Pigeon pea (Is) 43   211  ATTTTAATGTTTACACCCAAATCAGCTTCTCCTATTGTTTTAAAACTTTTAAAAAGTGCAATCGCAAACG 280
PPWBpr (EF183497)    211  ATTTTAATGTTTACACCCAAATCAGCTTCTCCTATTGTTTTAAAACTTTTAAAAAGTGCAATCGCAAACG 280
PPWBja (EF183496)    211  ATTTTAATGTTTACACCCAAATCAGCTTCTCCTATTGTTTTAAAACTTTTAAAAAGTGCAATCGCAAACG 280

                              290       300       310       320       330       340       350
                       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Periwinkle (Ma) 5    281  CTACTAATAACTTCAGTTTAGATGACAAAAATTTATATGTAAAAGAAATTTTTGTAAACGAAGGTTTACG 350
Orange (JD) 32       281  CTACTTTTAACTTCAGTTTAGATGACAAAAATTTATTTGTAAAATAATTTTTTGTAATCGAAGGTTTACG 350
Tabebuia (Ma) 2      280  CTACTAATAACTTCAGTTTAGATGACAAAAATTTATATGTAAAAGAAATTTTTGTAAACGAAGGTTTACG 349
Pigeon pea (Is) 43   281  CTACTAATAACTTCAGTTTAGATGACAAAAATTTATATGTAAAAGAAATTTTTGTAAACGAAGGTTTACG 350
PPWBpr (EF183497)    281  CTACTAATAACTTCAGTTTAGATGACAAAAATTTATATGTAAAAGAAATTTTTGTAAACGAAGGTTTACG 350
PPWBja (EF183496)    281  CTACTAATAACTTCAGTTTAGATGACAAAAATTTATATGTAAAAGAAATTTTTGTAAACGAAGGTTTACG 350

                              360       370       380       390       400       410       420
                       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Periwinkle (Ma) 5    351  TTTAACAAGACTTTTTCCTAGAGCAAAAGGGAGAGCAGATCGAATTAAAAAAAGAACTAGT-CATATTAC 419
Orange (JD) 32       351  TTTAACAAGACTTTTTCCTAGGGCAAAAGGGAGAGCAGATCGAATTAAAAAAAGAACTAGTGCATATTAC 420
Tabebuia (Ma) 2      350  TTTAACAAGACTTTTTCCTAGAGCAAAAGGGAGAGCAGATCGAATTAAAAAAAGAACTAGT-CATATTAC 418
Pigeon pea (Is) 43   351  TTTAACAAGACTTTTTCCTAGGGCAAAAGGGAGAGCAGATCGAATTAAAAAAAGAACTAGT-CATATTAC 419
PPWBpr (EF183497)    351  TTTAACAAGACTTTTTCCTAGGGCAAAAGGGAGAGCAGATCGAATTAAAAAAAGAACTAGT-CATATTAC 419
PPWBja (EF183496)    351  TTTAACAAGACTTTTTCCTAGGGCAAAAGGGAGAGCAGATCGAATTAAAAAAAGAACTAGT-CATATTAC 419

                              430       440       450       460       470       480       490
                       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Periwinkle (Ma) 5    420  AATAGTAGTTGCACCTCAGTTATCTGAAAACAAAGCAAAGGAGATAGTTAATAATGGGGCAAAAGAGTAA 489
Orange (JD) 32       421  AATAGTAGTCGCACCTCAGTTATCGGAAAACAAAGCAAAGGAGATAGTTAATAATGGGGCAAAAGAGTAA 490
Tabebuia (Ma) 2      419  AATAGTAGTTGCACCTCAGTTATCTGAAAACAAAGCAAAGGAGATAGTTAATAATGGGGCAAAAGAGTAA 488
Pigeon pea (Is) 43   420  AATAGTAGTCGCACCTCAGTTATCTGAAAACAAAGCAAAGGAGATAGTTAATAATGGGGCAAAAGAGTAA 489
PPWBpr (EF183497)    420  AATAGTAGTCGCACCTCAGTTATCTGAAAACAAAGCAAAGGAGATAGTTAATAATGGGGCAAAAGAGTAA 489
PPWBja (EF183496)    420  AATAGTAGTCGCACCTCAGTTATCTGAAAACAAAGCAAAGGAGATAGTTAATAATGGGGCAAAAGAGTAA 489

