Alma Mater Studiorum - Università di Bologna

DOTTORATO DI RICERCA IN SCIENZE AGRARIE

ECOLOGIA MICROBICA E PATOLOGIA VEGETALE

Ciclo XXVI

Settore Concorsuale di afferenza: 07/D1
Settore Scientifico disciplinare: AGR/12

IDENTIFICATION AND GENETIC DIVERSITY IN PHYTOPLASMAS ASSOCIATED WITH DISEASES OF CASSAVA AND OTHER AGRONOMIC RELEVANT CROPS IN SOUTH-EAST ASIA AND LATIN AMERICA

Presentata da: Dott. Juan Fernando Mejia

Coordinatore
Prof. Giovanni Dinelli

Relatore
Prof.ssa Assunta Bertaccini

Correlatore
Dr.ssa Elizabeth Alvarez

Esame finale anno 2014

“A mis amores Ana y Juan Martin, que este y otros sueños se sigan haciendo realidad, los amo con todo mi corazón”

INDEX

Summary

Identification and genetic diversity of phytoplasmas infecting tropical plant species, selected among those most agronomically relevant in South-east Asia and Latin America were studied. Correlation between evolutionary divergence of relevant phytoplasma strains and their geographic distribution by comparison on homologous genes of phytoplasma strains detected in the same or related plant species in other geographical areas worldwide was achieved. Molecular diversity was studied on genes coding ribosomal proteins, groEL, tuf and amp besides phytoplasma 16S rRNA. Selected samples infected by phytoplasmas belonging to diverse ribosomal groups were also studied by *in silico* RFLP followed by phylogenetic analyses. Moreover a partial genome annotation of a '*Ca.* P. brasiliense' strain was done towards future application for epidemiological studies.

Phytoplasma presence in cassava showing frog skin (CFSD) and witches' broom (CWB) diseases in Costa Rica - Paraguay and in Vietnam – Thailand, respectively, was evaluated. In both cases, the diseases were associated with phytoplasmas related to aster yellows, apple proliferation and "stolbur" groups, while only phytoplasma related to X-disease group in CFSD, and to hibiscus witches' broom, elm yellows and clover proliferation groups in CWB. Variability was found among strains belonging to the same ribosomal group but having different geographic origin and associated with different disease. Additionally, a dodder transmission assay to elucidate the role of phytoplasmas in CWB disease was carried out, and resulted in typical phytoplasma symptoms in periwinkle plants associated with the presence of aster yellows-related strains.

Lethal wilt disease, a severe disease of oil palm in Colombia that is spreading throughout South America was also studied. Phytoplasmas were detected in symptomatic oil palm and identified as '*Ca.* P. asteris', ribosomal subgroup 16SrI-B, and were distinguished from other aster yellows phytoplasmas used as reference strains; in particular, from an aster yellows strain infecting corn in the same country. Phylogeny based on parsimony analyses of ribosomal protein, groEL and amp genetic loci indicated a single monophyletic origin of aster yellows and the new '*Ca.* P. asteris' strains.

The close association of '*Ca*. Phytoplasma' species with cassava witches' broom, frog skin and oil palm lethal wilt diseases was confirmed since all symptomatic plants resulted to be

infected. New restriction enzymes for practical use in ribosomal group/subgroup identification are proposed. The implications of genetic variability detected in several strains is also discussed in relatioship with geographic strain distribution. The results of this research provid important information and tools that can be used to further study these disease epidemiology and insect vector identity.

Chapter 1

1 Introduction

Phytoplasma, formerly termed mycoplasma-like organism (MLOs), are minute cell wall-less prokaryotes reported in association with numerous insects' vectors and several hundred plant species, including commercial crops; roots and tubers, cereals, grain legumes, fruit crops, ornamental plants; timber and shade trees (Bertaccini, 2007; Bertaccini *et al*., 1992; Lee *et al*., 2007). Many of the economically important diseases are those of woody plants, including coconut lethal yellowing, peach X-disease, grapevine yellows, and apple proliferation. Discovered by Doi and co-worked in 1967, although their resemblance to animal and human mycoplasmas in morphology and ultrastructure, it remained uncertain whether phytoplasmas were members of the class *Mollicutes* until the late 1980's. Lim and Sears in 1989 showed that a MLO represented a new member of the class *Mollicutes* (Figure 1.1). The trivial name phytoplasma followed by designation of '*Candidatus* Phytoplasma' was adopted for their naming (IRPCM, 2004). Phytoplasma strains were initially differentiated and identified by their biological properties, such as symptomatology in infected plants, plant host and insect vector ranges, however the determination of biological properties was laborious and time-consuming, and often the results were inconclusive.

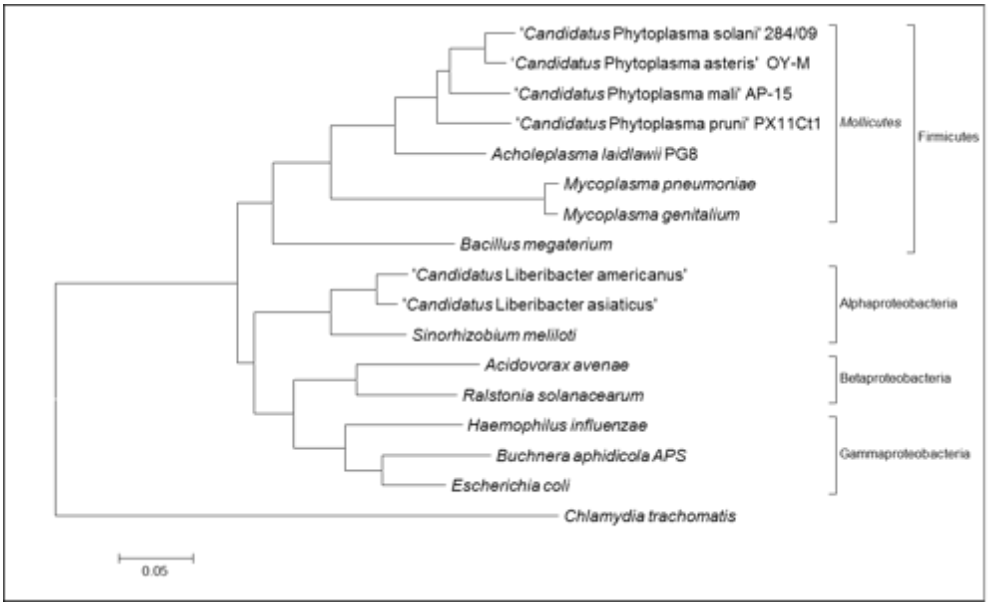


Figure 1.1. Phylogenetic distance tree constructed by the maximum parsimony method, within the 16S rRNA gene sequences of phytoplasmas with those of other bacteria obtained from GenBank. Phytoplasmas are currently classified in the *Mollicutes* class, along with mycoplasmas (based on Oshima *et al*., 2013).

Due to disturbances in the normal balance of growth regulators, plants infected by phytoplasmas exhibit an array of symptoms including virescence, development of green leaf like structures instead of flowers (phyllody), proliferation of axillary buds resulting in a witches' broom behavior, abnormal internodes elongation and generalized stunting (Figure 1.2) (Bertaccini, 2007).

Following the first cloning of phytoplasma DNA (Kirkpatrick *et al*., 1987), nucleic acid-based probes differentiating phytoplasmas in plants and vectors provided the first evidence of genetic differences among phytoplasma strains derived from different plant hosts and geographical locations. The application of PCR and nested-PCR assays allow to broadly detect phytoplasma presence, also in mixed infection, in field collected samples (Lee *et al.,* 1994; 1995).

First phytoplasma identification and classification systems proposed were based on specificity of vector transmission, on range of host plants and on symptom expression of a common host (periwinkle). Experimentally determined plant host ranges and ranges of insect vector species are broader than those observed in nature, and show a considerable amount of overlaps, therefore resulted to be unsuitable also for tentative classification. Development of antisera and of cloned probes showed clear evidence that phytoplasmas could be distinguished at molecular level; grouping was obtained on the basis of DNA detection by phytoplasma specific probes (Lee *et al*., 1992). The first comprehensive phytoplasma classification scheme was based on restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR) - amplified 16S rRNA (Lee *et al*., 1998a; 1998b; 2000), providing a reliable mean for the differentiation of a broad array of phytoplasmas. The PCR assays coupled with RFLP or sequence analyses on the 16S rDNA of phytoplasmas provide then a rapid and reliable mean for classification, opening the possibility to epidemiological studies on diseases associated with phytoplasma presence. Nevertheless, closely related strains (e.g. '*Candidatus* Phytoplasma asteris' strains) could be associated with different diseases and different symptoms (Lee *et al*., 2004a). To allow epidemiological studies, it is essential to identify and characterize the diverse strains that may be involved in a disease. The 16S rRNA gene-based system has deficiency in the differentiation of closely related strains. Several molecular markers, identified thus far, have shown improved resolving power in the delineation of these strains (Lee *et al*., 2010). Analysis of *rp, secY* or *tuf* gene sequences delineated biologically and/or ecologically distinct strains that often cannot be readily resolved based on the 16S rRNA gene alone (Lee *et al*., 2007; 2010a; Martini *et al*., 2007; Makarova *et al*., 2012).

In silico restriction analysis methods has led to the identification of putative new phytoplasma groups, since over the last few years, phytoplasmas have been discovered at an increasingly rapid pace in emerging diseases worldwide (Wei *et al*., 2007; 2008a; Zhao *et al*., 2009). At present, thirty-six '*Ca.* Phytoplasma' species, thirty-three ribosomal groups and 118 ribosomal subgroups, have been classified and associated with several hundred plant species diseases (Table 1.1).

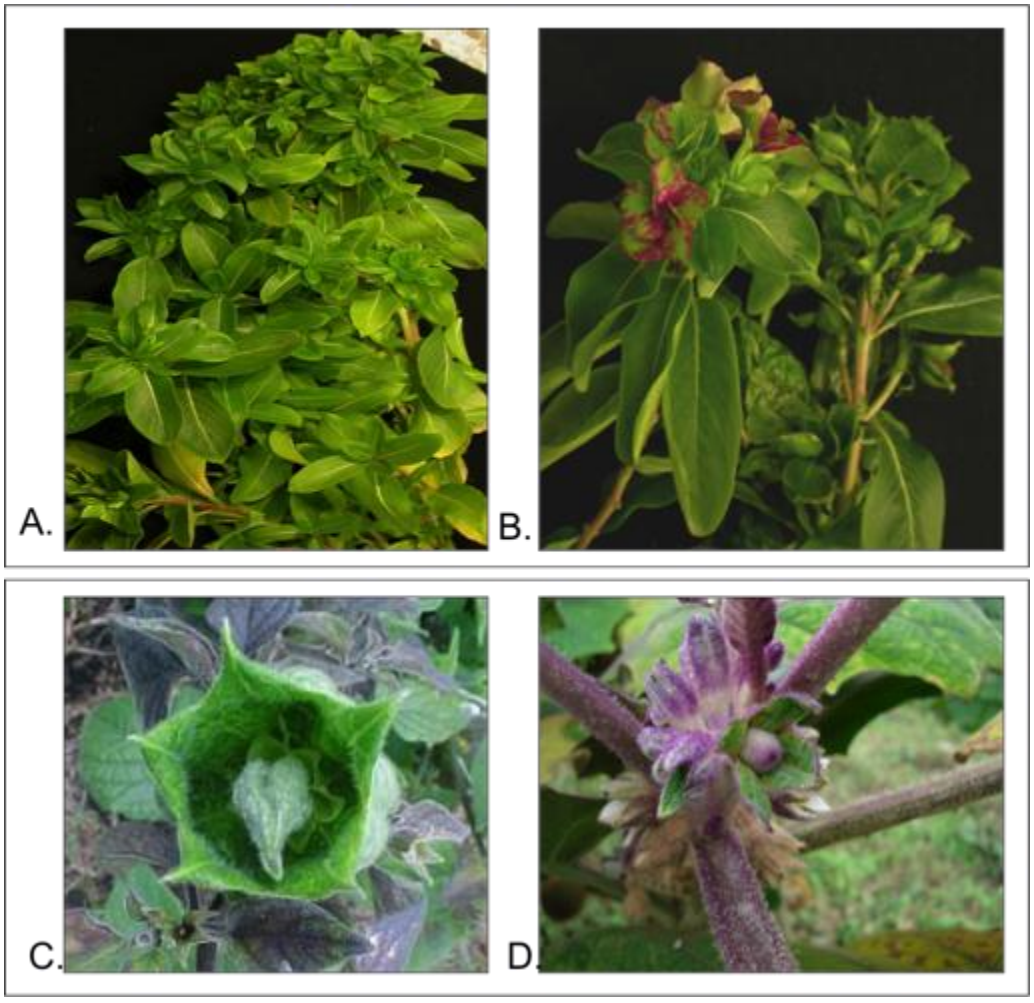


Figure 1.2 Symptoms associated with phytoplasma presence. A and B periwinkle virescence and witches' broom: virescence/phyllody, development of green leaf like structures instead of flowers, ssterility of flowers, proliferation of axillary buds resulting in a witches' broom appearance in an natural infected *Catharanthus roseus* in Palmira (Colombia). **C.** *Pysalis peruviana* witches' broom and **D.** *Solanum quitoense* machorreo phytoplasma: sterility of flowers, abnormal internodes elongation, generalized stunting.

Very recently the proof that phytoplasmas can now be grown on laboratory media was provided (Bertaccini *et al*., 2010; 2012; Contaldo *et al*., 2012; 2013). This is an important breakthrough in the study of the biology and on the advancement of phytoplasma-incited

plant diseases management. Since their discovery the proof of phytoplasma pathogenicity was lacking due mainly to the inability to isolate and grow them in culture. The inability to fulfill Koch's postulates severely restricted the understanding of the roles of phytoplasmas in disease etiology and plant–insect-phytoplasma interactions. The disappearance of symptoms in some cases after antibiotic (i.e. tetracycline) treatment provided evidence to support their association with diseases (Ishiie *et al*., 1967).

1.1 Phytoplasma-associated diseases worldwide

The geographical distribution and impact of phytoplasma diseases depends on the host range of the phytoplasma as well as the feeding behavior of the insect vector. Many phytoplasmas have restricted host ranges and oligophagous or monophagous insect vectors, which restrict their geographical distribution. Some have a broad range of plant hosts and polyphagous vectors and therefore have a wide distribution. As an example potato witches' broom and maize bushy stunt (MBS) are among the most widespread diseases in herbaceous hosts causing severe yield losses. While symptomatic potatoes are associated with the presence of phytoplasmas belonging to different groups mainly according to different growing areas (Girsova *et* al., 2008; Liefting *et al*., 2009; Eroglu *et al*., 2010; Hosseini *et al.*, 2011; Mejia *et al*., 2011); the corn resulted to be consistently infected in America by a molecularly distinguishable strain of aster yellows phytoplasmas (16SrI-B) and has been reported as the causal agent of maize bushy stunt (Harrison *et al.*, 1996; Bedendo *et al*., 1997; Ebbert *et al.*, 2001; Mejia *et al*., 2013), while similar symptoms in corn in Europe allow the detection of "stolbur" phytoplasmas (Duduk and Bertaccini, 2006). The most economically dangerous phytoplasmas reported worldwide belong to the ribosomal groups 16SrI, 16SrII, 16SrIII, 16SrIV, 16SrV, 16SrVI, 16SrIX, 16SrX, 16SrXI and 16SrXII (Table 1). In the figures 1.3 and 1.4 are described the main geographical areas affected by the most important phytoplasma diseases worldwide and overall situation in the tropical and subtropical area.

1.1.1 Aster yellows (16SrI)

Beside the phytoplasma diseases described above belonging to the aster yellow group (16SrI), such as causal agent of MBS in *Zea mays*, many others have been reported in several crops worldwide. Aster yellows phytoplasma is widely distributed in North America, it induces yellows and dwarfing in vegetables such as lettuce, carrot, onion, cabbage, celery, potato and tomato; small fruits, such as blueberry and strawberry; and ornamentals, such as China aster, *Oenothera* spp. and periwinkle in the USA, as well as clover in both the USA and Canada

(Lee *et al*., 2003; 2004a). Ribosomal subgroup 16SrI-A has also been also associated with grapevine yellows in Virginia (Davis *et al*., 1998) and it has been demonstrated to be one of the agents of sugarcane yellow leaf disease in Cuba (Arocha *et al*., 2005). In Europe, it also affect lettuce, onion, potato and tomato, is associated with clover phyllody and strawberry green petal, and also agent of various disorders of gladiolus, hydrangea, primula, anemone, ranunculus, chrysanthemum and poplar (Berges *et al*., 1997; Seruga *et al*., 2003; Lee *et al*., 2004a). In Italy and more recently in South Africa and in Chile, it has recently reported in grapevine with symptomatology indistinguishable from the one reported for grapevine yellows diseases (Alma *et al.*, 1996; Gajardo *et al*., 2009; Engelbrecht *et al*., 2010). In Asia aster yellows is responsible for marguerite yellows, phyllody in hydrangea and paulownia witches' broom (Takinami *et al*., 2013) (Figures 1.3 and 1.4). In Saudi Arabia, a 16SrI group was found associated with the Al-Wijam disease of date palm (Alhudaib *et al.,* 2008), while aster yellows phytoplasmas could be consistently associated with a lethal yellowing of oil palm (*Elaeis guinensis*) and woody host plants in Colombia (Perilla-Henao *et al*., 2012; Alvarez *et al*., 2014) and Brazil (Montano *et al*., 2007). Recently, it was also reported in citrus showing huanglongbing (yellow shoot disease) symptoms in Guangdong, China (Teixeira *et al*., 2009).

1.1.2 Peanut witches' broom (16SrII)

Witches' broom disease of lime (WBDL), associated with the presence of group 16SrII ('*Ca.* P. aurantifolia') (Table 1), is responsible for major losses of Mexican lime trees (*Citrus aurantifolia* L.); it was first observed in Oman where the destruction of 98% of Mexican lime trees occurred. The disease was then spreading in near areas such as United Arab Emirates, India and Iran where 30% of the Mexican lime trees have been destroyed (Mardi *et al*., 2011). The natural host range of this phytoplasma includes *Citrus aurantifolia*, *C. medica*, *C. limetta*, *C. lemon* and *C. jambhiri*. In the U.A.E. also sweet lime (*Citrus limettioides*) was reported as naturally infected. Among non-rutaceous plant hosts, periwinkle (*Catharanthus roseus*); *Achyranthes aspera*, *Amaranthus graecizans* and *Suaeda fruticosa* are reported as hosts (Moghal *et al*., 1998). The phytoplasma associated with the disease expanded its territory to other plants such as grapefruit and it is anticipated that it can become a serious threat to other horticultural productions. Members of this taxonomic group are also responsible for cotton phyllody in western Africa (Desmidts and Rassel, 1974) and faba bean phyllody in Sudan (Jones *et al*., 1984). Group 16SrII phytoplasmas are also present in Australia, where they were associated with a tomato big bud and sweet potato little leaf diseases (Schneider *et al*., 1999).

In Asia peanut, sun hemp and crotalaria witches' brooms (Yang, 1985; Sharma, 1990; Yang et al., 2008) are associated with phytoplasmas of this ribosomal group (Figures 1.3 and 1.4).

1.1.3 X-disease (16SrIII)

In North America, western-X disease, associated with phytoplasmas of the 16SrIII group, was the major phytoplasma disease to peach and cherry production in California (Granett and Gilmer, 1971; Kirkpatrick *et al*., 1995; Blomquist and Kirkpatrick, 2002), but they were reported in other crops, such as walnut, pecan and grapevine in Virginia (Davis *et al*., 1998; Lee *et al*., 2000). The phytoplasma was also detected in Italy, in declining cherry trees (Landi *et al*., 2007) and in myrtle plantations (Prota *et al*., 2007). In sour and sweet cherry infected by this agent in Europe leaf reddening and premature leaf drop, proliferation of branches and non-seasonal flowering were observed (Valiunas *et al*., 2009; Cieślińska, 2011). In South America its has been widely described in Argentina in daisy, garlic, summer squash, tomato and China tree (Galdeano *et al.,* 2013); in Colombia in cassava, 'uchuva', 'naranjilla', coffee, and 'nogal cafetero' (Alvarez *et al*., 2009; Mejia *et al*., 2010) and in Brazil in several plant species (Montano et al., 2007; Mello *et al*., 2011); in Africa, they are responsible of a sugarcane yellows (Cronjé *et al*., 1998). This phytoplasma group is reported in Asia but with minor relevance (Lee *et al*., 2000).

1.1.4 Coconut lethal yellows (16SrIV)

The group 16SrIV phytoplasma (Table 1) has been shown as specifically associated with the palm lethal yellowing (LY) disease vectored by *Myndus crudus* (American palm cixiid) and possibly also by *Cedusa* species of derbid planthoppers (Brown *et al.,* 2006). Phytoplasmas closely related to the 16SrIV group were reported in the date palm and other palm species (Harrison *et al.,* 2002b; 2009) and in weeds (Brown *et al.,* 2008a; 2008b) collected near symptomatic coconut palms. LY infects and kills coconut palms as well as many other palm species (Nejat *et al.,* 2012). Known since the 1800's it has long been of concern in Florida (USA) where it killed about 75% of the coconut palms in some areas before 1965. It was then detected in Mexico where many hundreds of thousands of coconut palms were killed and the local coconut industries were obliged to close (Harrison *et al*., 1992; 2002a; 2009; Oropeza *et al*., 2011; Ntushelo *et al*., 2012). However not only this ribosomal group is associated and/or responsible of this disease, very recently a novel taxon associated with lethal yellowing-type disease of coconut in Mozambique (lethal yellows disease Mozambique, LYDM) was reported, and described as new taxon i.e. '*Candidatus* Phytoplasma palmicola' (Harrison *et*

al., 2014). Another new taxon designed as '*Ca*. P. malaysianum' was also associated with coconut yellow decline and oil palm decline in Malaysia (Nejat *et al*., 2009; 2012).

1.1.5 Elm yellows (16SrV)

"Flavescence dorée" (FD) phytoplasma is a quarantine pathogen of grapevine belonging to the 16SrV ribosomal group (Boudon-Padieu, 2002). It is widespread in the main grapevine growing countries in Europe (Bertaccini *et al.*, 1995; Daire *et al.*, 1997; Martini *et al*., 1999); genetic studies indicated that phytoplasmas belonging to ribosomal subgroups 16SrV-C and 16SrV-D (Table 1) are involved with different geographic distributions (Figure 1.3). Both FD types resulted to be experimentally transmissible by the same vector *Scaphoideus titanus* (Mori *et al.*, 2002). Strains of FD subgroup 16SrV-D were detected in Northern Italy (Martini *et al*., 1999), France and Spain (Angelini *et al*., 2001; Torres *et al*., 2005) where the disease showed the highest epidemic outbreaks. Disease symptoms mainly involve plant decline, leaf rolling, shriveled grapes, unripened shoots and reddening or yellowing of leaves on red or white cultivars respectively. The severity and increasing presence of this disease has prompted extensive efforts for specific phytoplasma detection. The FD strain identification is achieved by studying the polymorphisms in *rpS3*, *SecY* genes as well as other genes (Angelini *et al*., 2001; Martini *et al.*, 2002; Arnaud *et al*., 2007; Botti and Bertaccini, 2007). However, in Europe other phytoplasmas of group 16SrV mainly infect alder, blackberry and *Spartium*. '*Ca*. P. ulmi' is responsible for yellows of elm species in North America and Europe (Lee *et al*., 2004b). In Asia, '*Ca*. P. ziziphi' is the agent of jujube witches' broom in China, Korea and Japan. Severe declines in cherry and peach are associated also with the presence of '*Ca.* P. zyziphi' in China and India. (Zhu *et al*., 1998; 2011; Jung *et al*., 2003a; Lee *et al*., 2004b) (Figures 1.3 and 1.4; Table 1). In both, Europe and USA elm yellows or elm witches' broom, were associated with '*Ca.* P. ulmi' presence, however the vectors are diverse and the two phytoplasmas appears to be differentiable on multi locus typing analyses (Jović *et al*., 2011). Forest trees are severely damaged by diseases associated with phytoplasmas also in other areas of the world (Bertaccini *et al*., 1996; Duduk *et al*., 2009; Franco *et al*., 2010). 16SrV ribosomal group is also reported, but has few hosts or impact in a restricted area in Colombia (Mejia *et al*., 2011).

1.1.6 Clover proliferation (16SrVI)

Phytoplasmas in 16SrVI group were first described as being associated with a disease of alsike clover in Canada, and later with phytoplasma diseases of tomato, as well as potato and elm, in North America (Chen and Hiruki, 1975; Lee *et al*., 1991; Shaw *et al*., 1993; Jacobs *et*

al., 2003). In Europe and the Middle East, this phytoplasma was recently associated with diseases of pepper and tomato in Spain, Jordan, Lebanon and in Austria in *Vaccinium myrtillus* (Anfoka *et al*., 2003; Castro and Romero, 2002; Choueiri *et al*., 2007; Fernandez *et al*., 2007). Phytoplasmas of this group are also responsible for the little leaf disease of eggplant in India and Bangladesh (Mitra, 1988; Schneider *et al*., 1995; Siddique *et al*., 2001). Recently, symptoms of abnormal proliferation of shoots resulting in formation of witches' broom growths were observed on diseased plants of passion fruit (*Passiflora edulis* f. *flavicarpa* Deg.) in Brazil associated with '*Ca.* P. sudamericanum' designed as new ribosomal subgroup16SrVI-I (Davis *et al.*, 2012).

1.1.7 Pigeon pea witches' broom (16SrIX)

The economic impact of the phytoplasma group 16SrIX is mostly restricted to the Middle East and is a major threat to almond production in Iran and Lebanon. The almond witches' broom disease was first reported as almond brooming disease in Fars province of Iran (Salehi and Izadpanah, 1995). A similar epidemic disease of almond was reported in Lebanon (Choueiri *et al*., 2007), and the phytoplasma agent was described as '*Ca.* P. phoenicium' (Verdin *et al*., 2003; Salehi *et al*., 2006; Zirak *et al*., 2009; 2010; Molino Lova *et al*., 2011) (Table 1). Production of almonds has been seriously affected since the 1990s in Lebanon and Iran, as the phytoplasma induces a lethal disease. Thousands of almond trees have died over the past 15 years in Lebanon, since the beginning of the first epidemic, which occurred in the south of the country (Abou-Jawdah *et al*., 2003). A related phytoplasma was also associated with *Knautia arvensis* (Knautia phyllody, KAP) detected in Italy (Marcone *et al*., 2001). Other members of this group are associated with pigeon pea witches' broom in North America; in particular subgroup 16SrIX-E was associated with juniper witches' broom disease in Oregon (Davis *et al*., 2010). The 16SrIX group phytoplasmas were also recently described as being associated with a yellowing disease of citrus in Brazil (Chen *et al*., 2008). In natural infected periwinkle the phytoplasma was also reported in Brazil and Colombia (Montano *et al*., 2007; Duduk *et al*., 2008).

1.1.8 Apple proliferation (16SrX)

Phytoplasma diseases of fruit trees were studied in deep in Europe for their relevant economic impact, they include apple proliferation (AP), pear decline (PD) and European stone fruit yellows (ESFY). Phylogenetic analyses showed that the 16S rDNA sequences of the phytoplasmas associated with these diseases are identical or nearly identical, however psyllid vector transmission and host range specificity allow to differentiate them in '*Ca.* P. mali',

'*Ca.* P. pyri' and '*Ca.* P. prunorum' (Seemüller and Schneider, 2004) (Table 1). AP is present in almost all European countries; very recently a link was demonstrated between the diverse '*Ca*. P. mali' strains colonizing apple trees and their pathogenicity degree (Seemüller *et al*., 2010). Apple is the main host of '*Ca.* P. mali' that was also detected in wild and ornamental *Malus* species, in hazelnut (*Corylus* spp.), cherry (*Prunus avium*), apricot (*P. armeniaca*) and plum (*P. domestica*). The psyllid *Cacopsylla picta* (Forster) and *C. melanoneura* are the responsible of the diffusion of AP in apple orchards in Italy and Germany. PD was firstly reported in the fifties in Western areas of North America, but today it is of relevant importance mainly in European pear orchards. In North America and UK the known vector is *Cacopsylla pyricola* (Foerster), but *Cacopsylla pyri* (L.) has been found as the main vector in other European regions. ESFY are affecting several stone fruit species inducing apricot, plum, and peach, but infect also several other *Prunus* species. ESFY disease is characterized by rapid and widespread diffusion especially when the conditions are favorable for host-plants and vectors.

1.1.9 Rice yellow dwarf (16SrXI)

The ribosomal group 16SrXI is present in Asia, where it is associated with rice yellow dwarf disease. Known phytoplasma phylogenetically related are those associated with sugarcane white leaf and sugarcane grassy shoot, annual bluegrass white leaf, Bermuda grass white leaf and *Brachiaria* grass white leaf (Jung *et al*., 2003b). This group seems to be absent from other rice producing areas in the world, but has been occasionally described in Europe on *Cirsium arvense* and on napier grass (*Pennisetum purpureum*) in Kenya (Foissac and Wilson, 2010). However, the 16SrXI group has been also associated also with Weligama wilt disease of coconut in Sri Lanka (Table 1.1, Figures 1.3 and 1.4) and in the same species; it was associated with Kalimantan wilt disease in Indonesia (Warokka, 2005).

1.1.10 "Stolbur" (16SrXII)

The "stolbur" phytoplasma, '*Ca*. P. solani' (Quaglino *et al*., 2013), a member of the group 16SrXII-A, infects a wide range of cultivated plants in Europe, Mediterranean basin and southern Russia such as the solanaceous crops, grapevine, celery, sugar beet, strawberry and lavender (Garnier, 2000; Ember *et al*., 2011). Grapevine yellows (GY) are widespread diseases associated with molecularly distinguishable phytoplasmas worldwide. One of these is "bois noir" (BN) associated with phytoplasmas belonging to ribosomal subgroup 16SrXII-A, that has symptoms undistinguishable from those of FD, and is widespread in all viticultural areas. BN phytoplasmas are transmitted to grapevine by *Hyalesthes obsoletus* Signoret

(Homoptera, *Cixiidae*) from *Convolvulus arvensis* L. (Maixner, 1994; Sforza *et al.*, 1998) and *Urtica dioica* L. (Alma *et al*., 2002). However, these phytoplasmas were also detected in other plants and auchenorrhyncha species that could be also involved in BN epidemiology. The usefulness of *tuf* gene polymorphism for epidemiological studies was clearly shown (Langer and Maixner, 2004; Mori *et al*., 2008). The phytoplasma is also reported, but has few hosts or impact in a restricted area in the north part of South America (Franco *et al*., 2010; Mejia *et al*., 2011) and in some cases of grapevine yellows in Chile (Gajardo *et al*., 2009).

Table 1.1. Classification of phytoplasmas based on RFLP analyses and/or sequencing of 16S rDNA.

16Sr group/subgroup	strain (acronym) **'*Candidatus* sp.'**	Genbank number
	16SrI: aster yellows (America, Europe, Asia, Africa)	
I-A	Aster yellows witches' broom (AYWB)	NC007716
I-A	Tomato big bud (BB)	L33760
I-B	Onion yellows mild strain (OY-M)	NC005303
I-B	Aster yellows (MAY) **'*Ca.* P. asteris'**	M30790
I-C	Clover phyllody (CPh)	AF222065
I-D	Paulownia witches' broom (PaWB)	AY265206
I-E	Blueberry stunt (BBS3)	AY265213
I-F	Aster yellows apricot - Spain (A-AY)	AY265211
I-I	Strawberry witches' broom (STRAWB1)	U96614
I-K	Strawberry witches' broom (STRAWB2)	U96616
I-L	Aster yellows (AV2192)	AY180957
I-M	Aster yellows (AVUT)	AY265209
I-N	Aster yellows (IoWB)	AY265205
I-O	Soybean purple stem (SPS)	AF268405
I-P	Aster yellows from *Populus* (PopAY)	AF503568
I-Q	Cherry little leaf (ChLL)	AY034089
I-R	Strawberry phylloid fruit (StrawbPhF)	AY102275
I-S	Mexican potato purple top phytoplasma (COAH10)	FJ914654
I-U	Mexican potato purple top phytoplasma (JAL6)	FJ914650
I-V	Mexican potato purple top phytoplasma (SON18)	FJ914642
I-W	Peach rosette-like disease (PRU0382)	HQ450211
I-Y	"Brote grande" of tomato **'*Ca.* P. lycopersici'**	EF199549
	16SrII: peanut witches' broom (America, Africa, Europe, Asia, Australia)	
II-A	Peanut witches' broom (PnWB)	L33765
II-B	Lime witches' broom (WBDL) **'*Ca.* P. aurantifolia'**	U15442
II-C	Faba bean phyllody (FBP)	X83432
II-D	Papaya mosaic (PpM) **'*Ca.* P. australasia'**	Y10096
II-E	Pichris echioides phyllody (PEY)	Y16393
II-F	Cotton phyllody (CoP)	EF186827
	16SrIII: X-disease (America, Europe, Asia)	
III-A	Peach X-disease (PX11CT1) **'*Ca.* P. pruni'**	JQ044392/JQ044393
III-B	Clover yellow edge (CYE)	AF173558

16Sr group/subgroup	strain (acronym) '***Candidatus* sp.**'	Genbank number
III-C	Pecan bunch (PBT)	GU004371
III-D	Goldenrod yellows (GR1)	GU004372
III-E	Spiraea stunt (SP1)	AF190228
III-F	Milkweed yellows (MW1)	AF510724
III-G	Walnut witches' broom (WWB)	AF190226/AF190227
III-H	Poinsettia branch-inducing (PoiBI)	AF190223
III-I	Virginia grapevine yellows (VGYIII)	AF060875
III-J	Chayote witches' broom (ChWBIII)	AF147706
III-K	Strawberry leafy fruit (SLF)	AF274876
III-L	Cassava frog skin disease (CFSD)	EU346761
III-M	Potato purple top (MT117)	FJ226074
III-N	Potato purple top (AKpot6)	GU004365
III-P	Dandelion virescence (DanV)	AF370119/AF370120
III-Q	Black raspberry witches' broom (BRWB7)	AF302841
III-T	Sweet and sour cherry (ChD)	FJ231728
III-U	Cirsium white leaf (CWL)	AF373105/AF373106
III-V	Passion fruit phytoplasma (PassWB-Br4)	GU292082
	16SrIV: coconut lethal yellows (America, Africa)	
IV-A	Coconut lethal yellowing (LYJ-C8)	AF498307
IV-B	Yucatan coconut lethal decline (LDY)	U18753
IV-C	Tanzanian coconut lethal decline (LDT)	X80117
	16SrV: elm yellows (Europe, America, Asia, Africa)	
V-A	Elm yellows (EY) **'*Ca.* P. ulmi'**	AY197655
V-B	Jujube witches' broom (JWB-G1) **'*Ca.* P. ziziphi'**	AB052876
V-C	«Flavescence dorée» (FD-C)	X76560
V-D	«Flavescence dorée» (FD-D)	AJ548787
V-E	Rubus stunt (RuS) **'*Ca.* P. rubi'**	AY197648
V-F	Balanite witches' broom (BltWB) **'*Ca.* P. balanitae'**	AB689678
	16SrVI: clover proliferation (Europe, America, Asia)	
VI-A	Clover proliferation (CP) **'*Ca.* P. trifolii'**	AY390261
VI-B	Strawberry multiplier disease (MC)	AF190224
VI-C	Illinois elm yellows (EY-IL1)	AF409069/AF409070
VI-D	Periwinkle little leaf (PLL-Bd)	AF228053
VI-E	*Centaurea solstitialis* virescence (CSVI)	AY270156
VI-F	Catharanthus phyllody phytoplasma (CPS)	EF186819
VI-H	Portulaca little leaf phytoplasma (PLL-Ind)	EF651786
VI-I	Passionfruit (WB-Br3) **'*Ca.* P. sudamericanum'**	GU292081
	16SrVII: ash yellows (America, Europe)	
VII-A	Ash yellows (AshY) **'*Ca.* P. fraxini'**	AF092209
VII-B	Erigeron witches' broom (ErWB)	AY034608
VII-C	Argentinian alfalfa witches' broom (ArAWB)	AY147038
	16SrVIII: Loofah witches' broom (Asia)	
VIII-A	Loofah witches' broom (LufWB)	AF086621
	16SrIX: pigeon pea witches' broom (Europe, Asia, America)	
IX-A	Pigeon pea witches' broom (PPWB)	AF248957
IX-B	Almond witches' broom (AlWB) **'*Ca.* P. phoenicium'**	AF515636

16Sr group/subgroup	strain (acronym) '***Candidatus* sp.**'	Genbank number
IX-C	Naxos periwinkle virescence (NAXOS)	HQ589191
IX-D	Almond witches' broom (AlWB)	AF515637
IX-E	*Juniperus* witches' broom	GQ925918
IX-F	Almond and stone fruit witches' broom (N27-2)	HQ407532
IX-G	Almond and stone fruit witches' broom (A1-1)	HQ407514
	16SrX: apple proliferation (Europe, America)	
X-A	Apple proliferation (AP) **'*Ca*. P. mali'**	AJ542541
X-B	European stone fruit yellows (ESFY) **'*Ca*. P. prunorum'**	AJ542544
X-C	Pear decline (PD) **'*Ca*. P. pyri'**	AJ542543
X-D	Spartium witches' broom (SpaWB) **'*Ca*. P. spartii'**	X92869
X-E	Black alder witches' broom (BAWB[BWB])	X76431
	16SrXI: rice yellow dwarf (Europe, Asia, Africa)	
XI-A	Rice yellow dwarf (RYD) **'*Ca*. P. oryzae'**	AB052873
XI-B	Sugarcane white leaf (SCWL)	X76432
XI-C	Leafhopper-borne (BVK)	X76429
	16SrXII: "stolbur" (Europe, Asia, America, Africa, Australia)	
XII-A	Stolbur (STOL11) **'*Ca*. P. solani'**	AF248959
XII-B	Australian grapevine yellows (AUSGY) **'*Ca*. P. australiense'**	L76865
XII-C	Strawberry lethal yellows (StrawLY)	AJ243045
XII-D	Japanese hydrangea phyllody **'*Ca*. P. japonicum'** (JHp)	AB010425
XII-E	Yellows diseased strawberry (StrawY)**'*Ca*. P. fragariae'**	DQ086423
XII-F	"Bois noir" (BN-Op121)	EU836651
XII-G	"Bois noir" (BN-Fc3)	EU836647
XII-H	Bindweed yellows (BY-S57/11) **'*Ca*. P. convolvuli'**	JN833705
	16SrXIII: Mexican periwinkle virescence (America)	
XIII-A	Mexican periwinkle virescence (MPV)	AF248960
XIII-B	Strawberry green petal (SGP)	U96616
	16SrXIV: Bermudagrass white leaf (Europe)	
XIV-A	Bermudagrass white leaf (BGWL) '***Ca*. P. cynodontis'**	AJ550984
XIV-B	Bermudagrass white leaf Iran	EF444485
	16SrXV: hibiscus witches' broom (America)	
XV-A	Hibiscus witches' broom (HibWB) **'*Ca*. P. brasiliense'**	AF147708
XV-B	Guazuma witches' broom (GWB)	HQ258882
	16SrXVI: sugarcane yellow leaf syndrome (America)	
XVI-A	Sugarcane yellow leaf syndrome **'*Ca*. P. graminis'**	AY725228
	16SrXVII: papaya bunchy top (America)	
XVII-A	Papaya bunchy top **'*Ca*. P. caricae'**	AY725234
	16SrXVIII: American potato purple top wilt (America)	
XVIII-A	American potato purple top wilt **'*Ca*. P. americanum'**	DQ174122
	16SrXIX: Chestnut witches' broom (Asia)	
XIX-A	Chestnut witches' broom **'*Ca*. P. castaneae'**	AB054986
	16SrXX: Rhamnus witches' broom (Europe)	
XX-A	Rhamnus witches' broom **'*Ca*. P. rhamni'**	AJ583009
	16SrXXI: Pinus phytoplasmas (Europe)	
XXI-A	Pinus phytoplasma (PinP) **'*Ca.* P. pini'**	AJ310849
	16SrXXII: Lethal yellowing-type (Africa)	
XXII-A	Coconut lethal yellowing-Nigerian Awka disease (LDN)	Y14175

16Sr group/subgroup	strain (acronym) '***Candidatus* sp.**'	Genbank number
XXII-A	Mozambique coconut (LYDM-185) **'*Ca.* P. palmicola'**	KF751388
XXII-B	Cape St. Paul wilt (CSPW)	KF419286
	16SrXXIII: -	
XXIII-A	Buckland valley grapevine yellows	AY083605
	16SrXXIV: -	
XXIV-A	Sorghum bunchy shoot	AF509322
	16SrXXV: -	
XXV-A	Weeping tea witches broom	AF521672
	16SrXXVI: -	
XXVI-A	Sugarcane phytoplasma D3T1	AJ539179
	16SrXXVII: -	
XXVII-A	Sugarcane phytoplasma D3T2	AY539180
	16SrXXVIII: -	
XXVIII-A	Derbid phytoplasma	AY744945
	16SXXIX: Cassia witches' broom (Asia)	
XXIX-A	Cassia witches' broom (CaWB) **'*Ca.* P. omanense'**	EF666051
	16SXXX: Salt cedar witches' broom (Asia)	
XXX-A	Salt cedar witches' broom **'*Ca.* P. tamaricis'**	FJ432664
	16SXXXI: Soybean stunt (America)	
XXXI-A	Soybean stunt (SoyST1c1) **'*Ca.* P. costaricanum'**	HQ225630
	16SXXXII: Malaysian periwinkle virescence and phyllody (Asia)	
XXXII-A	Malaysian p. virescence (MaPV) **'*Ca.* P. malaysianum'**	EU371934
XXXII-B	Malayan yellow dwarf phytoplasma (MYD)	EU498727
XXXII-C	Malayan oil palm phytoplasma (MOP)	EU498728
	16SXXXIII: *Allocasuarina muelleriana* phytoplasma (Australia)	
XXXIII-A	Allocasuarina phytoplasma **'*Ca.* P. allocasuarinae'**	AY135523

- the ribosomal group has no designation since only Genbank sequences were employed for its determination

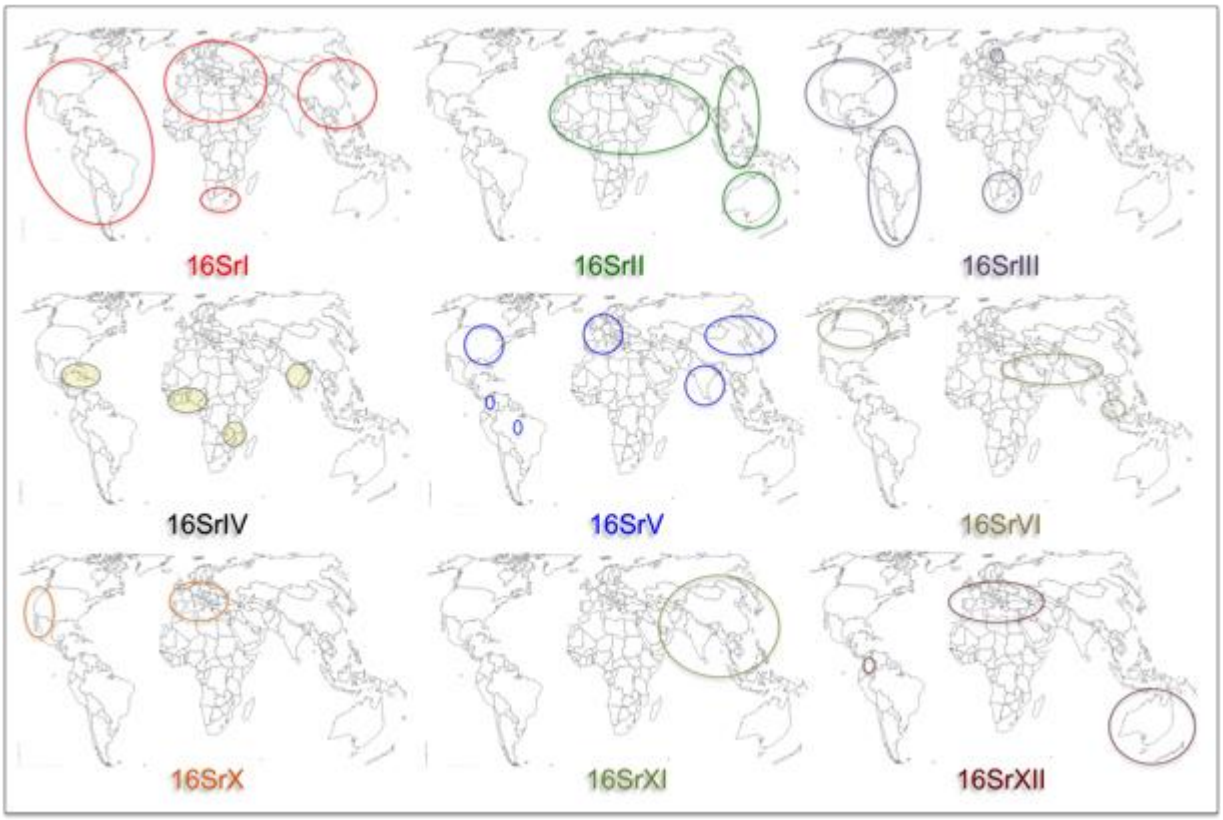


Figure 1.3. Geographical distribution of the most relevant phytoplasmas in agriculture worldwide (based on Foissac and Wilson, 2010).