                              500       510       520       530       540       550       560
                       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Periwinkle (Ma) 5    490  TCCTAATGGGTTAAGATTAGGTATTATCCAAAATTGGAAATCTCAATGGTATATTGAAGATAAACAAGTT 559
Orange (JD) 32       491  TCCTAATGGGTTAAGATTAGGTATTATCCAAAATTGGAAATCTCAACGGTATTTGGAAGATAAACAAGTT 560
Tabebuia (Ma) 2      489  TCCTAATGGGTTAAGATTAGGTATTATCCAAAATTGGAAATCTCAATGGTATATTGAAGATAAACAAGTT 558
Pigeon pea (Is) 43   490  TCCTAATGGGTTAAGATTAGGTATTATCCAAAATTGGAAATCTCAATGGTATATCGAAGATAAACAAGTT 559
PPWBpr (EF183497)    490  TCCTAATGGGTTAAGATTAGGTATTATCCAAAATTGGAAATCTCAATGGTATATCGAAGATAAACAAGTT 559
PPWBja (EF183496)    490  TCCTAATGGGTTAAGATTAGGTATTATCCAAAATTGGAAATCTCAATGGTATATCGAAGATAAACAAGTT 559
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                                       570       580       590       600       610       620       630
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Periwinkle (Ma) 5         560   CCTAATTTAGTTCATGAAGATTTTAAAATCCGAATTTTAATTAAACAGTTTTATATGAAAGGTGTTATTT 629
Orange (JD) 32            561   CCTAATTTAGTTCATGAAGATGTTAAAATCCGATTTTTAATTAAACAGTTTTATATAAAAGGCGTTATTT 630
Tabebuia (Ma) 2           559   CCTAATTTAGTTCATGAAGATTTTAAAATCCGAATTTTAATTAAACAGTTTTATATGAAAGGTGTTATTT 628
Pigeon pea (Is) 43        560   CCTAATTTAGTTCATGAAGATTTTAAAATCCGAATTTTAATTAAACAGTTTTATATAAAAGGCGTTATTT 629
PPWBpr (EF183497)         560   CCTAATTTAGTTCATGAAGATTTTAAAATCCGAATTTTAATTAAACAGTTTTATATAAAAGGCGTTATTT 629
PPWBja (EF183496)         560   CCTAATTTAGTTCATGAAGATTTTAAAATCCGAATTTTAATTAAACAGTTTTATATAAAAGGCGTTATTT 629

                                       640       650       660       670       680       690       700
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Periwinkle (Ma) 5         630   C---AGATATTGAAATAAAACGTTTAAAAAAATCTAATAATGAAGAAATTACT-ATTAATTTATTTA--- 692
Orange (JD) 32            631   CTTAAGATATTGAAATAAAACGTTTAAAAAAATCTAATAATGAAGAAATTAATCATTAATTTATTTAAAC 700
Tabebuia (Ma) 2           629   C---AGATATTGAAATAAAACGTTTAAAAAAATCTAATAATGAAGAAATTACT-ATTAATTTATTTA--- 691
Pigeon pea (Is) 43        630   C---AGATATTGAAATAAAACGTTTAAAAAAATCTAATAATGAAGAAATTACT-ATTAATTTATTTA--- 692
PPWBpr (EF183497)         630   C---AGATATTGAAATAAAACGTTTAAAAAAATCTAATAATGAAGAAATTACT-ATTAATTTATTTA--- 692
PPWBja (EF183496)         630   C---AGATATTGAAATAAAACGTTTAAAAAAATCTAATAATGAAGAAATTACT-ATTAATTTATTTA--- 692