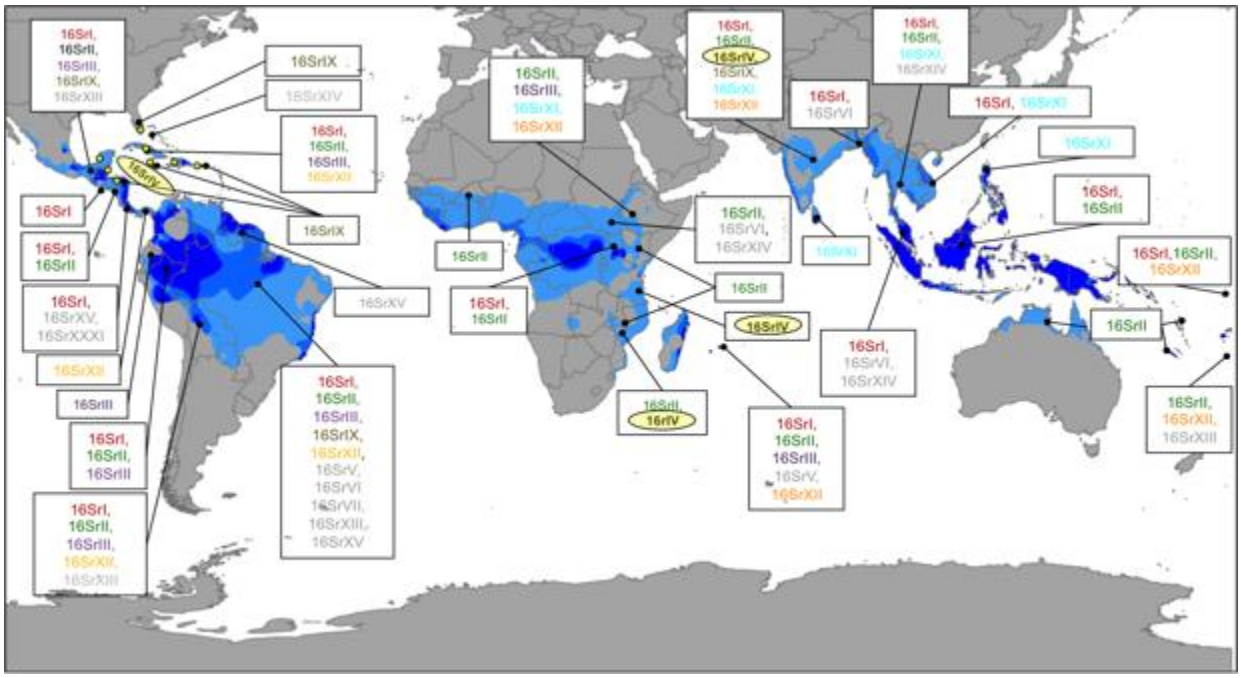


Figure 1.4. Geographic distribution of phytoplasma groups in the tropical and subtropical areas. Information was obtained using the NCBI database (National Center for Biotechnology information), each phytoplasma reported in each country is supported by a reference publication.

1.2 Phytoplasma genomics and plant interaction

The whole genome sequence for two strains of '*Ca*. P. asteris', two strains of '*Ca.* P. australiense', one strain of '*Ca*. P. mali' (Bai *et al*., 2006; Oshima *et al*., 2004; Tran-Nguyen *et al*., 2008; Kube *et al*., 2008; Andersen *et al*., 2013) are available, enabling to start the understanding of molecular mechanisms underlying pathogen-host interaction and also virulence (Suzuki *et al*., 2006; Oshima *et al.*, 2007; Hoshi *et al.*, 2009). Phytoplasmas have sizes variable from 200 to 800 nm, they are polymorphic because of the lack of cell wall, and survive and multiply in hysotonic habitats, such as plant phloem and insect emolymph. The phytoplasma chromosome is small (680-1,600 kb) and sequence analysis of 16S rDNA and other housekeeping genes indicate that are closely related to the *Acholeplasma* spp. (Figure 1.1), or to some of the mycoplasmas rather than to the *Spiroplasma* spp, that are the other Mollicutes reported as plant pathogenic (Lim and Sears, 1992; Gasparich *et al*., 2004;). Nevertheless, the genomes of 14 strains, including '*Ca*. P. asteris', '*Ca*. P. mali', '*Ca*. P. pyri', '*Ca*. P. pruni', and '*Ca.* P. australiense' were analyzed along with *Acholeplasma laidlawi*, to further determine their taxonomic relatedness. The results were in large agreement with the previously established 16S rDNA based classification schemes. Moreover, the taxonomic relationships within the '*Ca*. P. asteris', '*Ca*. P. pruni' and '*Ca*. P. mali', '*Ca*. P. pyri', that represent clusters of strains whose relatedness could not be determined by 16SrDNA analysis, could be comparatively evaluated with non-subjective criteria. '*Ca.* P. mali' and '*Ca.* P. pyri' were found to meet the genome characteristics for the retention into two different, yet strictly related species. Representatives of subgroups 16SrI-A and 16SrI-B belonging to '*Ca*. P. asteris' were also found to meet the standards used in other bacteria to distinguish separate species. However the genomes of the strains belonging to '*Ca*. P. pruni' were found more closely related, suggesting that their subdivision into '*Candidatus* species' should be approached with caution (Firrao *et al*., 2013).

Phytoplasmas have the smallest genome among bacteria and like other members of the *Mollicutes*, possess one circular or straight double-stranded DNA chromosome. Their genome has a low G+C content (21–28%), similar to mycoplasmas and to endosymbiotic bacteria. Many predicted genes are present in multiple copies; they contain two rRNA operons, and heterogeneity of these operons has been demonstrated in several phytoplasma a strains (Schneider and Seemüller, 1994; Liefting *et al*., 1996; Harrison *et al*., 2002a; Jung *et al*., 2003c; Davis *et al*., 2013).

Phytoplasma genomes contain large numbers of transposon genes and insertion sequences that are unique to these organisms and were named as variable mosaics (SVM) (Jomantiene and Davis *et al*., 2006; Jomantiene *et al*., 2007; Wei *et al*., 2008b) or potential mobile units, (PMUs) (Bai *et al*., 2006). PMUs and SVMs have similar compositions and contain similar genes. In the '*Ca.* P. asteris' AY-WB strain genome, PMUs are ~20 kb in size and consist of genes with similarities to *sigF, hflB, dnaG, dnaB, tmk, ssb, himA*, and the IS3 family insertion sequence tra5, organized in a conserved order (Bai *et al*., 2006). These genes are also found in multiple copies, singly or in clusters, in other phytoplasma genomes (Miyata *et al*., 2003; Davis *et al*., 2003a; Oshima *et al*., 2004; Lee *et al*., 2005; Jomantiene and Davis, 2006; Jomantiene *et al*., 2007; Arashida *et al*., 2008;) and exists as linear chromosomal and circular extrachromosomal elements in '*Ca.* P. asteris' AY-WB strain suggesting that they can have transposonic activity (Toruno *et al*., 2010). In the *'Ca.* P. asteris', onion yellows strain (OY); a mildly pathogenic, insect-transmitted line (OY-M) contains two types of plasmids (EcOYM and pOYM), each of which possesses a gene encoding the putative transmembrane protein, ORF3 that plays a role in the interactions of OY with the insect host of this phytoplasma (Ishii *et al*., 2009).

Differences in the chromosome size among phytoplasma species were reported; '*Ca*. P. cynodontis' and a tomato strain of the "stolbur" phytoplasma that belong to different 16Sr groups have the chromosomal size of 530 and 1,350 kb, respectively; or the rape virescence phytoplasma and the hydrangea phyllody from the same subgroup differ greatly in their genome size by 1,130 kb vs. 660 kb (Marcone *et al*., 1999). These differences are usually due to the occurrence of gene duplication and redundancy. In the genome of the onion yellows (OY) phytoplasma (Oshima *et al*., 2004), it was estimated that 18% of the total genes are multiple redundant copies of only five genes: *uvrD* (ATP-dependent DNA helicase, 3,117 nucleotides, 7 copies), *hflB* (ATP-dependent Zn protease, 1,551 nucleotides, 17 copies) , *tmk* (thymidylate kinase, 624 nucleotides, 6 copies), *dam* (DNA methylase, 660 nucleotides, 4 copies) , and *ssb* (single-stranded DNA-binding protein, 345 nucleotides, 15 copies), all of which are generally single copies (if they exist at all) in the other *Mollicutes* whose genome has so far been sequenced. In addition, 5 genes encoding elements of transporter systems have multiple copies, presumably not all functional. Multiple copies of insertion sequence-like elements are also present in the genome of the OY and other phytoplasma strains (Lee *et al*., 2005) and functional gene for thymidylate kinase (Miyata *et al*., 2003). Phytoplasmas lack many genes for standard metabolic functions but have a sec transport pathway (Bai *et al*.,

2006). Their genomes contain many genes encoding transporter systems, such as malate, metal- ion and amino-acid transporters, some of which have multiple copies. This suggests an import of many metabolites from the host cell, and a modification of the metabolic balance causing disease symptoms (Oshima *et al*., 2001a). Interestingly, an approximately 30-kb region was found to be tandem duplicated in the '*Ca.* P. asteris' OY-W strain genome (Oshima *et al*., 2007). Two sets of five glycolytic enzymes were encoded in this genomic region, which is a unique gene structure not identified in any other bacterial genomes. The gene organization of glycolytic genes of '*Ca.* P. asteris' AY-WB strain (Bai *et al*., 2006) is similar to that of '*Ca.* P. asteris' OY-M strain rather than '*Ca.* P. asteris' OY-W strain, suggesting that the duplication of glycolytic genes was specific to the latter strain. Glycolysis seems to be an important metabolic pathway in phytoplasmas, although the genes for glycolysis are completely absent in '*Ca.* P. mali' (Kube *et al*., 2008), which instead carries the gene for 2-dehydro-3-deoxyphosphogluconate aldolase leading to the hypothesis that pyruvate is formed independently from glycolysis in this phytoplasma (Kube *et al*., 2012). Phytoplasmas also lack the phosphotransferase system to import sugars essential for glycolysis but have a maltose ABC transport system able to import maltose, trehalose, sucrose and palatinose into phytoplasma cells. However, enzymes for converting these sugars to glucose-6-phosphate were not found (Christensen *et al.*, 2005). Phytoplasma genomes encode several genes necessary for folate biosynthesis; *folk*, *folP*, *folC*, and *folA* are encoded in the '*Ca.* P. asteris' OY-M strain genome, only *folA* is encoded in the '*Ca.* P. mali' and '*Ca.* P. australiense' genomes while in the '*Ca.* P. asteris' AY-WB strain genome, the *folA* gene is complete, but *folk* and *folP* are pseudogenes (Oshima *et al*., 2013).

Genome comparison between four different 16SrIII subgroups, reveled no differences significantly in their basic metabolism potential from the genomes of other wide-host-range phytoplasmas sequenced previously, but were distinct from strains of other species, as well as among each other, in genes encoding functions related to interactions with the host, such as membrane trafficking components, proteases, DNA methylases, effectors and several hypothetical proteins of unknown function, some of which are likely secreted through the Sec-dependent secretion system. The four genomes displayed a group of genes encoding hypothetical proteins with high similarity to a central domain of IcmE/DotG, a core component of the type IVB secretion system of Gram-negative *Legionella* spp. Conversely, genes encoding functional GroES/GroEL chaperones were not detected in any of the four drafts. The results also suggest a significant role of horizontal gene transfer among different

'*Candidatus* Phytoplasma' species in shaping phytoplasma genomes and promoting diversity (Saccardo *et al*., 2012). However although the use of draft sequences is the best way to evaluate the potential pathways of phytoplasma genomes, the availability of cultured strains should in the future backup of all virtual metabolic pathways hypotized under the sequencing proofs.

1.3 Interaction with hosts

Phytoplasmas are transmitted by insects to plant sieve elements, from which they spread systemically. Since they are pleomorphic and sufficiently small to pass through the sieve pores, they may be swept along with the assimilate flow from leaves to sugar consuming plant organs. Studies on the translocation of phytoplasmas after localized inoculation (Wei *et al*., 2004b) or the re-colonization of trees (Garcia-Chapa *et al*., 2003) provide evidence that the translocation of phytoplasmas cannot be explained only by assimilate flow. Active movement seems unlikely, considering the lack of genes coding for cytoskeleton elements or flagella (Christensen *et al*., 2005). Phytoplasmas are mainly spread by insects in the families *Cicadellidae* (leafhoppers), *Fulgoridae* (planthoppers), and *Psyllidae* (psyllids), which feed on the phloem tissues of infected plants, therefore their host range is strongly dependent upon the insect vectors (Weintraub and Beanland, 2006). Phytoplasmas may overwinter in insect vectors or in perennial plants, and interact in various ways with insect hosts: examples of both reduced and enhanced fitness of the phytoplasmas while they are in the vectors have been reported (Hogenhout *et al*., 2008). Some phytoplasma transmissions in insects have been reported to be transovarial, such as the insect/disease combinations *Scaphoideus titanus*/aster yellows, *Hishimonoides sellatiformis*/mulberry dwarf, *Matsumuratettix hiroglyphicus* (Matsumura)/sugarcane white leaf and *Cacopsylla pruni/*plum (Alma *et al*., 1997; Kawakita *et al*., 2000; Hanboonsong *et al*., 2002; Tedeschi *et al*., 2006). Phytoplasmas may also be transmitted from infected to healthy plants through the parasitic plant dodder (*Cuscuta* sp.). Experimental transmission of a phytoplasma from infected to healthy dodder of the same or different species, is one of the means by which experimental phytoplasma transmission is achieved. Phytoplasmas can also be spread via vegetative propagation such as grafting of infected plants onto healthy plants, propagation through cuttings, micropropagation and any other method to multiply plant material that avoids sexual reproduction.

Serological studies have recognized one or two abundant immuno-dominant proteins with trans- membrane domains that could play roles in *Mollicutes* recognition, adherence to plant

or insect host cells (a prerequisite for colonization and infection), pathogenicity and triggering of host resistance responses (Suzuki *et al*., 2006; Hoshi *et al*., 2009). The occurrence of major surface epitopes that are unique to each phytoplasma species suggests that these proteins are key participants in specific interactions with the host cells. Microarray analysis of *'Ca*. P. asteris' strain OY-M revealed that the expression of approximately 33% of the genes changes during host switching between plant and insect, suggesting phytoplasma dramatically alters gene expression in response to its host and may use transporters, secreted proteins, and metabolic enzymes in a host-specific manner (Oshima *et al*., 2011). The genes encoded in the PMU of '*Ca.* P. asteris' AY-WB strain are more highly expressed in insects than in plants, most likely due to increased production of the extrachromosomal circular type of PMU during insect infection (Toruno *et al*., 2010). The immunodominant membrane protein is a major portion of the total cellular membrane proteins in most phytoplasmas, and genes encoding immunodominant membrane proteins were isolated from several phytoplasma groups. They are classified into three distinct types: i) immunodominant membrane protein (Imp), in the phytoplasmas causing sweet potato witches' broom (SPWB) (Yu *et al*., 1998), apple proliferation (AP) (Berg *et al*., 1999), European stone fruit yellows (ESFY), pear decline (PD), and peach yellow leaf roll (PYLR) (Morton *et al*., 2003); ii) immunodominant membrane protein A (IdpA), in the phytoplasma causing western X-disease (WX) (Blomquist *et al*., 2001); iii) antigenic membrane protein (Amp), in the phytoplasmas causing aster yellows (AY), clover phyllody (CPh) (Barbara *et al*., 2002), and onion yellows (OY) (Kakizawa *et al*., 2004) and iv) putative membrane protein (vmp1), in the "stolbur" phytoplasmas (STOL) (Cimerman *et al.,* 2009). A high expression of the Amp protein was confirmed in AY, CPh, and OY phytoplasmas (Barbara *et al*., 2002; Kakizawa *et al*., 2004) and this protein was shown to be exported via the Sec protein secretion system, accompanied by the cleavage of its N-terminal signal sequence (Kakizawa *et al*., 2004).

The Amp of the OY phytoplasma forms a complex with an insect microfilament that was correlated with the phytoplasma-transmitting capacity of leafhoppers, suggesting that the interaction between Amp and insect microfilament complexes plays a major role in determining the transmissibility of phytoplasmas (Kube *et al*., 2012). Morton *et al*., 2003 isolated genes encoding Imps, and found that the sequence identities of these genes in several phytoplasmas were not correlated with that of the 16S rDNA gene, suggesting that the variability of immunodominant membrane proteins reflects some factors other than evolutionary time. *Imp* gene may have an important role in host–phytoplasma interactions,

like many positively selected proteins; the accumulation of Amp was calculated as about 10-fold greater than that of Imp and this level was consistent with the 'immunodominant' property of Amp in AY-group phytoplasmas. Since phytoplasmas are intracellular parasites of plants and insects, their ability to adapt to two diverse environments is of considerable interest (Oshima *et al*., 2011).

1.4 Virulence and pathogenicity

Phytoplasmas live and multiply in functional phloem sieve tube elements, the main effect of their presence is the impairment of the sieve tube function. Several studies have shown that inhibition of phloem transport occurs in phytoplasma-infected plants, which, in turn, leads to an accumulation of abnormal amounts of carbohydrates in source leaves, i.e. mature leaves, and a marked reduction of these essential energy- storage compounds in sink organs, i.e. young leaves and roots. Changes in photosynthate translocation along with other impaired physiological functions, including reduced photosynthesis, stomata conductance and root respiration, altered secondary metabolism and disturbed plant hormone balance, possibly mediated by phloem dysfunction, could account for symptoms exhibited by infected plants (Marcone, 2010). However, very little is known about phytoplasma virulence. Sequenced phytoplasmas possess none of the known virulence genes (such as *hrp*) found in other phytopathogenic bacteria (Oshima *et al*., 2004). Because they lack most of the common metabolic pathways, it has been speculated that they must assimilate a wide range of materials from the host cells, probably with detrimental effects to the hosts. In onion yellows (OY) phytoplasma the comparative study of a mild strain (OY-M, causing mild proliferation and yellowing) and of a severe strain (OY-W, causing yellowing, stunting, proliferation and witches' broom) showed that five glycolytic genes were duplicated in the severe strain. It was previously reported that the phytoplasma population of OY-W had a higher titer than the one of OY-M (Oshima *et al*., 2011a). In view of these findings, the higher consumption of the carbon source may affect the growth rate of the phytoplasma and may also directly or indirectly produce more severe symptoms (Oshima *et al*., 2007).

TENGU (virulence factor, "tengu-su" inducer), a small peptide of 38 residues, was shown to be a virulence factor secreted by phytoplasmas that induces dwarfism and witches' broom in the host plant (Sugawara *et al*., 2013). Differential gene expression between plant and insect hosts have been reported in '*Ca.* P. asteris' strain OY-M, in which TENGU is more highly expressed in plant hosts than in insect hosts (Hoshi *et al*., 2009). It was also shown that

“tengu” induces witches' broom and dwarfism when expressed in transgenic plants of *Nicotiana benthamiana* and *Arabidopsis thaliana*. Although, the localization of phytoplasma is restricted to the phloem, TENGU protein was detected in the apical buds by immunohistochemical analysis, suggesting that TENGU was transported from the phloem to other cells. Microarray analysis detected that auxin-responsive genes were down regulated in the TENGU-transgenic plants as compared with control plants (Hoshi *et al*., 2009).

Phytoplasmas possess two secretion systems, the *YidC* system for the integration of membrane proteins, and the Sec system for the integration and secretion of proteins into the host cell cytoplasm (Kakizawa *et al*., 2001; Wei *et al*., 2004a; Kakizawa *et al*., 2004; Lee *et al*., 2012). Antigenic membrane protein (Amp), a major surface membrane protein of phytoplasmas (Barbara *et al*., 2002), has been reported to be a substrate of the Sec system. Amp has a Sec signal sequence at its N-terminus, which is cleaved in ‘*Ca.* P. asteris’ strain OY-M (Kakizawa *et al*., 2004), suggesting that the phytoplasma Sec system utilizes recognition and cleavage of a signal sequence, as in other bacterial Sec systems. In addition to SAP11, SAP54 of ‘*Ca.* P. asteris’ AY-WB strain was reported to code in the ‘*Ca.* P. asteris’ AY-WB strain genome more than 56 genes encoding predicted secreted proteins. Among those SAP11 was studied in deep: it contains eukaryotic nuclear localization signals and localizes in plant cell nuclei (Bai *et al*., 2006); SAP11-expressing plants exhibit crinkled leaves and produce many stems morphological changes in *Arabidopsis thaliana* flower organ development and also show altered root architecture, similarly to the symptoms of phytoplasma-infected plants. Furthermore also SAP54 was reported among the 56 genes encoding predicted secreted proteins (Bai *et al*., 2006; MacLean *et al*., 2011; Lu *et al*., 2014). These morphological changes are paralleled by an accumulation of cellular phosphate (Pi) and an increase in the expression levels of Pi starvation-induced genes and miRNAs. In addition to the Pi starvation responses, SAP11 suppresses also salicylic acid-mediated defense responses and enhances the growth of a bacterial pathogen (Lu *et al*., 2014). Moreover, the fecundity of insect vectors was increased on SAP11-expressing versus normal plants (Sugio *et al*., 2011b). Although the molecular mechanisms remain unknown, it is assumed that proteins secreted by phytoplasmas may interfere with the function of genes involved in flower development (Sugio *et al*., 2011a; 2012). The identification of TENGU, SAP11, and SAP54 suggest that phytoplasma could aggressively induce symptoms by secretion of effector proteins and modification of plant-gene activity (Hoshi *et al*., 2009; Himeno *et al*., 2011; Sugio *et al*., 2011b).

Phytoplasma infection can lead to the production of defense proteins, an increase in phenolic compounds and an overproduction of hydrogen peroxide in host plants (Junquera *et al*., 2004; Musetti *et al*., 2000; 2005; Miura *et al*., 2012). Because of the large array of symptoms in diseased plants, nutrient depletion is probably not restricted to sugars, but also includes other compounds. A reduction in the concentration of photosynthetic pigments and of total soluble proteins (Bertamini and Nedunchezhian, 2001; Bertamini *et al*., 2002; Musetti *et al*., 2005), as well as alterations in the hormone balance (Jagoueix-Eveillard *et al*., 2001; Maust *et al*., 2003), amino-acid transport (Lepka *et al*., 1999) and the occurrence of folate and endopetidase gene homologues in phytoplasma genomes (Davis *et al*., 2003b) were described as potentially affecting host plants infected with different phytoplasmas.

1.5 Prevention and control

Phytoplasmas are responsible for numerous crop diseases worldwide, out of which two kinds of epidemiological situations can be distinguished: epidemic and non-epidemic. Many diseases are not epidemic on the crop itself, meaning an infected plant is an epidemiological dead-end host for the phytoplasma. This is particularly true when the phytoplasma reservoir consists of wild plants and when the insect vector is living in the wild component of the ecosystem and not developing on the crop. In this case, the phytoplasma insect vector may occasionally feed on the cultivated plant, causing monocyclic epidemics. The economic impact of such epidemics is directly linked to the abundance and infectivity of the insect populations and corresponds to the yield loss. Such diseases are generally not considered as quarantine diseases, and control measures only rely on the prophylactic reduction of weeds identified as the main reservoirs for the phytoplasmas (Foissac and Wilson, 2010). When the crop itself acts as the main reservoir and if the insect vector completes its life cycle on the infected crop, then the situation resulting from the transmission from plant to plant by the insect, into or between cultivated plots, corresponds to polycyclic epidemics. Most of the diseases that are spread this way are usually classified as quarantine diseases. This can occur on annual plants when there are no time gaps between cropping periods, but occurs mostly in perennial, woody crops. Phytoplasma associated with these diseases are molecularly distinguishable in most of the cases at the 16Sr DNA level (Table 1), therefore epidemiological studies can be carried out in order to eliminate infected plants and to prevent further epidemic spreading.

The main limitation to the real application of these procedures that can be very successful in

eliminating or reducing the impact of phytoplasmas diseases is that agricultural-related problems are not under consideration in many countries worldwide for opposite reasons (over production or not qualified production); people working in this field are not always aware of the risk connected with the trading or the maintenance in field of phytoplasma infected plants. Conventional strategies for phytoplasma containment are based on pesticide application against insect vectors, the use of resistant plants and the elimination of the pathogen from propagation materials. Moreover, other strategies such as the use of transgenic plants expressing phytoplasma-directed antibodies, antimicrobial peptides or elicitins have been proved partially ineffective. Recently, the availability of full genome sequences allowed improving the knowledge of phytoplasma-hosts interaction, opening perspectives for developing new phytoplasma control strategies. Current studies evidenced that a promising approach could be the use of natural or induced resistance and biocontrol agents such as endophytes and arbuscular mycorrhizal fungi. Interestingly, biocontrol agents could be employed as phytoplasma vector pathogens, symbiotic microorganisms able to reduce vector competence, phytoplasma antagonists or inducers of plant defense response (Bianco *et al*., 2011).

Outbreaks of phytoplasma disease epidemics can be controlled either by controlling the vectors, or by eliminating the pathogens from infected plants by meristem tip culture, antibiotics or by other chemicals (Bertaccini, 2007). Insect vector control using pesticides is the method of choice for limiting outbreaks of phytoplasma diseases. Even apart from environmental considerations, however, the efficacy of chemical control is far from complete, and phytoplasma diseases continue to be severe in several areas of the world, despite extensive use of insecticides (Firrao *et al*., 2007). On the other hand, removal of sources of *inoculum* is efficient for reducing diseases spread by monophagous vectors. Examples are the disease/vector systems of FD/*Scaphoideus titanus* and PD/*Cacopsylla pyri*, however it is difficult to achieve results when wild reservoir plants are sources of phytoplasma contamination for polyphagous leafhoppers such as *Hyalesthes obsoletus* or *Dalbulus maydis*, or when reservoirs and/or vectors are unknown. Similarly, it is easier to control monophagous insects reproducing on affected crops than insects that are also able to live on wild plants. Interference with the colonization of insects by phytoplasmas, or with the phytoplasma nutrient uptake in the plant phloem is primary target for plant protection without resorting to pesticides. Plant resistance elicitor benzothiadiazole (BTH) (Bion) was tested for its capacity to induce systemic resistance against chrysanthemum yellows phytoplasma (CY) infection in

the *Chrysanthemum carinatum* plant exposed to CY-infective *Macrosteles quadripunctulatus* leafhoppers. BTH application delayed symptom development and phytoplasma multiplication in treated plants compared with the control ones (D'Amelio *et al*., 2010).

"Bois noir"-infected grapevines can recover through spontaneous or induced symptom remission. Five elicitors (chitosan, two glutathione-plus-oligosaccharine formulations, benzothiadiazole, and phosetyl-Al) were evaluated towards their efficiency in recovery inducing, and recovery induction was observed with benzothiadiazole and the two glutathione-plus-oligosaccharine formulations. The plants that recovered naturally or after elicitor's stimulation showed qualitative and quantitative parameters of production not different from healthy plants. The application of resistance inducers promoted the recovery of BN-infected grapevines with no adverse effects on the plants. Therefore, grapevine could be used as a model species to test this innovative strategy to contain phytoplasma diseases (Romanazzi *et al.*, 2013).

To reduce the infectivity of vector populations it is first necessary to identify the barriers that limit phytoplasmas from colonizing the vectors. Alternatively, phytoplasma nutrient uptake from host plant phloem may be targeted to reduce phytoplasma multiplication, and symptom expression in the host. Control of epidemic outbreak by controlling the vector or by eliminating the pathogen from the infected plants resulted in some cases quite ineffective under field conditions: the first because it is impossible to eliminate all vectors from environments, and the second because the use of antibiotics is very expensive, not allowed in several countries, and not always effective for long-time. Therefore the only real way to control phytoplasma infection is to prevent the outbreaks by producing clean material or by finding phytoplasma resistant varieties. The use of tolerant varieties can be suggested only under restricted and defined environmental conditions (Loi *et al*., 1995; Parani *et al*., 1996; Sinclair *et al*., 1997a; 1997b; Carraro *et al*., 1998; Kison and Seemüller, 2001; Bisognin *et al*., 2008).

Chapter 2

2 Witches' broom and frog skin diseases of cassava in South-East Asia and Latin America

2.1 Cassava in Asia and Latin America

Cassava (*Manihot esculenta* Crantz), along with maize, sugarcane and rice, constitute the most important source of energy in the diet of tropical countries of the world, its energy yields on a per hectare basis are far superior to those of rice and maize. Is an important crop in regions at latitudes between 30º N and 30º S, and from sea level up to 1800 meters above sea level. In South America, particularly in Brazil, cassava is known as "mandioca" (or "manioc" in English). The English name "cassava" may have derived from the word "casabi", which, among the Arawak Indians, signifies "roots", or else came from the word "cazabe", which is a cake or dry biscuit produced by the indigenous populations of the Amazon Basin. In English, cassava is also known as "tapioca" (Ceballos, 2012).

Cassava is one of the world's most important crops, ranking fifth in importance in the tropics and first in sub-Saharian Africa. Unlike many other crops, cassava can be grown with minimum inputs, tolerating poor soils and long dry periods, has its origin in South America where it has been domesticated by the indigenous Indian population at least 5000 years ago, and has since been extensively cultivated in the tropics and subtropics of the continent (Allem, 2002; Olsen, 2002). After the discovery of the Americas, European traders took the crop to Africa as a potentially useful food crop; later it was also taken to Asia to be grown as a food security crop and for the extraction of starch. Thus, in the 19th century cassava became an important food crop in southern India, as well as on Java island of Indonesia and in the southern Philippines, while in Malaysia and parts of Indonesia it was also used for extraction of starch. After the Second World War it became an important industrial crop in Thailand, mainly to produce starch for local consumption, dried chips, and later pellets for the rapidly growing European animal feed market (Howeler, 2011).

Cassava is the second most important food crop in the least-developed countries (LDCs), and the fourth most important in developing countries. World production of cassava roots was estimated between 2011 and 2012 at 262,585,741.40 tonnes. Africa was the largest producer with the 57% (149,479,840.00), followed by Asia with 30% (80,744,002.60) and only 13% (80,744,002.60) in Latin America and the Caribbean (FAOstat, 2014) (Figure 2.1).

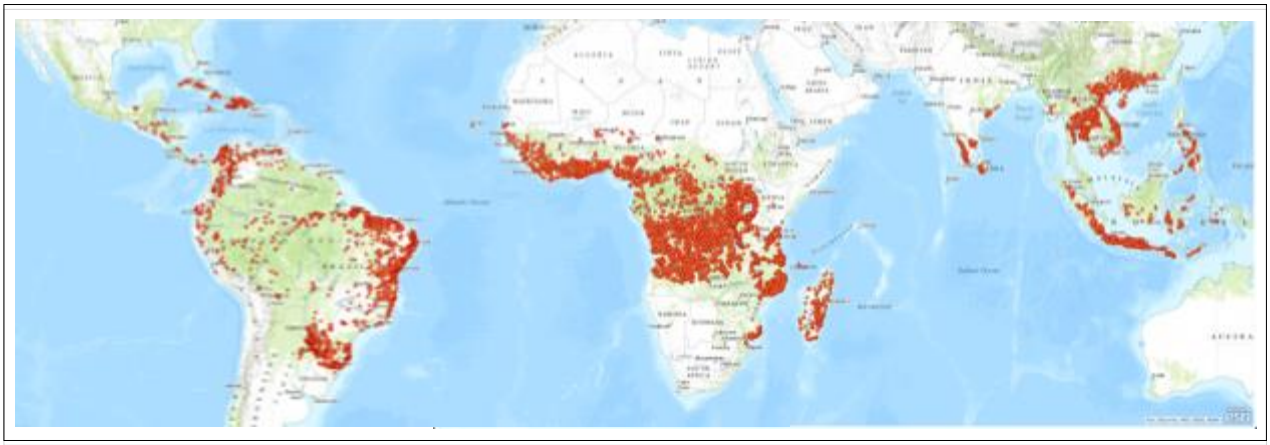


Figure 2.1. Cassava distribution in Latin America, Africa and Asia, each dot on the map represent 500 ha of land in cassava cultivation (Source: Carter *et al.* 1992 – CIAT, http://gisweb.ciat.cgiar.org/RTBMaps).

In Thailand and Vietnam, the yield increases achieved during the past ten years are mainly due to a concerted effort to distribute widely the new high-yielding and high-starch varieties, as well as to the adoption of improved cultural practices, such as more balanced fertilizer use and soil conservation measures. In Thailand, new varieties are now planted in nearly 100% of the area, while 80-90% of farmers apply chemical fertilizers; in Vietnam the new varieties are now planted in about 60% of the cassava area while about 80% of farmers apply chemical and/or organic manures. These two factors combined nearly doubled yields in Vietnam over the past ten years (Howeler, 2011). However, while in Africa between 2008 - 2012 the production was increased, in America and Asia it was dramatically fold down (Figure 2.2).

Phytoplasma associated diseases are long time know worldwide as inducing severely economic damages on a variety of cultivated and wild plants. The increasing threat of phytoplasma diseases worldwide comes both from emerging diseases in Latin America, Asia, Africa and the Caribbean mainly in sugarcane, corn, cassava, coconuts, papaya, and vegetables and from devastating epidemics in the rest of the world in grapevines, citrus, forest trees, oil-seed crops, alfalfa, stone and pome fruits. In both cases diseases have the potential to spread to other crop species throughout the world and/or to have an important impact on global trade. There are concerns that climate changes resulting from global warming may facilitate the spread of these phytoplasma diseases to new areas and to additional crops, particularly if the vectors become more widespread and able to survive during warmer winters. Phytoplasmas usually do not kill in short time the host plant from which they are strongly metabolic dependent, however unusually cold conditions kill infected plants, while

under tropical conditions asymptomatic plant presence is frequent with severe epidemiological consequences (Bertaccini, 2008).

Figure 2.2. Cassava production in Africa, Asia and Latin America that are the main cassava producing countries, 2008-2012. Source: FAOstat, January 2014.

The recent establishment of several exotic pests and diseases in cassava in the South East Asia and the continuously spread and/or distribution of disease such as frog skin (CFSD) in Latin America and the Caribbean regions is broadly identified as the most important threat to its continued production. The full list of these organisms in SE Asia is unknown, but preliminary surveys have confirmed the presence of several species of neotropical mealybugs, whiteflies, and mites. Severe cases of cassava bacterial blight (CBB), brown leaf spot disease and cassava witches' broom (CWB) have also been documented, several of which, if unchecked, appear likely to cause major declines in production in the livelihoods, in particular areas in some countries - particularly for the marginalized poor in relatively remote areas. During

2009/2010, in Thailand only, cassava losses to these pests and diseases have been estimated at US$145 million with reductions in production of at least 20% (Thai Tapioca Development Institute, personal communication, 2010). Many farmers in the SE Asia have even abandoned their cassava crops, with the planted area beginning to decline. Thus, the pest and disease is not only placing a huge strain on Thailand´s and Vietnam´s cassava trade and on the large number of poor cassava farmers, but also on the many laborers employed in the cassava industry. With cassava a relatively recent introduction to Southeast Asia, unlike other parts of the world, cassava in this region has been largely pest and disease free. However, since witches' broom disease was first reported in Thailand in 2008 it has spread across the region - a trend attributed by researchers to unchecked, cross-border movement of infected cassava planting material.

The main objectives of this investigation were the detection, classification, identification and evaluation of the genetic variability in CFSD and CWB associated phytoplasmas, in cassava varieties infected in the field and from different regions in Latin America and SE Asia. Adaptation and improvement of detection techniques to develop a sensitive diagnostic method was also carried out.

2.2 Cassava phytoplasma diseases

Phytoplasmas have been reported to infect cassava in several countries worldwide; these include the phytoplasma associated with cassava frog skin disease (CFSD) (Alvarez *et al*., 2009; Souza *et al*., 2014), and other phytoplasmas phylogenetically related to aster yellows (16SrI) and X-disease (16SrIII) groups associated with cassava witches' broom disease (CWB), reported in the territory of Wallis and Futuna, Cuba and Brazil (Davis *et al*., 2005; Arocha *et al*., 2009; Flores *et al*., 2013; Frison and Feliu, 1991).

Two different types of symptoms have been reported:

(1) CFSD symptoms in the roots are a woody aspect and a thickened peel that is cork-like, fragile, and opaque. The peel also presents lip-like slits that, when they join, create a net-like or honeycomb pattern. In advanced stages of the disease, the sclerenchyma and parenchyma are brown instead of white, cream, or pink. In many cases, roots may be very thin and the bases of stems very thick. Generally, the aerial parts of diseased plants are more vigorous and better developed than those of healthy plants;

(2) CWB symptoms are associated with stunting and excessive proliferation of branches; shoots have small leaves and shortened internodes, without distortion or chlorosis, proliferation of shoots from the cutting with generally weak growth; a few weak, stunted shoots germinate from the cuttings which never reach normal size. Moderate temperatures (between 13 and 20°C) favor the disease, and at higher temperatures the symptoms disappear.

Cassava frog skin disease (CFSD) is regarded as one of the constraints for cassava as it directly affects the production of root diseases, causing losses in crop yield of 90% or more (Nolt *et al*., 1992; Calvert and Cuervo, 2002). Recently, it has been reported with increasing frequency in Colombia, Costa Rica, Brazil, Panama, Paraguay, Peru and Venezuela (Chaparro and Trujillo, 2001; Calvert and Cuervo, 2002; E. Alvarez and J.M. Pardo, personal communication). CFSD was reported for the first time in 1971, in the Department of Cauca, southern Colombia. Its origin could be the Amazon region of Brazil and Colombia, affecting different cassava varieties cultivated by indigenous communities (Alvarez *et al*., 2012). In the 1980s, the disease occurred in most cassava-growing regions of Colombia and has continuously spread. As an example, the disease was reported in Panama between 1999 and 2000 and it was established that the affected plants were grown from stem cutting imported from Costa Rica. The planting material had all phytosanitary certificates required by the regulatory authorities (Calvert and Thresh, 2001).

In Colombia and Brazil CFSD has been associated with a phytoplasma identified as group 16SrIII by restriction fragment length polymorphism (RFLP) and sequence analyses of amplified rDNA products. RFLP analyses indicated that CFSD strains from Colombia differed from all phytoplasmas described previously in group 16SrIII and, on this basis, they were tentatively assigned to new ribosomal and ribosomal protein subgroups 16SrIII-L and rpIII-H, respectively (Alvarez *et al*., 2009). In Brazil, the phytoplasma was classified belonging to the subgroup 16SrIII-A (Oliveira *et al.,* 2014; Souza *et al*., 2014). Additional pathogens have been also reported associated with the disease. While in Brazil phytoplasmas and dsRNA virus (possible reovirus) are reported as co-infecting the symptomatic plants, in Colombia, a three newly described viruses belonging to the families *Secoviridae*, *Alphaflexiviridae* and *Luteoviridae* were found in cassava plants showing CFSD symptoms in severe roots (Calvert *et al.,* 2008; Carvajal *et al*., 2013; Souza *et al*., 2014).

In Costa Rica there are few studies on the incidence of this disease in cassava production. Rodríguez *et al*. (1997), conducted an analysis of the current situation of frog skin in the

Northern Region. According to this study, 70.1% of farmers were aware of the disease and a 30.29% was reported in their plantations; the disease has been more common in the regions of La Fortuna and Pital. A 72.59% of producer's associated the disease with reduced production of cassava and 49.31% associated it also to the use of infected cuttings. The variety "Little tree" is reported as more susceptible; moreover in preliminary experiments (S. Torres, personal communication, 2010) a reduction in the yield of 100% was observed when cuttings from diseased plants were used. In addition, CFSD incidence between 30% and 50% in La Fortuna region has been reported.

In Paraguay the frog skin is not officially reported, however, between the years 2001-2002 and 2007 - 2008 a 15% of reduction in the average yield per hectare was recorded. This reduction is empirically attributed to degradation but, according to J. Caballero (personal communication, 2010), this statement may hide the frog skin disease and lack of knowledge about it made it not possible to diagnose it. Symptoms of the disease in most varieties only are expressed in roots and are only observed when the plants are harvested. There are no typical symptoms in branches and leaves, but a few varieties (e.g. "La Reina" - CM 6740-7, "Secundina" - Mcol 2063) showing mosaic symptoms and curly leaves are hardly distinguishable under field conditions and can be easily confused by symptoms caused by mites, thrips, trace element deficiencies or toxicity of herbicides, in addition, the symptoms disappear at relatively high temperatures (over 30°C).