                                       710       720       730       740       750       760       770
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Periwinkle (Ma) 5         693   CTTCTAAAATTGGATT--AATACAAGGTATAGACAACAAAACAAAAAATAAA--TTATTGCAAAAAATTG 758
Orange (JD) 32            701   CTTCTAAAATTGGATTGAAATACAAGGTATAGACAATAAAACAAAAAATAAATTTATTGCAAAAAATTG 770
Tabebuia (Ma) 2           692   CTTCTAAAATTGGATT--AATACAAGGTATAGACAACAAAACAAAAAATAAA--TTATTGCAAAAAATTG 757
Pigeon pea (Is) 43        693   CTTCTAAAATTGGATT--AATACAAGGTATAGACAATAAAACAAAAAATAAA--TTATTGCAAAAAATTG 758
PPWBpr (EF183497)         693   CTTCTAAAATTGGATT--AATACAAGGTATAGACAATAAAACAAAAAATAAA--TTATTGCAAAAAATTG 758
PPWBja (EF183496)         693   CTTCTAAAATTGGATT--AATACAAGGTATAGACAATAAAACAAAAAATAAA--TTATTGCAAAAAATTG 758

                                       780       790       800       810       820       830       840
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Periwinkle (Ma) 5         759   AAAAATTAATAAATAAAAAAGTATTGATAAA--TGTTTTTGAAGTAAAAGCATTAGATAAAATAGCTAGT 826
Orange (JD) 32            771   AAAAAATAATAAATAAAAGGGTATTGATAAATTTGTTTTTGAAGTAAAAGCATTAGATAAAATAGCTAGT 840
Tabebuia (Ma) 2           758   AAAAAATAATAAATAAAAAAGTATTGATAAA--TGTTTTTGAAGTAAAAGCATTAGATAAAATAGCTAGT 825
Pigeon pea (Is) 43        759   AAAAAATAATAAATAAAAAAGTATTGATAAA--TGTTTTTGAAGTAAAAGCATTAGATAAAATAGCTAGT 826
PPWBpr (EF183497)         759   AAAAAATAATAAATAAAAAAGTATTGATAAA--TGTTTTTGAAGTAAAAGCATTAGATAAAATAGCTAGT 826
PPWBja (EF183496)         759   AAAAAATAATAAATAAAAAAGTATTGATAAA--TGTTTTTGAAGTAAAAGCATTAGATAAAATAGCTAGT 826

                                       850       860       870       880       890       900       910
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Periwinkle (Ma) 5         827   TTAGTGGCACAAAATATTGTTTATTCAATTGCAACAAAGAAGTTA--TTTTCGTGCAGTACAAAAAATTT 894
Orange (JD) 32            841   TTAGGGGCGCAAAATATTGTTTATTCAATTCCAACAAAGAAGTTATTTTTTCGCGCAGTACAAAAAATTT 910
Tabebuia (Ma) 2           826   TTAGTGGCACAAAATATTG-TTATTCAATTGCAACAAAGAAGTTA--TTTTCGTGCAGTACAAAAAATTT 892
Pigeon pea (Is) 43        827   TTAGTGGCACAAAATATTG-TTATTCAATTGCAACAAAGAAGTTA--TTTTCGCGCAGTACAAAAAATTT 893
PPWBpr (EF183497)         827   TTAGTGGCACAAAATATTG-TTATTCAATTGCAACAAAGAAGTTA--TTTTCGCGCAGTACAAAAAATTT 893
PPWBja (EF183496)         827   TTAGTGGCGCAAAATATTG-TTATTCAATTGCAACAAAGAAGTTA--TTTTCGCGCAGTACAAAAAATTT 893