CFSD presence constantly delayed the work focused on the search for improving yields of varieties and ensures production stability. These problems are related mainly to the insufficient knowledge of the disease presence, therefore, an efficient methodology for the detection of pathogens involved, will allow the selection of disease-free planting materials for commercial purposes. Cassava witches' broom (CWB) is an important disease of *M. esculenta* Crantz that is widely cultivated across South East Asia. This disease, known as "superbrotamiento" in Spanish, has been reported in Brazil, Venezuela, Mexico and in the Amazonian region of Peru. The disease is mechanically transmitted, through the use of cuttings taken from diseased plants. Several types of symptoms are described (Lozano *et al*., 1981; Jayasinghe *et al*., 1984).

Recently cassava plants with symptoms suggestive of phytoplasma diseases were observed in the southern and northern regions of Asia. In 2010, more than 60,000 ha were affected in Vietnam with crop losses as high as 80%, and reductions in yield and starch content reached 30%. The disease was observed in Quang Ngai, Dong Nai, Yen Bai provinces of Vietnam;

Rayong and Chachoengsao in Thailand in the southern and northern regions, also affecting the availability of clean planting material and associated with relevant economic yield losses.

Cassava witches' broom disease, threatens up to 40 million smallholder farmers in the region who depend on the crop for their livelihood. Named after the broom-like leaf proliferation it causes at the top of cassava plants, the disease has already swept through Vietnam, Thailand, Cambodia, Lao, China and Philippines, resulting in dramatic reductions in cassava root starch content and in parts of Vietnam, up to 80% per cent yield losses.

2.3 Materials and Methods

2.3.1 Plant samples

Sampling in Latin America was performed during 2011 in nine different localities in the provinces of Guanacaste, Alajuela and Limòn in Costa Rica and in one locality in San Pedro province in Paraguay, in which cassava plants showed typical CFSD symptoms. In South East Asia, sampling was also conducted during the 2009, 2010, 2011 and 2012 growing seasons (generally in November), in three provinces of Chacheoengsao and Rayong in Thailand and in ten different localities belonging to Yen Bai and Dong Nai provinces in Vietnam, in which cassava plants showed symptoms of CWB (Figure 2.3, Table 1).

In Latin America, a total of 202 samples corresponding to 37 plants (182 samples) from Costa Rica and 10 plants (20 samples) from Paraguay, showing symptoms typically associated with CFSD, were collected. The roots of all plants were examined and classified according to a scale of symptom severity reported by Alvarez *et al*. (2009; 2013) (Figure 2.4). In South East Asia, a total of 190 samples were collected corresponding to 40 plants (48 samples) in south of Thailand and 60 plants (142 samples) in south and north of Vietnam. In general the symptoms observed as associated with phytoplasmas were bud proliferation; shoots with short internodes and small leaves; dieback in some branches; bud proliferation in branch apices; advanced symptoms consisting in leaf drying and dieback of branches. In Thailand in few plants, also symptoms caused by mealybugs (*Phenacoccus manihoti*) presence were observed in mixed infection. Particularly, in the north of Vietnam also stem and roots vascular necrosis and/or stunting were observed together with bacterial blight, root rots, blight and brown leaf spot, but no typical frog skin disease symptoms were observed (Figures 2.5 and 2.6).

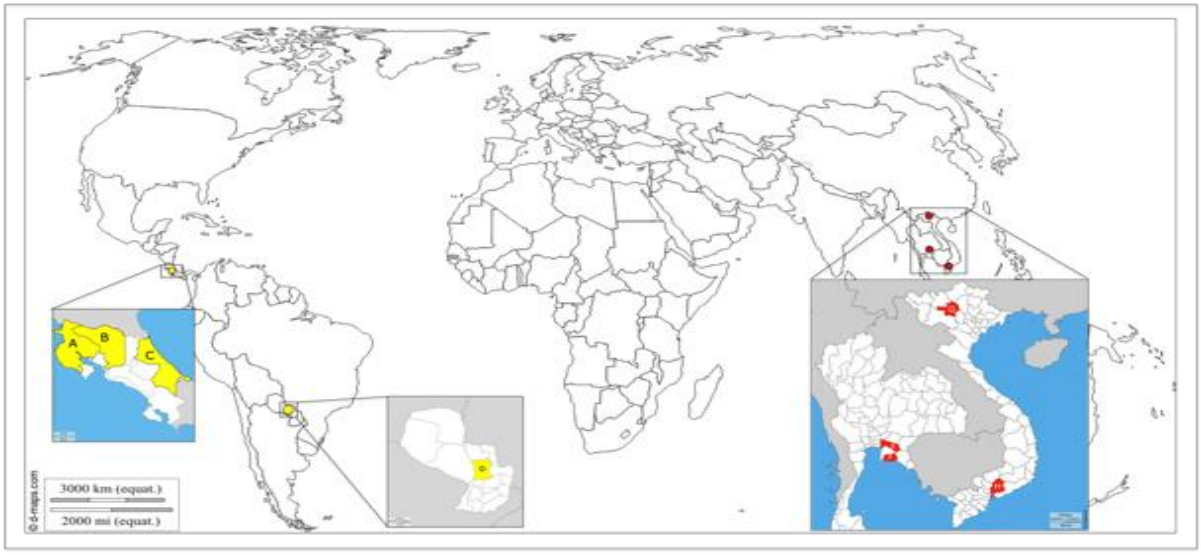


Figure 2.3. Geographic localization of the provinces in Latin America and South East Asia where the CFSD and CWB samples were collected, respectively. In Latin America, provinces of Guanacaste (A), Alajuela (B) and Limòn (C) in Costa Rica; San Pedro (D) in Paraguay. In South East Asia, provinces of Chacheoengsao (E) and Rayong (F) in Thailand and Yen Bai (G) and Dong Nai (H) in Vietnam.

2.3.2 DNA extraction

Total plant and pathogen DNA from cassava samples collected from symptomatic and asymptomatic plants from Costa Rica (Cassava Costa Rica Frog Skin Disease, CCFSD), Paraguay (Cassava Paraguay Frog Skin Disease, CPFSD), Thailand (Cassava Thailand Witches' broom, CThaiWB) and Vietnam (Cassava Vietnam Witches' Broom, CVWB) showing similar and typical frog skin and witches' broom diseases symptoms were extracted using the methodology described by Gilbertson and Dellaporta, (1983) and Prince *et al*. (1993) and were stored at -20ºC until further processing (see appendix A). The tissues included buds, leaves, midribs, petioles; young stem, field cuttings and tuberous roots; in the majority of these tissues it was possible differentiate primary and secondary phloem to improve the phytoplasma DNA extraction (see appendix A).

Table 2.1. Description of samples collected in four provinces and ten localities in Latin America associated with CFSD (cassava frog skin disease).

Strain$^{\Psi}$	Genotipe	Province*	Tissue**	Symptoms severity***
CCFSD_1-2*	Valencia	B1	Roots (SP)	4
CCFSD_1-3	Valencia	B1	Petiols	4
CCFSD_1-4	Valencia	B1	Midribs	4
CCFSD_14-5	Señorita	B3	Leaves	4
CCFSD_15-4	Señorita	B3	Midribs	4
CCFSD_16-5	Señorita	B3	Leaves	4
CCFSD_16-6	Señorita	B3	Buds	4
CCFSD_17-3	Señorita	B3	Petiols	4
CCFSD_17-4	Señorita	B3	Midribs	4

Strain[Ψ]	Genotipe	Province*	Tissue**	Symptoms severity***
CCFSD_17-5	Señorita	B3	Leaves	4
CCFSD_19-3	Valencia	B5	Petiols	4
CCFSD_19-4	Valencia	B5	Midribs	4
CCFSD_19-5	Valencia	B5	Leaves	4
CCFSD_2-5	Valencia	B1	Leaves	4
CCFSD_21-1	Mejorada	B2	Roots (PP)	4
CCFSD_21-4	Mejorada	B2	Midribs	4
CCFSD_21-6	Mejorada	B2	Buds	4
CCFSD_22-2	Valencia	B6	Roots (SP)	4
CCFSD_22-3	Valencia	B6	Petiols	4
CCFSD_22-4	Valencia	B6	Midribs	4
CCFSD_23-2	Valencia	B6	Roots (SP)	4
CCFSD_23-4	Valencia	B6	Midribs	4
CCFSD_23-6	Valencia	B6	Buds	4
CCFSD_27-2	Valencia	A2	Roots (SP)	4
CCFSD_3-1	Valencia	B1	Roots (PP)	4
CCFSD_3-2	Valencia	B1	Roots (SP)	4
CCFSD_31-1	Señorita	A1	Roots (PP)	3
CCFSD_31-4	Señorita	A1	Midribs	3
CCFSD_31-5*	Señorita	A1	Leaves	3
CCFSD_31-6	Señorita	A1	Buds	3
CCFSD_34-1	Valencia	C1	Roots (PP)	4
CCFSD_34-2	Valencia	C1	Roots (SP)	4
CCFSD_5-2	Valencia	B4	Roots (SP)	4
CCFSD_6-1	Valencia	B4	Roots (PP)	1
CCFSD_9-2	Valencia	B4	Roots (SP)	4
CCFSD_9-4	Valencia	B4	Midribs	4
CCFSD_9-5	Valencia	B4	Leaves	4
CPFSD_B1(2)	Unknown	D1	Roots (SP)	2,5
CPFSD_B1(5)	Unknown	D1	Roots (SP)	2,5
CPFSD_B2(1)	Unknown	D1	Roots (SP)	2,5
CPFSD_B2(2)	Unknown	D1	Roots (SP)	2,5
CPFSD_B3(1)	Unknown	D1	Roots (SP)	2,5
CPFSD_B3(2)	Unknown	D1	Roots (SP)	2,5
CPFSD_E1(1)	Unknown	D1	Roots (SP)	2,5
CPFSD_E1(2)	Unknown	D1	Roots (SP)	2,5
CPFSD_E3(1)	Unknown	D1	Roots (SP)	2,5
CPFSD_E3(2)	Unknown	D1	Roots (SP)	2,5
CPFSD_E4(1)	Unknown	D1	Roots (SP)	2,5
CPFSD_E4(2)	Unknown	D1	Roots (SP)	2,5
CPFSD_E5(1)	Unknown	D1	Roots (SP)	2,5
CPFSD_E5(2)	Unknown	D1	Roots (SP)	2,5
CPFSD_E6(1)	Unknown	D1	Roots (SP)	2,5
CPFSD_E6(2)	Unknown	D1	Roots (SP)	2,5
CPFSD_P(1)	Unknown	D1	Roots (SP)	2,5
CPFSD_P(2)	Unknown	D1	Roots (SP)	2,5
CPFSD_PT(1)	Unknown	D1	Roots (SP)	2,5
CPFSD_PT(2)	Unknown	D1	Roots (SP)	2,5

[Ψ]Strain designation by country and disease: CCFSD, Cassava Costa Rica Frog Skin Disease, CPFSD, Cassava Paraguay Frog skin Disease. Number after an underscore means the same plant. *****Province, localities, collector or farmer as are indicated in Figure 2.3: A1, Guanacaste, Fortuna, PCI-ROJAS; A2, Guanacaste, Fortuna, Rogelio Solano; B1, Alajuela, Aguas Zarcas, Mamirmi; B2, Alajuela, Los chiles, PCI-ROJAS; B3, Alajuela, Los lagos, TEC; B4, Alajuela, Pital, TEC; B5, Alajuela, Santa Rosa, PCI-ROJAS; B6, Alajuela, TEC; C1, Limòn, Guapiles, PCI-ROJAS; D1, San Pedro, Chorè. **Tissue: Stem (FC), stem field cuttings; Roots (SP), roots secondary phloem; Roots (PP), roots primary phloem. ***Scale of symptom severity currently used to evaluate cassava frog skin disease based on Alvarez *et al*., 2009; the scale severity is described in Figure 2.4.

Table 2.1a. Description of samples collected in four provinces and eight localities in South East Asia associated with CWB (cassava witches' broom).

Strain*	**Genotipe crop age (months)**	**Province***	**Tissue****	**Symptoms descriptions*****
CThaiWB_47	Rayong 9	I	L/M/P	*in vitro*
CThaiWB_1	Rayong 7(8)	F1	L/M/P	(a)
CThaiWB_2	Rayong 7(8)	F1	L/M/P	(b)
CThaiWB_3a	Rayong 7(8)	F1	L/M/P	(b)
CThaiWB_3b	Rayong 7(8)	F1	L/M/P	(b)
CThaiWB_4	Rayong 7(8)	F1	L/M/P	(d)
CThaiWB_5	Rayong 7(8)	F1	L/M/P	(c)
CThaiWB_6	Rayong 7(8)	F1	L/M/P	(c)
CThaiWB_7	Rayong 7(8)	F1	L/M/P	(d)
CThaiWB_8	Huay Bong 60 (10-11)	F2	L/M/P	(e)
CThaiWB_9	Huay Bong 60 (10-11)	F2	L/M/P	(e)
CThaiWB_10	Huay Bong 60 (10-11)	F2	L/M/P	(e)
CThaiWB_11	Huay Bong 60 (10-11)	F2	L/M/P	(b)
CThaiWB_12	Huay Bong 60 (10-11)	F2	L/M/P	(c)
CThaiWB_13	Kasetsart 50 (6)	E1	L/M/P	(c)
CThaiWB_14	Rayong 5 (6)	E1	L/M/P	(e)
CThaiWB_15	Rayong 5 (6)	E1	L/M/P	(e)
CThaiWB_16	Ragong 7 (8)	F1	Roots (SP)	(d) (f)
CThaiWB_17	Rayong 5 (6)	E1	Roots (SP)	(e) (f)
CThaiWB_18	Rayong 5 (6)	E1	Roots (SP)	(f)
CThaiWB_19	Rayong 5 (6)	E1	Roots (SP)	(e) (f)
CThaiWB_20	Kasetsart 50 (6)	E1	Roots (SP)	(c) (f)
CThaiWB_21	Rayong 7(8)	F1	Roots (SP)	(c) (f)
CThaiWB_22	Huay Bong 60 (10-11)	F2	Roots (SP)	(g)
CVWB_36	KM140 (8)	H3	L/M/P	(a)
CVWB_37	KM140 (8)	H3	L/M/P	(a)
CVWB_38	KM140 (8)	H3	L/M/P	(a) (f) (g)
CVWB_39	KM140 (8)	H3	Roots (SP)	(a) (f) (g)
CVWB_40	KM140 (8)	H3	L/M/P	(a) (g)
CVWB_41	KM140 (8)	H3	Roots (SP)	(a) (g)
CVWB_44	KM140 (8)	H3	L/M/P	(c) (f)
CVWB_45	KM140 (8)	H3	Roots (SP)	(c) (f)
CVWB_30	SM937-26 (8)	H3	L/M/P	(a) (h)
CVWB_31	SM937-26 (8)	H3	L/M/P	(a) (h) (j)
CVWB_32	SM937-26 (8)	H3	Roots (SP)	(a) (h) (j)
CVWB_33	SM937-26 (8)	H3	L/M/P	(a) (f) (j)
CVWB_34	SM937-26 (8)	H3	Roots (SP)	(a) (f) (j)
CVWB_35	Unknow (8)	H3	Roots (SP)	(a) (f) (g)
CVWB_46	SM937-26 (8)	H3	L/M/P	(c)
CVWB_23	Unknow (8)	H1	L/M/P	(e)
CVWB_24	KM140 (8)	H1	L/M/P	(a)
CVWB_25	KM94 (8)	H1	L/M/P	(a) (h)
CVWB_26	SM937-26 (8)	H1	L/M/P	(a) (h) (j)
CVWB_27	KM94 (8)	H1	L/M/P	(a) (h) (j)
CVWB_28	SM937-26(8)	H1	Roots (SP)	(a) (h) (j)
CVWB_29	KM94(8)	H1	Roots (SP)	(a) (h) (j)
CVWB_42	SM937-26 (8)	H1	L/M/P	(c) (f) (j)
CVWB_43	SM937-26 (8)	H1	Roots (SP)	(f) (j)
CVWB_1V	KM 94 (5-6)	H3	Stem (FC)	(a)
CVWB_2V	KM140 (5-6)	H3	Stem (FC)	(a)
CVWB_3V	KM94 (5-6)	H2	Stem (FC)	(a)
CVWB_10V	KM140 (5-6)	H2	Stem	(a)
CVWB_14V	KM2112 (5-6)	H2	Buds	(a)

Strain*	**Genotipe crop age (months)**	**Province***	**Tissue****	**Symptoms descriptions*****
CVWB_6V	KM140 (5-6)	H3	Stem (FC)	(a)
CVWB_8V	KM140 (5-6)	H2	Stem (FC)	(a)
CVWB_4V	KM94 (5-6)	H3	Stem (FC)	(a)
CVWB_7V	KM140 (5-6)	H3	Stem (FC)	(a)
CVWB_13LV	KM94 (5-6)	H2	L/M/P	(a)
CVWB_13SV	KM94 (5-6)	H2	Stem	(a)
CVWB_12V	KM2112 (5-6)	H2	Stem	(a)
CVWB_5V	KM94 (5-6)	H2	Stem	(a)
CVWB_11V	KM94 (5-6)	H2	Stem	(a)
CVWB_12AF	KM2112 (7)	H2	Stem	(a)
CVWB_12AN	KM2112 (7)	H2	Midribs	(a)
CVWB_12ANB	KM2112 (18)	H2	Midribs, down plant	(a)
CVWB_12ANM	KM2112 (18)	H2	Midribs, middle plant	(a)
CVWB_12ANMix	KM2112 (18)	H2	Midribs, mix plant	(a)
CVWB_12ANU	KM2112 (18)	H2	Midribs, upper plant	(a)
CVWB_12AP	KM2112 (7)	H2	Petiols	(a)
CVWB_12BF	KM2112 (7)	H2	Stem	(a)
CVWB_12BN	KM2112 (7)	H2	Midribs	(a)
CVWB_12BNB	KM2112 (18)	H2	Midribs, down plant	(a)
CVWB_12BNM	KM2112 (18)	H2	Midribs, middle plant	(a)
CVWB_12BNMix	KM2112 (18)	H2	Midribs, mix plant	(a)
CVWB_12BNU	KM2112 (18)	H2	Midribs, upper plant	(a)
CVWB_12BP	KM2112 (7)	H2	Petiols	(a)
CVWB_1-1	KM94 (6-8)	G1	L/M/P	(a) (i)
CVWB_2-1	KM94 (6-8)	G1	L/M/P	(a) (i)
CVWB_2-2	KM94 (6-8)	G1	L/M/P	(a) (i)
CVWB_2-2-1*	KM94 (6-8)	G1	L/M/P	(a) (i)
CVWB_2-4	KM94 (6-8)	G1	L/M/P	(a) (i)
CVWB_2-6	KM94 (6-8)	G1	L/M/P	(a) (i)
CVWB_3-3	KM94 (6-8)	G1	L/M/P	(a) (i)
CVWB_3-6	KM94 (6-8)	G1	L/M/P	(a) (i)
CVWB_5-12	KM94 (6-8)	G1	L/M/P	(a) (i)
CVWB_5-15	KM94 (6-8)	G1	L/M/P	(a) (i)
CVWB_6-3	KM94 (6-8)	G1	L/M/P	(a) (i)
CVWB_6-5	KM94 (6-8)	G1	L/M/P	(a) (i)
CVWB_7-7	KM94 (6-8)	G1	L/M/P	(a) (i)
CVWB_7-8	KM94 (6-8)	G1	L/M/P	(a) (i)
CVWB_13-1*	KM94 (6-8)	G1	L/M/P	(a) (i)
CVWB_15*	KM94 (6-8)	G1	L/M/P	(a) (i)

[Ψ]Strain designation by country and disease: CThaiWB, cassava Thailand witches' broom; CVWB, cassava Vietnam witches' broom. Number after an underscore means the same plant. *****Province, localities, collector or farmer as are indicated in Figure 2.3: E1, Chacheoengsao, Plaeng Yao, farmer; F1, Rayong Field Crop Research Center (RFCRC); F2, Rayong, farmer; G1, Yen Bai; H1, Dong Nai, Tay Hoa, Trang Bom; H2, Dong Nai, Trang Bom; H3, Hung Loc Agricultural Research Center (HLARC); I, Bangkok, Kasetsart University. **Tissue: Stem (FC), stem field cuttings; Roots (SP), roots secondary phloem; L/M/P, Leaf/Midribs/Petioles. ***Description of symptoms observed under field conditions in Vietnam and Thailand. (a) Short internodes, small-yellow leaves and witches' broom in the middle and/or lower parts of plant, mealybug not present; (b) Short internodes, small-yellow leaves and witches' broom in the middle part of the plant, mealybugs on buds; (c) within focus, plants apparently healthy; (d) Severe incidence of mealybugs on shoots and apices; those shoots not appearing normal or having typical symptoms of mealybug attack appear to suffer from phytoplasmas; (e) Small leaves and witches broom in the upper and middle part of the plant, mealybugs not present; (f) roots without symptoms, apparently healthy; (g) roots with net-like symptoms; (h) dieback in branch; (i) stem and roots vascular necrosis and/or stunting; (j) roots with abnormal dried and yellowing of pulp.