                                       920       930       940       950       960       970       980
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Periwinkle (Ma) 5         895   CAGCTCAAAAGGTTTTAAAAAGCGGAACTAAAGGGGCTAAAATAATTCTTTCAGGCCGGTTAGGAGGAGC 964
Orange (JD) 32            911   CAGCTCAAAAAGTTTTAAAGAGCGGAGCTAAAGGTGTTAAAATAATTCTTTCAGGCCGTTTAGGAGGAGC 980
Tabebuia (Ma) 2           893   CAGCTCAAAAAGTTTTAAAGAGCGGAGCTAAAGGTGTCAAAATAATTCTTTCAGGCCGTTTAGGAGGAGC 962
Pigeon pea (Is) 43        894   CAGCTCAAAAAGTTTTAAAGAGCGGAGCTAAAGGTGTTAAAATAATTCTTTCAGGCCGTTTAGGAGGAGC 963
PPWBpr (EF183497)         894   CAGCTCAAAAAGTTTTAAAGAGCGGAGCTAAAGGTGTTAAAATAATTCTTTCAGGCCGTTTAGGAGGAGC 963
PPWBja (EF183496)         894   CAGCTCAAAAAGTTTTAAAGAGCGGAGCTAAAGGTGTTAAAATAATTCTTTCAGGCCGTTTAGGAGGAGC 963

                                       990      1000      1010      1020      1030      1040      1050
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Periwinkle (Ma) 5         965   TGAAATTGCTCGTAACCGAACTATTACTTAAGGTTTAAGCCCTTTAAATACCTTTAAAGCTGAAATTGAT 1034
Orange (JD) 32            981   TGAAATTGCTCGTAGCGAAACTATTTCTTTAGGTTTAGTCCCTTTAAATACTTTTAGAGCTGATATTGAT 1050
Tabebuia (Ma) 2           963   TGAAATTGCTCGTAGCGAAACTATTTCTTTAGGTTTAGTCCCTTTAAATACTTTTAGAGCTGATATTGAT 1032
Pigeon pea (Is) 43        964   TGAAATTGCTCGTAGCGAAACTATTTCTTTAGGTTTAGTCCCTTTAAATACTTTTAGAGCTGATATTGAT 1033
PPWBpr (EF183497)         964   TGAAATTGCTCGTAGCGAAACTATTTCTTTAGGTTTAGTCCCTTTAAATACTTTTAGAGCTGATATTGAT 1033
PPWBja (EF183496)         964   TGAAATTGCTCGTAGCGAAACTATTTCTTTAGGTTTAGTCCCTTTAAATACTTTTAGAGCTGATATTGAT 1033

                                      1060      1070      1080      1090      1100      1110      1120
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Periwinkle (Ma) 5         1035  TAGGTTTTTGAAGAGGCGCATACTACTTATGGTGTTTTAGGTGTTAAAGTATGGATTTTTCATGGAGAAG 1104
Orange (JD) 32            1051  TATGCTTTTGAAGAGGCGCATACTACTTATGGTGTTTTAGGTGTTAAAGTATGGATTTTTCATGGAGAAG 1120
Tabebuia (Ma) 2           1033  TATGCTTTTGAAGAGGCGCATACTACTTATGGTGTTTTAGGTGTTAAAGTATGGATTTTTCATGGAGAAG 1102
Pigeon pea (Is) 43        1034  TATGCTTTTGAAGAGGCGCATACTACTTATGGTGTTTTAGGTGTTAAAGTATGGATTTTTCATGGAGAAG 1103
PPWBpr (EF183497)        1034  TATGCTTTTGAAGAGGCGCATACTACTTATGGTGTTTTAGGTGTTAAAGTATGGATTTTTCATGGAGAAG 1103
PPWBja (EF183496)        1034  TATGCTTTTGAAGAGGCGCATACTACTTATGGTGTTTTAGGTGTTAAAGTATGGATTTTTCATGGAGAAG 1103
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                                 1130      1140      1150      1160      1170      1180      1190
                             ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Periwinkle (Ma) 5       1105 TTTTGTCTAATAAAACTATTGCAGATACAAGACAATTTTTTGCACAAACACAAGAAACAAAAAAACACTT 1174
Orange (JD) 32          1121 TTTTGTCTAATAAAACTATTGCAGATACAAGACAATTTTTTGCACAAACACAAGAAACAAAAAAACACTT 1190
Tabebuia (Ma) 2         1103 TTTTGTCTAATAAAACTATTGCAGATACAAGACAATTTTTTGCACAAACACAAGAAACAAAAAAACACTT 1172
Pigeon pea (Is) 43      1104 TTTTGTCTAATAAAACTATTGCAGATACAAGACAATTTTTTGCACAAACACAAGAAACAAAAAAACACTT 1173
PPWBpr (EF183497)       1104 TTTTGTCTAATAAAACTATTGCAGATACAAGACAATTTTTTGCACAAACACAAGAAACAAAAAAACACTT 1173
PPWBja (EF183496)       1104 TTTTGTCTAATAAAACTATTGCAGATACAAGACAATTTTTTGCACAAACACAAGAAACAAAAAAACACTT 1173

                                 1200      1210      1220      1230      1240      1250      1260
                             ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Periwinkle (Ma) 5       1175 TGTTCGAAGATATCCGCAAAAGAATTTTAAGAAAAATACATCTTAAGTTATTAAAGAGGTGAAAAAATTA 1244
Orange (JD) 32          1191 TGTTCGAAGATATCCGCAAAAGAATTTTAAGAAAAATACATCTTAAGTTATTAAAGAGGTGAAAAAATTA 1260
Tabebuia (Ma) 2         1173 TGTTCGAAAATATCCACAAAAGAA-TTTAAGAAAAATACATCTTAAGTTTTTAAAGAGGTGAAAAAATTA 1241
Pigeon pea (Is) 43      1174 TGTTCGAAGATATCCGCAAAAGAA-TTTAAGAAAAATACATCTTAAGTT-TTAAAGAGGTGAAAAAATTT 1241
PPWBpr (EF183497)       1174 TGTTCGAAGATATCCGCAAAAGAATTTTAAGAAAAATACATCTTAAGTTATTAAAGAGGTGAAAAAATTA 1243
PPWBja (EF183496)       1174 TGTTCGAAGATATCCGCAAAAGAATTTTAAGAAAAATACATCTTAAGTTATTAAAGAGGTGAAAAAATTA 1243

                                 1270
                             ....|....|....|....
Periwinkle (Ma) 5       1245 TGTTAATGCCAAAAAGAAC 1263
Orange (JD) 32          1261 TGTTAATGCCAAAAAGAAC 1279
Tabebuia (Ma) 2         1242 TGTTAATGCCAAAAAGAAC 1260
Pigeon pea (Is) 43      1242 CCTTAATGCCAAAAAGAAC 1260
PPWBpr (EF183497)       1244 TGTTAATGCCAAAAAGAAC 1262
PPWBja (EF183496)       1244 TGTTAATGCCAAAAAGAAC 1262
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Appendix 4. Cycle threshold value (Cq) and Melting temperature (Tm) from qPCR assay to amplify a small region (102bp) of the 16S rDNA gene. Specific primers were designed to improve detection of phytoplasmas in various plant samples.

Sample Name	**Cq[1]**	**Tm[2]**
Positive control[3]	21.19	82.3
Healthy plant[4]	33.32	75.4
Pigeon pea (Is) 43	17.05	82.3
Tabebuia (Ma) 2	17.77	82.0
Periwinkle (Ma) 5	21.09	82.3
Negative control[5] (MW)	33.62	75.4
Orange (JD) 32	15.64	82.3
Tabebuia (Ma) 3	32.01	81.7
Standard[6] (1:10)	**26.24**	**82.3**
Standard[6] (1:100)	**27.51**	**82.3**
Standard[6] (1:1000)	**26.48**	**82.3**

[1] Cq or quantification cycle is the cycle number at which the fluorescent signal crosses the threshold.
[2] Tm or melting temperature is the temperature at which 50% of dsDNA is disassociated or in its single-stranded form (melted)
[3] Pigeon pea (JD) 33 sample, positive control
[4] Healthy periwinkle plant, negative control
[5] Molecular water used as negative control
[6] Dilutions series 1:10; 1:100; 1:1000 from the positive sample Pigeon pea (JD) 33

Appendix 5. Cycle threshold value (Cq) and Melting temperature (Tm) from qPCR assay to amplify a small region (102bp) of the 16S rDNA gene. Specific primers were designed to improve detection of phytoplasmas in DNA obtained from citrus asymptomatic and symptomatic samples with Citrus greening disease.