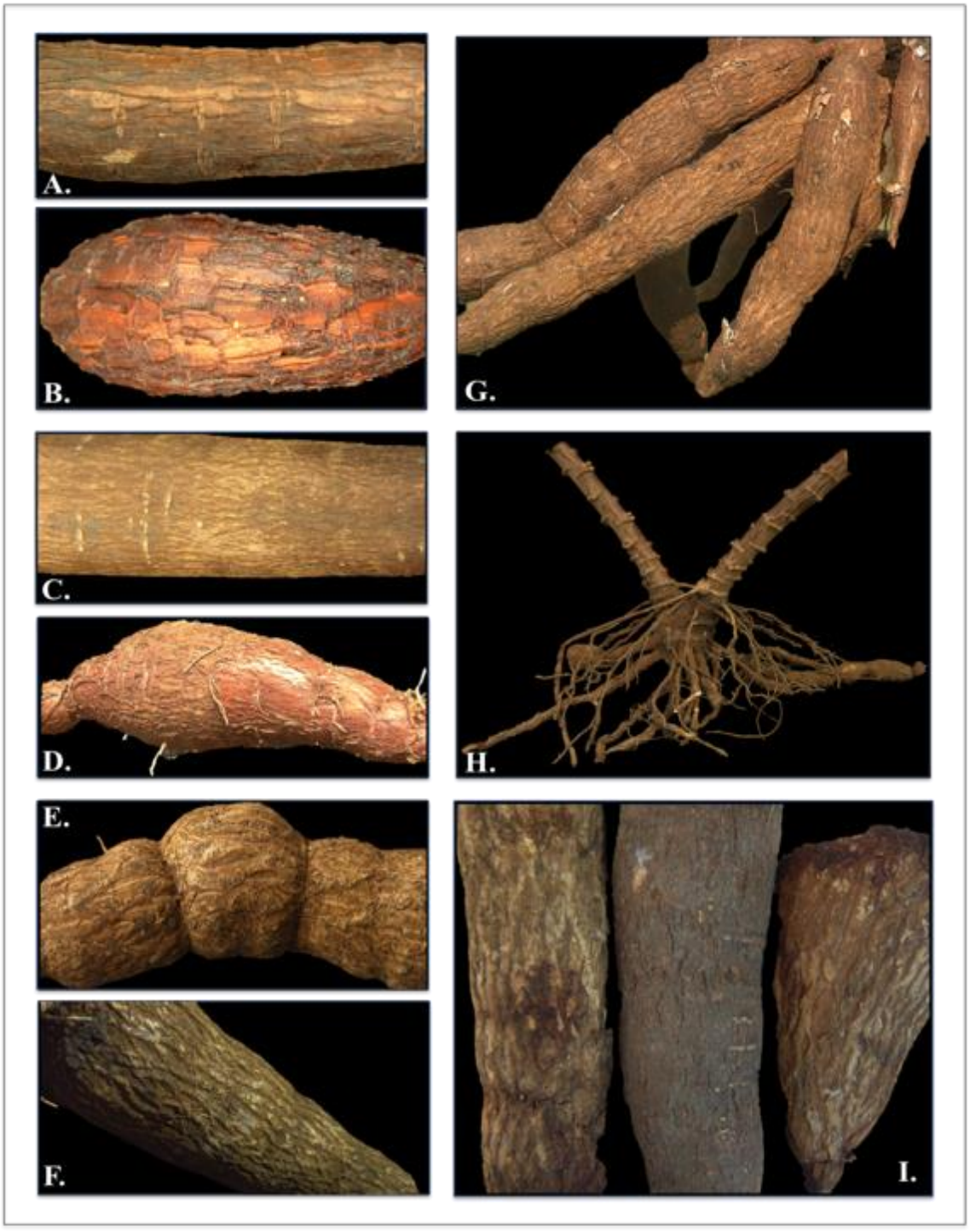


Figure 2.4. Scale of symptom severity currently used to evaluate cassava frog skin disease based on Alvarez *et al*. 2009 [grade (severity %)]. **A** and **B**. Healthy plant [0 (0.1)] = roots are filled; no symptoms in roots and roots have a thin and flexible peel. **C** and **D**, Very mild and mild [1-2 (10 - 35)] = Roots are filled, fissures or lip-like splits in a limited number - many of roots; roots are slightly opaque with an inflexible and brittle peel. **E.** Moderate [3 (65)]= Many fissures or lip-like splits in all root parts (basal, interemediate, and distal zones); some reduction of root size; roots are opaque with a brittle peel. **F, G** and **H.** Severe and very severe [4-5 (90-100)]= Reticulations or honeycombing in several-many roots, moderate-severe reduction of root diameter/volume; roots have a pronounced woody or fibrous character and a thick, cork-like, and brittle peel. **I.** Symptomatology between two different genotypes from two different regions; in the middle, variety CM 4843-1 (small and poorly defined lips-like); left and right variety CM 6740-7 (protuberant and clearly defined lips-like).

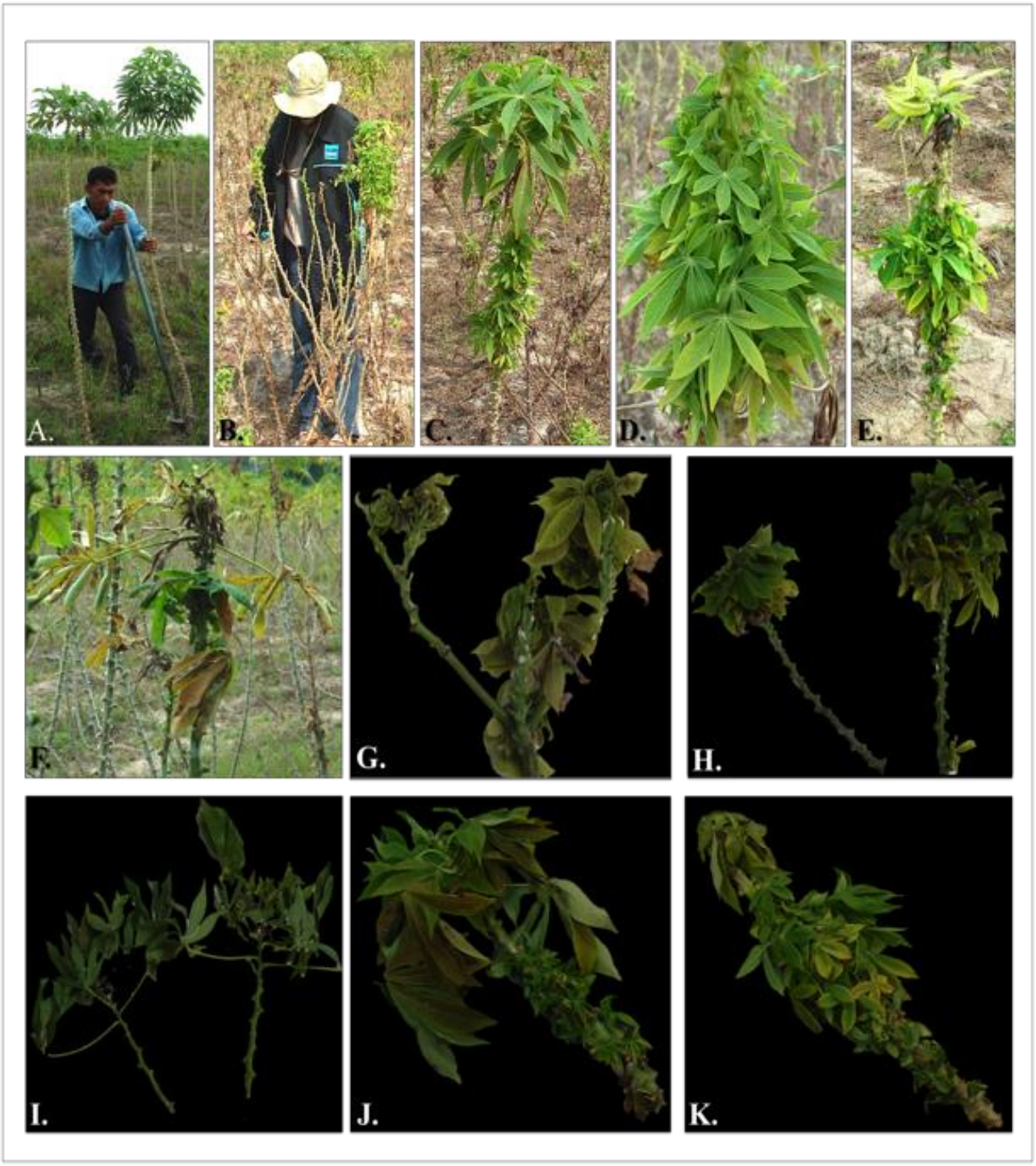


Figure 2.5. Symptoms observed in cassava plants in Thailand associated with phytoplasmas and mealybugs. **A.** Healthy cassava plant; **B, C** and **D.** Plants with exaggerated bud proliferation; shoot proliferation and/or usually rachitic branches growing from single stake; and shoots with short internodes and small leaves with chlorosis; **E**. Mixed symptoms caused by mealybugs (upper part plant) and associated with phytoplasmas (middle part plant); **F** and **G.** Single infection caused by melybugs; **H.** CThaiWB_15, Healthy buds, no mealybugs present, no leaf deformation, small leaves in the lower parts of the branch and witches' broom of axillary buds; **I.** Healthy cassava buds; **J.** CThaiWB_7, Severe incidence of mealybugs on shoots and apices; those shoots not appearing normal or having typical symptoms of mealybug attack appear to suffer from phytoplasmas; **K.** CThaiWB_4, symptoms in buds caused by mealybugs and, in the lower parts of the branch, small leaves and witches' broom associated with phytoplasmas.

Figure 2.6. Symptoms observed in cassava plants in Vietnam associated with phytoplasma disease. **A.** *Left*: infected cassava plant with short internodes and shoot proliferation, *Right*: Healthy plant; **B, C, E,** and **F.** Phytoplasma disease on cassava plants in Dong Nai plants with exaggerated shoot proliferation and small leaves in branch apices and middle part; shoots with short internodes and small leaves that sometimes showed chlorosis; dieback in some branches; and advanced symptoms with leaves drying and later dieback of branches; **D** and **G**. Phytoplasma disease on cassava plants in Yen Bai with chlorosis/yellowing, leaves drying and later dieback of branches. Cassava field cuttings and tuberous roots in Yen Bai showing dark vascular necrosis, **H, I** and **J.** Roots, *Left*: infected, *Right*: healthy; **K** and **L.** field cuttings**,** *Left*: healthy, *Right*: infected.

2.3.3 PCR and RFLP analysis on the 16S rDNA and *tuf* genes

For the detection and identification of phytoplasmas, PCR assays were performed using different universal and specific primers combinations to amplifying 16S rDNA region, in particular in direct PCR assays R16mF2/R16mR1, P1/P7 or P1AP7A primers pairs were carried out, followed by nested or semi-nested PCR assays on these templates diluted 1:30 using the universal primers R16F2n/R16R2, R16F1/B6(m23SR), B5(16R723f)/P7, 16R738f/16S1232r (=M1/M2) and the group specific primers R16(I)F1/R1, R16(III)F2/R1, R16(V)F1/R1, M1/V1731, R16(CJ)F1/R1 and R16(X)F1/R1. Moreover amplifications were carried out using R16F2n/R16R2 primers in direct PCR assays followed by nested-PCR assays with R16(I)F1/R1 and M1/M2 primer pairs. To increase sensibility and specificity, a second nested was also implemented. Further analyses were performed on *tuf* gene to avoid the presence of false negative (*Bacillus* spp) sometimes obtained by 16Sr DNA amplification and because it was shown to have more high genetic variability in phytoplasma molecular characterization than other genes; two pairs of primer cocktails were used, Tuf340/Tuf890 and Tuf400/Tuf835 in direct and nested PCR assays respectively. PCR and electrophoresis conditions are described in Appendix B and C.

In each experiment a CFSD and phytoplasma and DNA from the following reference strains in periwinkle: STOL-PO ("stolbur", 16SrXII-A), AV2192 (aster yellows, 16SrI-B), CA (carrot phyllody, 16SrI-C) and from healthy cassava plant were used as positive and negative controls, respectively.

For restriction fragment length polymorphism (RFLP) analyses, the fragments amplified 16S rDNA region using R16F2n/R16R2, B5(16R723f)/P7, R16(I)F1/R1, R16(X)F1/R1, R16(III)F2/R1, M1/M2 and for *tuf* gene using Tuf400/Tuf835 primer pairs were subjected to digestion with *Rsa*I, *Ssp*I, *Hha*I, *Mbo*II, *Tsp509*I and *Tru1*I restriction enzymes according to the manufacturer's instructions (Appendix C and D).

2.3.4 Phylogenetic and sequencing analysis

Several of the fragments amplified by the primer pairs R16mF2/R16mR1, R16F2n/R16R2, R16(I)F1/R1, M1/M2 and Tuf400/Tuf835 were sequenced directly after purification with Nucleospin extract II kit (Macherey-Nagel, Germany) at a private company (Macrogene, NL). Phylogenetic analyses were carried out on 16S rDNA and tuf sequences from 17 cassava witches' broom and 17 cassava frog skin disease strains and from several '*Candidatus*'

phytoplasma strains using *A. laidlawii* as the outgroup. The sequences were edited, assembled and analyzed following the methodologies described in the appendix E.

2.3.5 *In silico* restriction and single nucleotide polymorphisms (SNPs) analysis

For virtual restriction analysis and single nucleotide polymorphisms calculations, publicly available 16S rDNA and elongation factor sequences from different phytoplasmas groups and subgroups (Lee et al., 1998a; 1998b; 2000; Zhao *et al*., 2009, Makarova *et al*., 2012, Davis *et al*., 2013), were retrieved from GenBank and aligned with the 16S ribosomal DNA and tuf sequences from the same CFSD and CWB strains selected for phylogenetic analysis, the software and parameters used for those studies are described in appendix E.

2.3.6 Transmission assay by dodder (*Cuscuta* sp.)

To elucidate the pathogenicity role of phytoplasmas in CWB disease, a transmission study was conducted. The assay was carried out under insect-proof greenhouse conditions at 25°C to 30°C and 50 to 90% relative humidity (RH) using a 'Ca. P. asteris' naturally infected potted cassava plants as sources of inoculum, and the ectoparasite *Cuscuta* spp. (dodder) as bridge to healthy periwinkle plants [*Catharanthus roseus* (L.) G. Don]. Infected donor plants were of the susceptible cassava genotype KM2112, which showed severe symptoms of little leaf, chlorosis and witches' broom under field conditions, in Dong Nai province (Vietnam). The following treatments were carried out in six replication per trial: (1) from infected cassava (CVWB_12A) to healthy periwinkle plants; (2) from infected cassava (CVWB_12B) to healthy periwinkle plants (3) AY infected periwinkle to healthy periwinkle plants; (4) healthy periwinkle plants to healthy periwinkle plants. Transmission using dodder plants grown from seed germinated in insect-proof greenhouse was performed for about 2 months from a single CWB symptomatic source plant to six of 6-week-old periwinkle seedlings per trial. The plants were visually inspected every week for 3 months for the appearance of symptoms. *Cuscuta* spp. sexual seed was germinated in soil and in Petri dishes with Wathman® paper. Once germinated, the seeds were connected with the cassava plants corresponding to each treatment with each other through the long extensions issued by the parasite. Periwinkle seedlings were obtained from commercial seed. The presence and identity of phytoplasmas was assessed in all the plants before and after transmission experiments using nested-PCR assays with R16F2n/R16R2 followed by R16(I)F1/R16(I)R1 primers. For positive samples, the RFLP analyses method described above was applied for phytoplasma identification.

2.3.7 '*Candidatus* Phytoplasma brasiliense' partial genome annotation

For future epidemiological studies and to know and elucidate the role of the hibiscus witches' broom related strains associated with cassava phytoplasma disease, enriched DNA of '*Ca.* P. brasiliense', strain SuV, belonging to ribosomal group 16SrXV provided by dr. Xavier Foissac, INRA ("Institute national de la recherche agronomique"), France was used in a shotgun sequencing of two batches of samples. The sequenced genome was conducted in The Genome Analysis Centre (TGAC) sequencing unit (www.tgac.ac.uk), the metagenome analysis and the sequences assembling was conducted in the Humboldt University of Berlin, Germany by the Professor Michael Kube. Al this activity was carried out as a part of the WG4 under the COST project FA0807.

Selected contigs were provided for processing via the genome annotation platform iANT developed by LIPM ("Laboratoire des Interactions Plantes-Microorganismes") at INRA, France (https://iant.toulouse.inra.fr/bacteria/annotation/cgi/phytoplasma.cgi). The genome sequence annotation consisted in two steps: (1) syntaxic annotation, which consisted in describing structural elements such as structural RNAs or coding sequences (CDS), (2) functional annotation upon which a function is assign to the structural element. The predicted protein sequences were compared to protein databases to look for similarity. iANT implements automatic prediction of structural RNAs and coding sequences with frameD (CDS). Predicted proteins was compared to Uniprot and reference genomes and searched for protein domains (interPro) and transmembrane segments. Protein domains hits and similarity alignments were presented in a CDS comprehensive page with several links to Uniprot and interPro or other iANT annotation platforms. Additional annotations consisted in the attribution of a description for the protein product, a gene name if applicable, a functional class, an EC number for metabolic activities and Gene Ontology terms. All annotations can be retrieved after searching the annotation fields and compared to the iANT database of the annotated genome.

2.4 Results and discussion

2.4.1 Plant samples

In growing regions of both Latin America and South-East Asia cassava plants showing symptoms suggestive of a phytoplasma disease were observed during the four consecutive years of the survey.

In Latin America, diseased plants from Costa Rica showed mild, moderate and severe symptoms in roots, while in Paraguay only mild symptoms were observed (Table 2.1 and Figure 2.4). In South-East Asia, in particular Northern and Southern Vietnam, diseased plants in Dong Nai areas showed mainly shoot proliferation, short internodes, small and yellow leaves; bud proliferation in branch apices, advances symptoms consisting in leaf drying and dieback of branches were also observed (Figure 2.5A and B), while also dark vascular tissue in the stems and in the roots were observed in Yen Bai (Figure 2.6H - L).

In the three different regions of Thailand single and mixed infections associated with phytoplasma presence and also caused by mealybugs were observed (Figure 4.2E and K); typical symptoms of insect damage were leaf deformation and severe bud damage, with shoot recovery but without short internodes and/or small leaves (Figure 2.5F and G); for symptoms associated with phytoplasmas plants showed excessive bud and shoot proliferation and/or rachitic branches; shoots with short internodes and small leaves that show no deformation but sometimes yellowing or chlorosis (Figure 2.5B-D and J) (Table 2.1) were also observed.

A total of 137 samples from CFSD and 166 of CWB were prepared. Based on localities, genotype and plant tissues, 57 DNAs samples (17 plants) from CFSD and 92 (50 plants) from CWB were selected for processing targeting the 16S rRNA gene to verify phytoplasma presence.

2.4.2 RFLP and phylogenetic analysis on the 16S rDNA and *tuf* genes

Phytoplasma direct PCR assays with the primer pair P1/P7 followed by nested PCR with the primer pair R16F2n/R16R2 were less successful and useful than the PCR assays with the primer sets R16mF2/R16mR1 and P1A/P7A followed by R16F2n/R16R2 and/or R16F1/B6 (30% to 50% of positive results, respectively). However, the use of direct PCR assays with primers P1/P7 and R16F2n/R16R2 followed by semi-nested and nested PCR with the primer set B5/P7 and M1/M2 allowed obtaining better results (30% to 75% of positive results). Nested and semi-nested PCR reactions with M1/M2, R16F2n/R16R2 and B5/P7 primer pairs resulted in amplification of the expected fragment length of about 500 bp, 1,245 bp and 1,109 bp respectively from 14 out of the 17 plants collected from symptomatic CFSD plants and from 38 of 50 symptomatic CWB plants corresponding to 21 and 40 phytoplasma strains, respectively; no amplification was obtained from asymptomatic samples (Table 2.1, Table 2.2a and 2.2b).

Table 2.2a. Results of phytoplasma strain detection and classification by RFLP and sequence analyses on 16Sr and tuf amplicons from cassava plants showing CFSD from Costa Rica and Paraguay.

				16Sr group classification		
Sample[a]	**Genes**	**RFLP**	**Sequence**	**Single and mixed infection**	**Country**	**Disease**
CCFSD_1-2	16Sr	XII	-	XII	I, III, X, XII	I, III, X, XII
CCFSD_1-4	16Sr	I	-	I		
CCFSD_3-1	16Sr	I	-	I		
CCFSD_6-1	16Sr	I and X-A	-	I and X-A		
	Tu	I and III	I and III	I and III		
CCFSD_9-4	16Sr	-	I	I		
CCFSD_15-4	16Sr	I	-	I		
CCFSD_16-6	16Sr	I and X-B	I and X-B	I and X-B		
CCFSD_17-5	16Sr	I	-	I		
CCFSD_22-2	16Sr	I and XII	-	I and XII		
CCFSD_22-4	16Sr	I	-	I		
CCFSD_23-2	16Sr	X-A	X-A	X-A		
CCFSD_23-4	16Sr	I	-	I		
CCFSD_19-4	16Sr	XII	-	XII		
CCFSD_19-5	16Sr	I	-	I		
CCFSD_21-4	16Sr	XII	-	XII		
	Tu	XII	-	XII		
CCFSD_31-1	16Sr	-	I	I		
	Tu	XII	XII	XII		
CCFSD_34-1	16Sr	I	-	I		
CCFSD_27-2	16Sr	I	-	I		
	Tu	III and XII	III and XII	III and XII		
CPFSD_B1(2)	16Sr	-	I	I	I, X	
	Tu	-	I	I		
CPFSD_B2(1)	16Sr	I	I	I		
CPFSD_B2(2)	16Sr	-	X-A	X-A		

[a]Strain designation by country and disease: CCFSD, Cassava Costa Rica Frog Skin Disease, CPFSD, Cassava Paraguay Frog skin Disease. Number after an underscore means the same plant.