Sample Name	**Cq[1]**	**Tm[2]**
Citrus sample	31.98	75.1
Citrus sample	30.30	75.1
Citrus sample	33.11	74.8
Citrus sample	29.52	75.4
Healthy plant		86.8
Citrus sample	32.34	79.0
Citrus sample	32.32	78.7
Citrus sample	31.56	74.8
Citrus sample	33.62	75.1
Negative control[3]		
Citrus sample	31.80	75.1
Citrus sample	33.18	75.1
Citrus sample	33.85	74.8
Negative control		
Citrus sample	31.80	74.5
Citrus sample	31.65	79.0
Citrus sample	31.77	78.7
Citrus sample	32.13	74.8
Citrus sample	28.17	79.9
Citrus sample	31.89	78.7
Citrus sample	32.07	78.7
Citrus sample	31.80	74.8
Citrus simple	31.20	75.1
Citrus sample	31.70	78.7
Citrus sample	29.64	75.1
Citrus sample	31.44	75.1
Citrus sample	31.76	78.7
Citrus sample	19.53	79.0
Positive control[4]	**22.46**	**82.3**
Standard[5] (1:100)	**25.37**	**82.3**
Standard[5] (1:1000)	**30.67**	**82.3**
Standard[5] (1:10000)	**28.91**	**82.3**

[1] Cq or quantification cycle is the cycle number at which the fluorescent signal crosses the threshold.
[2] Tm or melting temperature is the temperature at which 50% of dsDNA is disassociated or in its single-stranded form (melted)
[3] Molecular water used as negative control
[4] Pigeon pea (JD) 33 sample, positive control
[5] Dilutions series 1:10; 1:100; 1:1000 from the positive sample Pigeon pea (JD) 33

Appendix 6. Cycle threshold value (Cq) and Melting temperature (Tm) from qPCR assay to amplify a small region (102bp) of the 16S rDNA gene. Specific primers were designed to improve detection of phytoplasmas in DNA obtained from citrus asymptomatic and symptomatic samples with Citrus greening disease.

Sample name	**Cq[1]**	**Tm[2]**
American Aster Yellows	37.17	74.5
Apple proliferation (AP)	28.94	80.8
Cactus phytoplasma (CACT)	34.80	81.4
Elm yellows (EY)	**22.56**	**82.3**
Poinsettia branching factor (JR1)	**28.13**	**82.3**
Tomato big bud (TBB)	**28.43**	**82.3**
Alder yellows (ALY)	**28.98**	**82.3**
Faba bean phyllody (FBPSA)	**28.71**	**82.3**
Beet leafhopper transmitted (BLTVA)	**34.18**	**82.3**
Stolbur (STOL)	**31.00**	**82.3**
Pichris echioides yellows (PEY)	**30.66**	**82.3**
Peach X disease (CX)	35.99	82.9
Ash yellow (ASHY 4)	37.12	83.2
Pseudomonas saccharophila	39.71	79.3
Sphingomonas phyllosphaerae	32.11	79.0
Archaea	32.20	76.0
Positive control[3]	**26.45**	**82.3**
Standard[4] (1:10)	**29.93**	**82.3**
Standard[4] (1:100)	**30.54**	**82.3**
Standard[4] (1:1000)	**33.03**	**82.3**
Standard[4] (1:10000)	**35.01**	**82.3**
Negative control[5]	33.42	75.7

[1] Cq or quantification cycle is the cycle number at which the fluorescent signal crosses the threshold.
[2] Tm or melting temperature is the temperature at which 50% of dsDNA is disassociated or in its single-stranded form (melted)
[3] Pigeon pea (JD) 33 sample, positive control
[4] Dilutions series 1:10; 1:100; 1:1000 from the positive sample Pigeon pea (JD) 33
[5] Molecular water used as negative control