Table 2.2b. Results of phytoplasma strain detection and classification by RFLP and sequences analyses on 16Sr and tuf amplicons in plants showing CWB disease from Thailand and Vietnam.

				16Sr group classification		
Sample	**Genes**	**RFLP**	**Sequence**	**Single and mixed infection**	**Country**	**Disease**
CThaiWB_1	16Sr	-	VI	VI	I, III, VI, XV	I, III, V, VI, X, XII, XV
CThaiWB_2	16Sr	XV	-	XV		
CThaiWB_3	16Sr	I, VI and XV	XV	I, VI and XV		
CThaiWB_4	16Sr	XV	-	XV		
CThaiWB_15	16Sr	VI and XV	XV	VI and XV		
CThaiWB_9	16Sr	VI and XV	VI	VI and XV		
	Tu	I	I	I		

Sample	Genes	RFLP	Sequence	16Sr group classification		
				Single and mixed infection	Country	Disease
CThaiWB_22	16Sr	-	VI	VI		
	Tu	III	-	III		
CThaiWB_6	16Sr	I and XV	-	I and XV		
CThaiWB_11	16Sr	XV	-	XV		
CThaiWB_12	16Sr	XV	XV	XV		
CThaiWB_13	16Sr	I, VI and XV	-	I, VI and XV		
CVWB_24	16Sr	XII	-	XII		
	Tu	XII	XII	XII		
CVWB_31	16Sr	I	-	I		
CVWB_33	16Sr	VI	-	VI		
CVWB_38	16Sr	I and XV	XV	I and XV		
CVWB_39	16Sr	I and VI	VI	I and VI		
	Tu	XII	-	XII		
CVWB_40	16Sr	XV	-	XV		
CVWB_29	16Sr	V-B	V-B	V-B		
CVWB_35	16Sr	V-B	-	V-B		
CVWB_42	16Sr	XV	-	XV		
CVWB_43	16Sr	VI and XV	-	VI and XV		
CVWB_46	16Sr	VI	-	VI		
CVWB_2V	16Sr	V	-	V		
CVWB_5V	16Sr	VI	-	VI		
CVWB_6V	16Sr	I	-	I		
CVWB_7V	16Sr	I	-	I		
CVWB_8V	16Sr	I	-	I		
CVWB_10V	16Sr	I	-	I		
CVWB_12V	16Sr	I	-	I		
CVWB_13L	16Sr	-	III	III		
CVWB_12AN	16Sr	I	-	I		
CVWB_12AP	16Sr	I	-	I		
	Tu	I and XII	I and XII	I and XII		
CVWB_12ANU	16Sr	I	-	I		
CVWB_12BNMix	16Sr	I	I	I		
CVWB_2-2-1*	16Sr	X-A		X-A		
CVWB_2-2	16Sr	I		I		
CVWB_2-6	16Sr	I		I		
CVWB_3-3	16Sr	I	-	I		
CVWB_3-6	16Sr	-	I	I		
CVWB_5-15	16Sr	I		I		
CVWB_6-3	16Sr	X-A	X-A	X-A		
CVWB_15	16Sr	I and X		I and X		

I, III, V, VI, X, XII, XV

[a]Strain designation by country and disease: CThaiWB, Cassava Thailand Witches' broom; CVWB, Cassava Vietnam Witches' Broom. Number after an underscore means different samples of the same plant.

The restriction profiles obtained with amplicons R16F2n/R16R2, B5/P7 and M1M2 using *Tru*I restriction enzyme, allowed different groups of profiles to be distinguished within and

between the diseases and regions evaluated. The identified profiles were identical and referable to the profile of phytoplasma reference strains related to aster yellows (16SrI), X-disease (16SrIII), elm yellows (16SrV), clover proliferation (16SrVI), apple proliferation (16SrX), "stolbur" (16SrXII) and hibiscus witches' broom (16SrXV), already reported (Lee *et al*., 1998, Montano *et al*., 2001) and after *in silico* comparison with several sequences of the strains references with the 16Sr ribosomal groups sequences of deposited in GenBank. Following this methodology an endophytic *Bacillus* spp. led to some false positive results, was also identified (Figure 2.7).

In particular amplification of the 16S ribosomal groups –I, -XII and -XV detected in CWB and CFSD (16SrXV was detected only in CWB samples) was obtained using R16(I)F1/R1 primers pair that yield the expected 1,095 bp bands (Figure 2.8). Additionally, nested PCR reactions on P1/P7 and R16F2n/R2 amplicons were performed with primer pair, specific for phytoplasmas belonging to X-disease [R16(III)F2/R1], elm yellows and jujube witches' broom [R16(V)F1/R1 and M1/V1731, R16(CJ)F1/R1] and apple proliferation [R16(X)F1/R1], and resulted in expected length fragment amplification, about 1,100 bp for R16(X)F1/R1, R16(V)F1/R1 and R16(CJ)F1/R1 and 973 bp for M1/V1731. Nested PCR with primers specific for 16SrIII phytoplasma group [R16(III)F2/R1] gave no amplification from any of the cassava samples collected in the four countries evaluated, while bands of the expected lengths (about 800 bp) were obtained only from corresponding positive controls (data not shown).

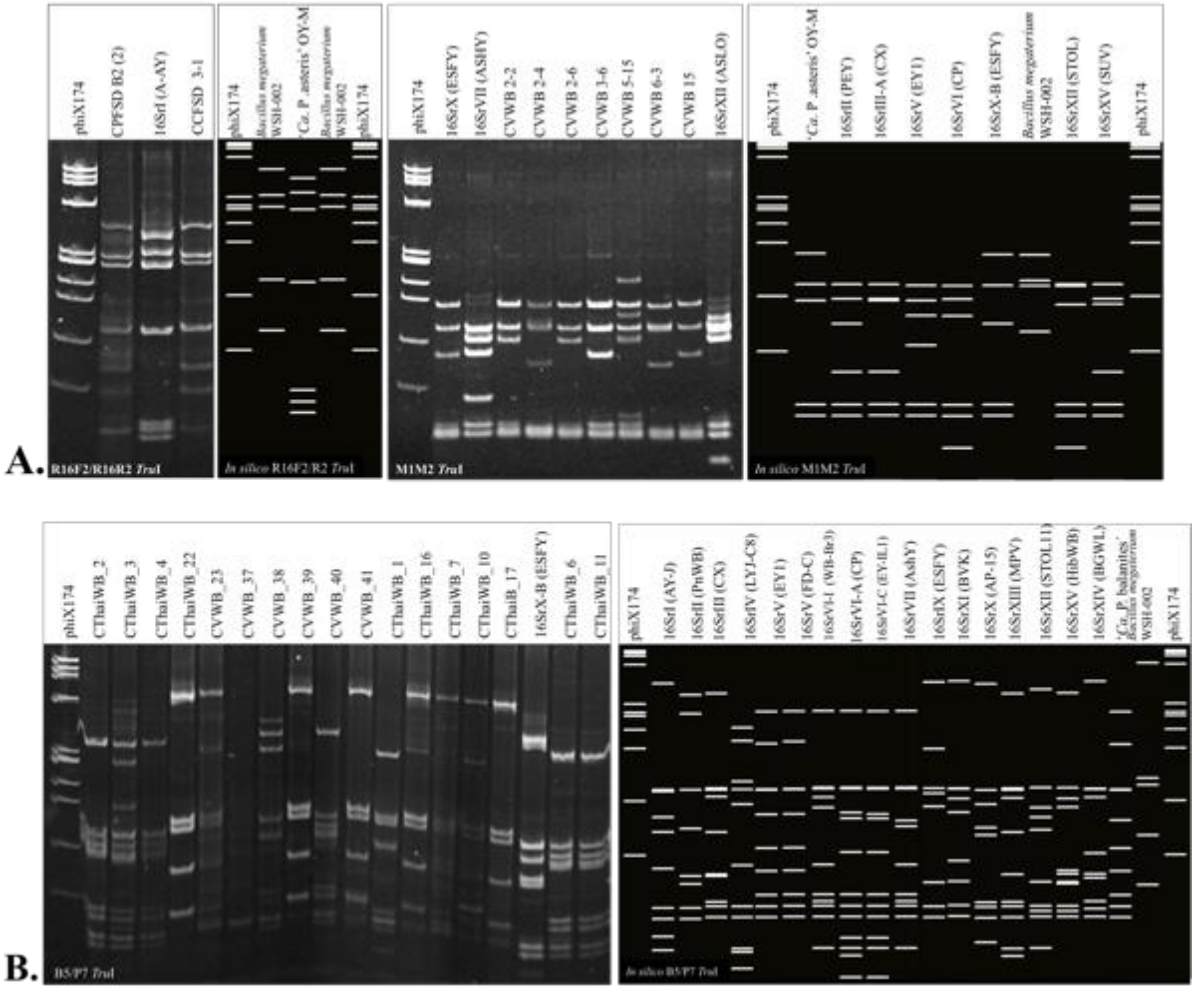


Figure 2.7. Differential profiles obtained after actual (left) and *in silico* (right) RFLP analyses with R16F2n/R16R2, M1/M2 and B5/P7 amplicons with the restriction enzyme *Tru*I, in preliminary phytoplasma differentiation. **A.** Typical R16F2n/R16R2 and M1/M2 restriction profile for *Bacillus* sp., detected in Costa Rica (CCFSD_3-1), Paraguay [CPFSD_B2(2)] and Vietnam (CVWB_2-4, CVWB_6-3) samples and profiles of two phytoplasmas belonging to 16SrI and 16SrX in Vietnam samples: CVWB_2-2, CVWB_2-6, CVWB_3-6 and CVWB_15, respectively. **B.** Restriction profiles of phytoplasmas belonging to 16SrXV (CThaiWB_2, CThaiWB_4, CThaiWB_38, CVWB_40, CThaiWB_6 and CThaiWB_11) and *Bacillus* sp. (CThaiWB_22, CVWB_39, CVWB_41, CThaiWB_16, CThaiWB_7, CThaiWB_10 and CThaiWB_17) obtained with B5/P7 amplicons in several samples from Vietnam and Thailand. phiX174, marker ΦX174 *Hae*III digested: fragment sizes in base pairs from top to bottom: 1353; 1078; 872; 603; 310; 281; 271; 234; 194; 118 and 72. Sample abbreviations and accession numbers for the reference strain are as indicated in Table 1.1.

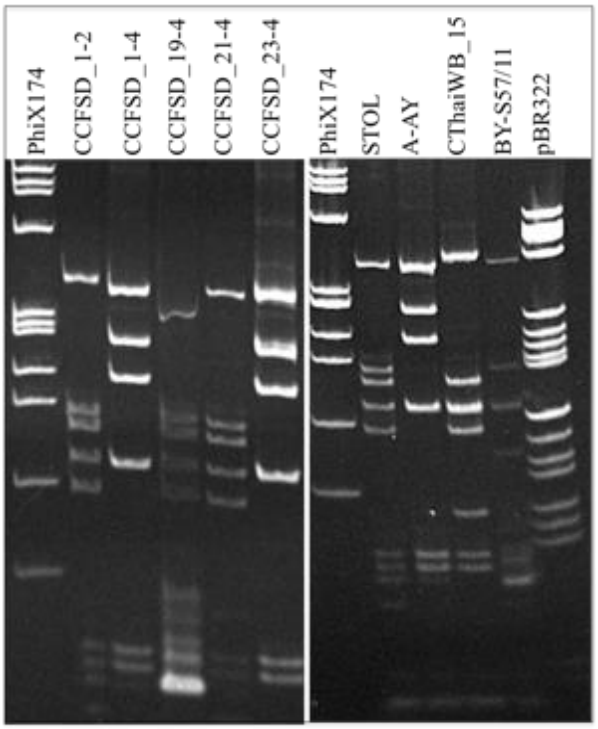


Figure 2.8. Polyacrylamide gel 6.7% showing the *Tru*I RFLP patterns of phytoplasma 16S rDNA obtained on R16(I)F1/R16(I)R1 amplicons after nested PCR assays from cassava samples identified belonging to the groups 16SrI (CCFSD_1-4 and CCFSD_23-4), 16SrXII (CCFSD_1-2, CCFSD_19-4, CCFSD_21-4), 16SrXV (CThaiWB_15) and from phytoplasma reference strains. Cassava sample acronyms are as reported in Table 1. Reference strains acronyms: STOL (16SrXII-A), "stolbur"; A-AY (16SrI-F), aster yellows from apricot; BY-S57/11, '*Ca.* P. convolvuli' (kindly provided by Dr. B. Duduk). phiX174, marker ΦX174 *Hae*III digested, fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72; pBR322, marker pBR322 *Bsu*RI digest, fragment sizes in base pairs from top to bottom: 587, 540, 502, 458, 434, 267, 234, 213, 192, 184, 124, 123, 104, 89, 80, 64, 57, and 51.

In the 57 samples from CFSD from the two Latin American countries processed with nested-PCR assays using specific and general primer pairs phytoplasmas belonging to 16Sr groups -I, -X and -XII were identified in the 64%, 14% and 22% of samples from Costa Rica. The 16SrI and –XII phytoplasmas were also identified in the 75% and 25% of samples from Paraguay (Table 2.2a, Figure 2.9). The results allow the association of symptoms (roots slightly opaque with an inflexible and brittle peel with many fissures or lip-like splits in all root parts and reduction of their diameter/volume) with phytoplasmas belonging to groups 16SrIII and -XII in Costa Rica and 16SrI and -X in both countries. Mixed infection was also detected, 16SrI with 16SrX and –XII but only in samples from Costa Rica (Table 2.2a).

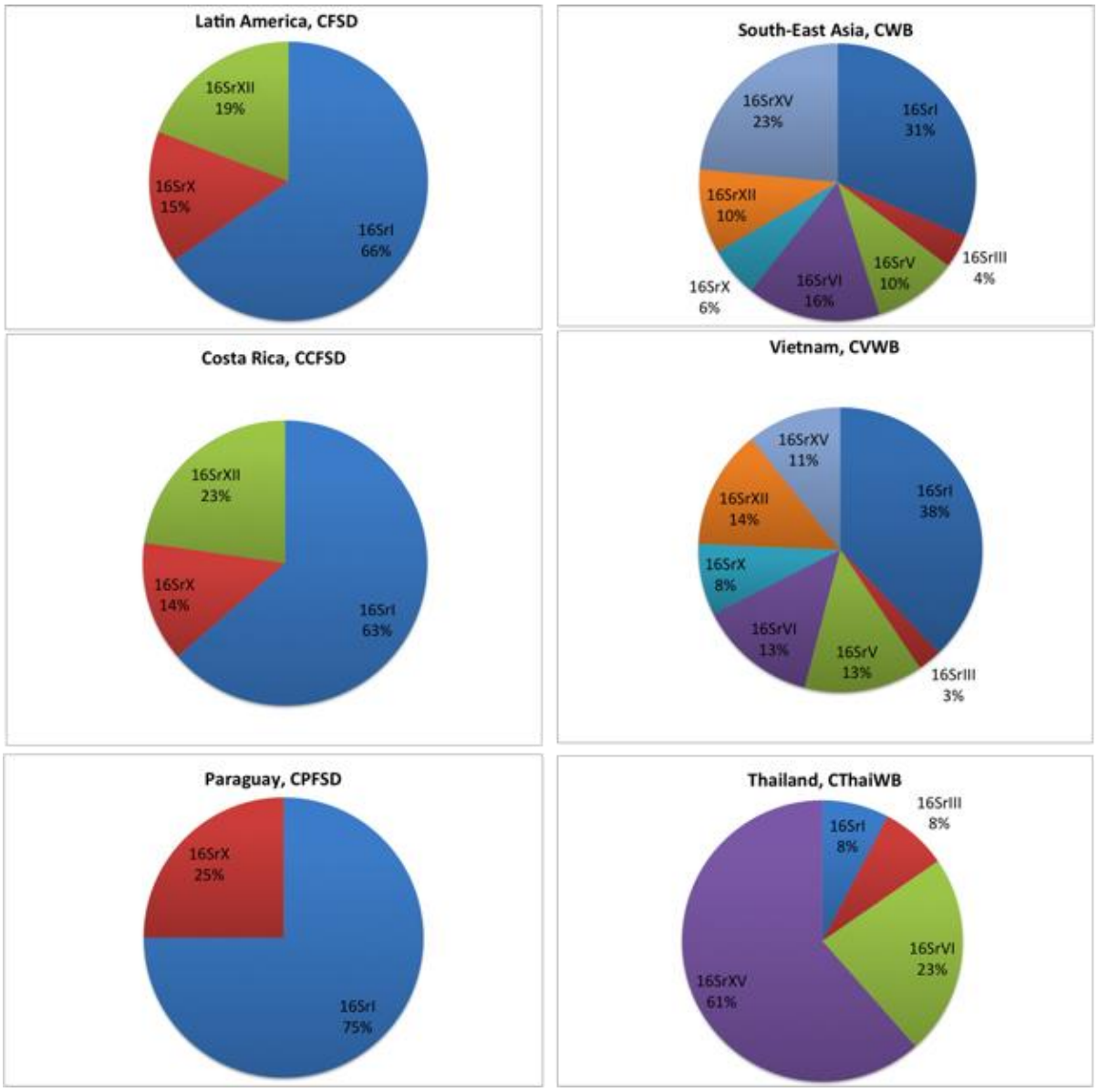


Figure 2.9. Percentages of ribosomal phytoplasma groups detected in Latin America and South-East Asia associated with CFSD and CWB, in Costa Rica (CCFSD), Paraguay (CPFSD), Vietnam (CVWB) and Thailand (CThaiWB).

The 92 samples from CWB evaluated from South-East Asia using specific and universal sets of primers showed the presence of phytoplasmas belonging to 16SrI in the 38%, -III in 3%, -V and -VI in 13%, –X in 8%, –XII in 14% and –XV in 11% of samples from Vietnam; whereas 16Sr groups -I, -III, -VI and -XV were identified in the 8%, 8%, 23% and 61% of samples from Thailand (Figure 2.9). The results allow the association of symptoms (high bud proliferation, shoots with short internodes, and small leaves) with phytoplasmas belonging to groups 16SrV, V-B, -X and -XII in Vietnam and 16SrI, -III, -VI and -XV in both countries. Mixed infection was also detected; 16SrVI with 16SrI and 16SrXV phytoplasmas in both countries, and with 16SrXII just in Vietnam. Phytoplasmas were detected in roots, small

leaves, midribs of symptomatic plants and also in plants with big bud symptoms associated with mealybugs presence and presenting witches' broom in the lower parts of branches in Thailand (Table 2.1 and 2.2b).

Moreover, both *in silico* and real RFLP analyses showed the presence of profiles differentiable from each others and from those of reported strains in the majority of the phytoplasma groups detected. SNPs analysis confirmed the presence of further polymorphism among and between Latin American's and South-East Asian phytoplasma strains belonging to 16SrI, -X and –XII groups and also in the phytoplasma strains belonging to 16SrV, -VI and -XV only detected in Asia. The phytoplasma identity was also confirmed by phylogenetic analyses of the most relevant phytoplasma ribosomal groups identified.

Further analyses performed on tuf gene to avoid the presence of false positive (*Bacillus* spp) sometimes obtained by 16Sr DNA amplification were also carried out. PCR with the barcode primers Tuf400/Tuf835 resulted in amplification of the expected fragment length of about 450 bp from all symptomatic cassava samples tested. Sequence and RFLP analyses with *Tru*I and *Tsp509*I restriction enzyme on these amplicons differentiated five groups of profiles related to different tuf and ribosomal groups. Comparisons of RFLP patterns of the PCR products with patterns published for tuf (Table 2.6) from selected reference phytoplasmas confirmed that the phytoplasmas found in cassava belong to aster yellows group (16SrI) in both diseases; in Vietnam and Thailand some strains were affiliated to 16SrI-C subgroup and in some cases in mixed infection. Moreover X-disease-related (16SrIII) phytoplasmas were identified in Costa Rica, Vietnam and Thailand samples; and "stolbur" (16SrXII) phytoplasmas in Costa Rica and Vietnam samples. X-disease phytoplasmas were also detected in mixed infection with 16SrI and 16SrXII in some Costa Rica samples (Figure 2.16).

Table 2.6. Results of phytoplasma strain differentiation by RFLP analyses on *tuf* gene amplicons compared with phytoplasmas reference strains (identical letters indicates identical profiles).

Strain acronyms	Tuf profiles[a]		16Sr group
	***Tru*I**	***Tsp509*I[b]**	
CHRYM	A	A	I-A
AY1	A	A	I-B
KVE	A	B	I-C
SEPT	A	A	II-A
WBDL	A	B	II-B
PEP	A	C	II-F
CR	B	D	III-B
SPI	C	E	III-E
RuS	D	F	V-E
FD-VE	D	F	V-D
FD-AS	D	F	V-C

Strain acronyms	Tuf profiles[a]		16Sr group
	*Tru*I	*Tsp509*I[b]	
LUM	E	G	VI
ASHY-4	F	H	VII-A
Erigeron	M	M	VII-B
AP-15	G	I	X-A
LNp	H	I	X-B
BVK	A	L	XI-C
ASLO	I	C	XII
BA	I	C	XII
Turnera	I	A	XIII
SUV	L	C	XV
CVWB_2-2	A	A	I
CVWB_6V	A	A	I
CVWB_12V	A	A	I
CCFSD_9-4	A	A	I
CCFSD_16-6	A	A	I
CThaiWB_1	A	B	I-C
CThaiWB_9	A	B	I-C
CVWB_12AN	A	B	I-C
CVWB_40	A	B	I-C
CVWB_29	A	B	I-C
CVWB_22	B	-	III
CCFSD_21-4	I	C	XII
CCFSD_31-1	I	C	XII
CVWB_24	I	C	XII
CVWB_39	I	C	XII
CCFSD_6-1	A + B	A + D (?)	I + III
CCFSD_27-2	I + B	C + (?)	XII + III
CVWB_12AP	A+I	A+C	I +XII

[a]RFLP profiles of reference strains are from Contaldo *et al*., 2011. [b] (+) two phytoplasma in mixed infection, (?)phytoplasma groups assigned sequencing.

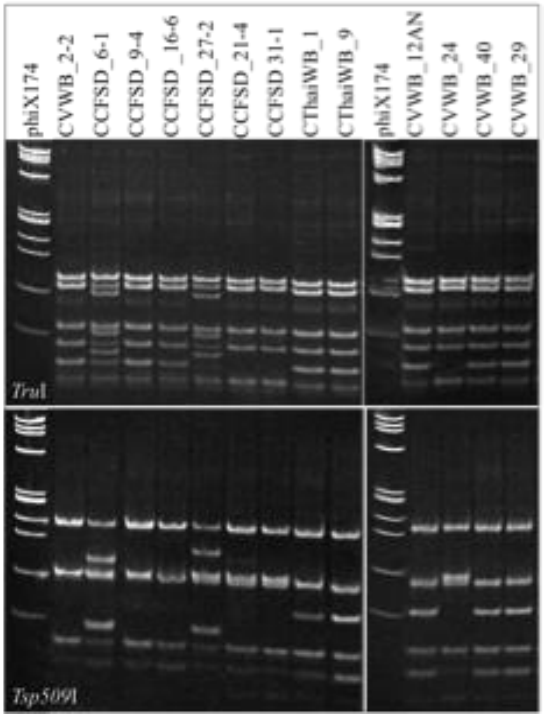


Figure 2.16. Polyacrylamide gel 6.7% showing the *Tru*I and *Tsp509*I RFLP patterns of *tuf* gene obtained with Tuf400/Tuf835 primer pair in nested PCR from cassava samples. 16SrI (CVWB_2-2, CCFSD_9-4, CCFSD_16-6), 16SrI-C (CThaiWB_1, CThaiWB_9, CVWB_12AN, CVWB_40, CVWB_29), 16SrXII (CCFSD_21-4, CCFSD_31-1, CVWB_24). Mixed infection of 16SrIII with 16SrI (CCFSD_6-1) and 16SrXII (CCFSD_27-2). phiX174, marker ΦX174 HaeIII digested, fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

2.4.2.1 Aster yellows-related strains, 16SrI

The majority of the symptomatic plants tested was positive to phytoplasma presence in Costa Rica, Paraguay and Vietnam and showed aster yellows-related phytoplasma presence. The restriction profiles obtained with amplicons R16F2n/R16R2 using *Hha*I and *Tru*I restriction enzymes (Figure 2.10), as well as with B5/P7, R16(I)F1/R16(I)R1 and M1/M2 amplicons allowed different profiles to be distinguished; one of them was indistinguishable from the reference strains aster yellows from France (AY-J), periwinkle virescence from Italy (NA) and primula green yellows from UK (PrG), which belongs to ribosomal subgroup 16SrI-B, while the other profiles were different from the reference strains and never reported before. In particular, restriction analyses with *Tru*I on R16(I)F1/R16(I)R1 amplicons allowed four different groups of phytoplasma profiles to be distinguished in Costa Rica and Paraguay samples, three in the samples CCFSD_9-4, CCFSD_31-1, CPFSD_B1(2) and other one in CCFSD_16-6 (Figure 2.11). In Vietnam samples, analyses showed that the two strains CVWB_3-6 [CWB-V1] and CVWB_12BNMix [CWB-V2] were distinguishable for the presence of a SNP that is also a differential *Tru1*I restriction site distinguishing phytoplasmas in both R16(I)F1/R16(I)R1 and M1/M2 amplicons (Figure 2.12). In several cases the five latter phytoplasmas were also present in mixed infection in the same plant as showed in the Figure 2.11 for CCFSD_9-4 and Figure 2.12 for CVWB_3-3, CVWB_3-6 and CVWB_12ANU, CVWB_12BNMix.

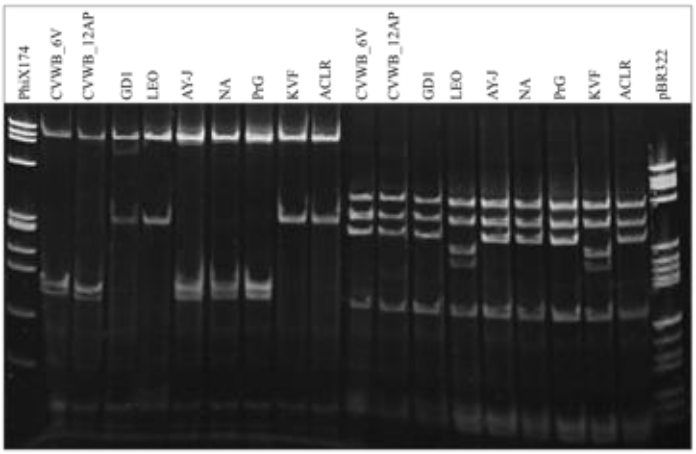


Figure 2.10. Polyacrylamide gel 6.7% showing the *Hha*I (left) and *Tru1*I (right) RFLP patterns of phytoplasma16S rDNA R16F2n/R16R2 primer pair products in nested PCR from two cassava samples and from phytoplasma reference strains in periwinkle. Sample acronyms: GD1 (16SrI-A), grey dogwood stunt; LEO (16SrI-C), *Leontodon* yellows; AY-J (16SrI-B), aster yellows; NA (16SrI-B), periwinkle virescence; PrG (16SrI-B), primula green yellows; KVF (16SrI-C), clover phyllody; ACLR [A-AY] (16Sr-F), aster yellows apricot; phiX174, marker ΦX174 *Hae*III digested; fragment sizes as above; pBR322, marker pBR322 *Msp*I digest, fragment sizes in base pairs from top to bottom: 622, 527, 404, 307, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110, 90, 76, and 67.

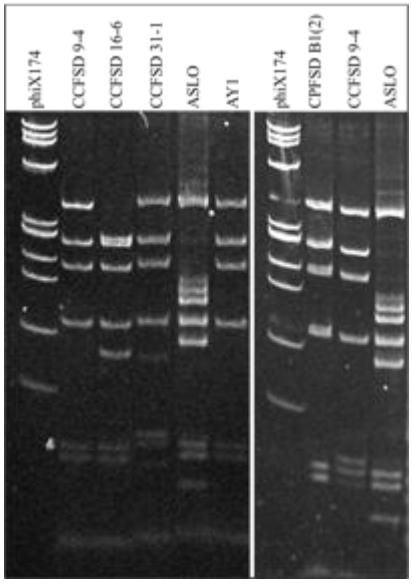
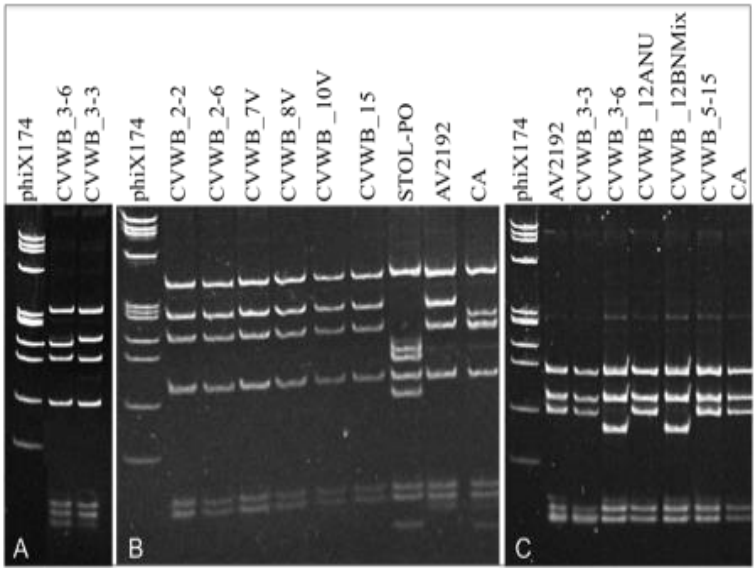


Figure 2.11. RFLP analyses of 16S rDNA amplicons obtained in nested PCR with R16(I)F1/R16(I)R1 primers using the endonuclease *Tru1*I. Three different 16SrI patterns obtained from CFSD-associated phytoplasmas from Costa Rica (CCFSD_9-4, CCFSD_16-6 and CCFSD_31-1) and one strain from Paraguay [CPFSD_B1(2)]. References strains AY1 (16SrI-B), Maryland aster yellows; ASLO (16SrXII-A), "stolbur" from aster from Slovenia; phiX174, marker ΦX174 *Hae*III digested; fragment sizes as above.

Figure 2.12. Restriction fragment length polymorphism analysis of the 1,100 bp fragments amplified in nested-PCR assays with primers R16(I)F1/R16(I)R1 (A and B) and M1/M2 (C) and digested with *Tru1*I from cassava samples CVWB_2-2 to CVWB_15 collected in Vietnam. In plants CVWB_3 and CVWB_12 two profiles were obtained in different samples collected from the same plant. References strains in periwinkle: STOL-PO ("stolbur", 16SrXII-A), AV2192 (aster yellows, 16SrI-B), and CA (carrot phyllody, 16SrI-C); phiX174, marker ΦX174 *Hae*III digested; fragment sizes as above.



Sequencing of 16S rDNA of selected cassava samples. The sequences of two strains obtained by direct sequencing of R16(I)F1/R16(I)R1 and/or R16F2n/R16R2 amplicons CVWB_3-6 and CVWB_12BNMix (1,096 and 1,103 bp, respectively) from Vietnam, were deposited in the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) under accession number KF897511 and KF897512. Furthermore, among the Costa Rica samples the strains CCFSD_9-4, CCFSD_16-6, CCFSD_31-1 and CCFSD23-4 (1,097 bp and 1,055 respectively); and from Paraguay CPFSD_B1(2) and CPFSD_B2(2) (1,004 bp and 1,013 bp respectively), were sequenced. Using the Blast tool from GenBank, the 16Sr DNA sequence of samples CCFSD_9-4, CCFSD_16-6, CCFSD_31-1, CPFSD_B1(2) and CVWB_3-6

showed the highest identity value of 99% (1095/1097, 1092/1097, 1093/1097, 1038/1041 and 1092/1097, respectively) with aster yellows phytoplasma strains from India and Texas (USA) (KF826901, KF573456, respectively), while the same region of sample CCFSD23-4, CPFSD_B2(1) and CVWB_12BNMix showed the highest identity value of 100% and 99% (1097/1097, 1045/1046 and 1096/1097, respectively) with aster yellows phytoplasma strains from Texas (USA) and Lithuania (KF573449 and JQ772016, respectively). Phylogenetic comparison of the 16S rRNA gene alone of the previous described samples with 23 representative strains of phytoplasmas from aster yellows ribosomal group indicated that the majority of phytoplasmas detected in cassava samples can be enclosed in the 16SrI-B ribosomal subgroups as defined by Lee *et al*. (1998a; b). The DNA sequence chromatogram of sample CCFSD23-4 and a reference strain PhyArg clearly indicated the presence of ambiguous bases, and this sequence was therefore employed for *in silico* cloning (Figure 2.14) and, after sequencing of the cloned amplicons, employed for phylogenetic analyses (see below).

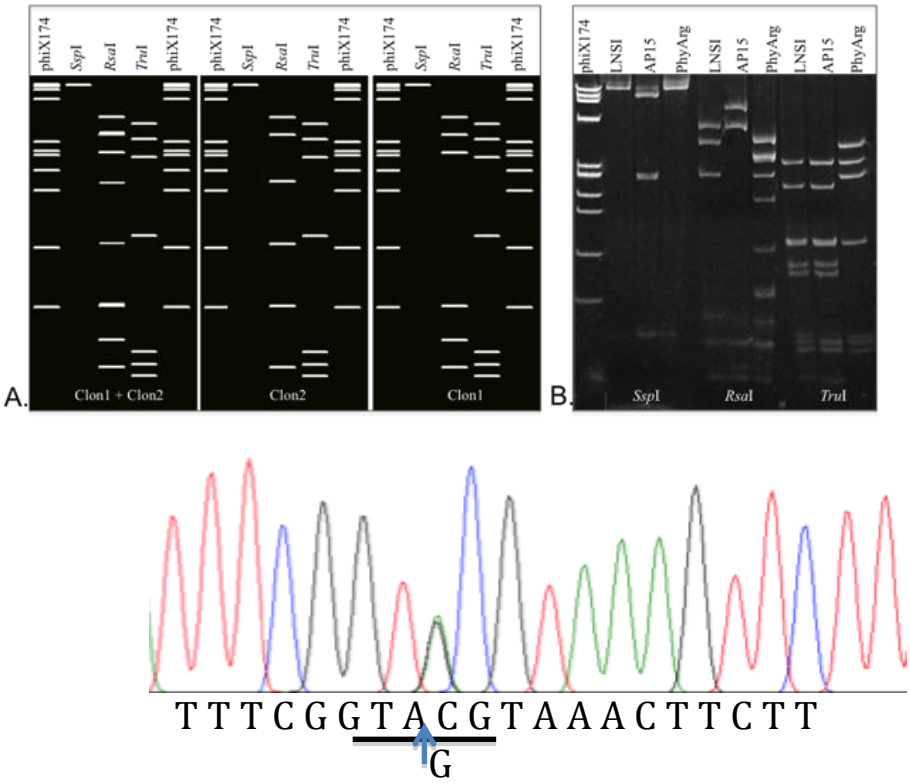


Figure 2.14. Evidence of sequence heterogeneity in the 16S rDNA sequence of aster yellows phytoplasma PhyArg used as reference strain in this study. **A.** *In silico* RFLP patterns of *Rsa*I digestion of DNA from clone 1 and clone 2. Clon1 + Clon2, strain PhyArg. **B.** Actual RFLP of strain PhyArg, confirming the interoperon heterogeneity shown with *Rsa*I restriction. **C.** Chromatogram of PCR-amplified rDNA from reference strain PhyArg. Double base-call is indicated by two peaks representing bases A and G, respectively. The recognition site for restriction enzyme *Rsa*I (position 428) is underlined; the location of enzyme cutting is indicated by an arrow.

Table 2.3. Aster yellows-related reference phytoplasma strains ('*Ca.* P. asteris') employed for characterization on 16S rDNA and tuf.

Phytoplasma associated disease (acronym)	Geographic origin	GenBank accession numbers		16SrI subgroup[b]
		16S rDNA	*Tuf*[a]	
New Jersey aster yellows (NJ-AY)	NJ, USA	HM590622	JQ824265	A
Plantago virescence (PVM)	Germany	AY265216	-	A
Hydrangea phyllody (HYDP)	Belgium	-	JQ824283	A
Chrysanthemum yellows (CHRYM)	Germany	-	JQ824240	A
Carrot yellows (ca2006/1)	Serbia	EU215424	-	A
Grey dogwood stunt (GD1)	NY, USA	DQ112021	JQ824243	A
Cassava witches' broom (CWFWB)	Wallis and Futuna	AY787139	-	A
Maryland aster yellows (AY-1)	USA	-	JQ824205	B
Periwinkle virescence (NA)	Italy	HM590621	-	B
Primula green yellows (PrG)	UK	HM590623	-	B
Oilseed rape virescence (RV)	France	HM590625	JQ824246	B
Carrot yellows (ca2006/9)	Serbia	EU215426	-	B
Primrose virescence (PRIVA)	Germany	AY265210	-	B(L)
Papaver phyllody (PAP)	Italy	-	JQ824212	B
Aster yellows (AV2192)	Germany	AY180957	JQ824275	B(L)
Aster yellows (AVUT)	Germany	AY265209	JQ824285	B(M)
Aster yellows (AY-J)	France	HM590616	JQ824215	B
Maize bushy stunt (MBS Col)	Colombia	HQ530152	Unpublished	B
Phyllody Argentina (PhyArg rrnA)	Argentina	Unpublished	-	B
Vietnamese cassava (VCP17)	Kon Tum, Vietnam	JQ973105	-	B
Vietnamese cassava (VCP34)	Dong Nai, Vietnam	JQ973106	-	B
Leontodon yellows (LEO)	Italy	HM590620	-	C
Achillea yellows (ACH)	Italy	-	JQ824213	
Carrot yellows (CA)	Italy	HM448473	JQ824226	C
Clover phyllody (KVE)	France	AY265217	JQ824248	C
Clover phyllody (KVF)	France	HQ530150	AJ271317	C
Clover phyllody (KVG)	Germany	-	JQ824279	C
Clover phyllody (KVM)	France	-	AJ271318	C
Potato purple top (PPT)	France	HQ530151	-	C
Cassava witches' broom (CCubWB)	Cuba	EU328256	-	C
Blueberry stunt (BBS3)	MI, USA	AY265213	-	E
Aster yellows apricot ACLR (A-AY)	Spain	AY265211	JQ824251	F

[a]Reference sequences from *tuf* were retrieved from Makarova *et al*., 2012.[b]Letters indicate RFLP subgroups in the 16SrI group.

Among the sequences described (Table 2.3) fourteen, all belonging to phytoplasmas enclosed in 16SrI-B subgroup clade, were selected for further evaluation to estimate the evolutionary divergence (Table 2.4). The number of base differences per sequence between sequences is shown. The analysis involved nine cassava samples. Calculation of the number of base substitutions per site values of these 16S rDNA sequences plus those of the two operons of sample CCFSD_23-4 and PhyArg showed the overall average of 2.59 varied from 0 to 10.00. The value of comparison between the two sequences obtained after *in silico* cloning from samples CCFSD_23-4 and PhyArg was 3.00 in both cases (Table 2.4).

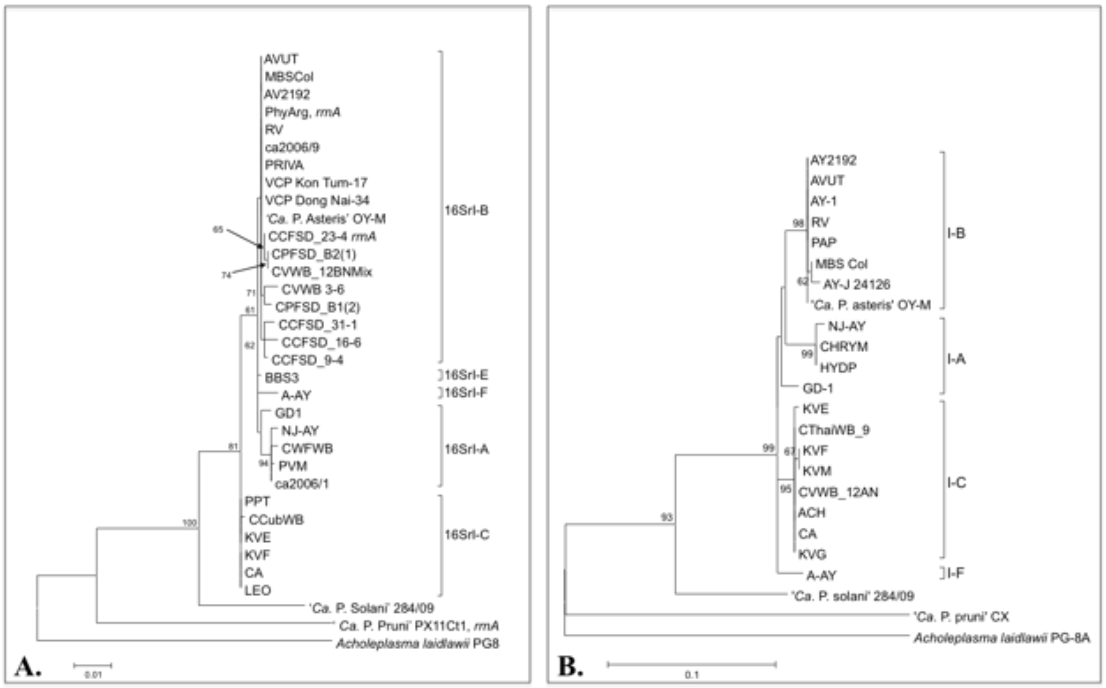


Figure 2.13. Phylogenetic trees constructed by maximum parsimony analysis of (A) 16S rDNA gene sequences and (B) *tuf* gene sequence from selected CWB and CFSD strains. Reference strains employed are described in Table 2.3. '*Ca.* P. asteris' strain OY-M (NC_005303); '*Ca*. P. pruni' (JQ044393 for 16Sr and JQ824211 for *tuf* gene); '*Ca*. P. solani' strain 284/09 (NC_022588) and *A. laidlawii* PG-8A (NC_010163). Numbers on the branches are bootstrap values obtained for 1,000 replicates (only values above 60% are shown). Letters in square brackets and bars, represent diverse RFLP subgroups in the 16S rDNA gene and *tuf* of aster yellows strains.

In silico and SNPs analyses of the 16S rRNA gene alone of samples CVWB_3-6, CVWB_12BNMix, CCFSD_9-4, CCFSD_16-6, CCFSD_31-1, CCFSD23-4, CPFSD_B1(2), CPFSD_B2(2) and 14 strains of aster yellows phytoplasmas (Table 2.5; Figure 2.14) confirmed that the majority of phytoplasma detected in cassava sample were most closely related to aster yellows phytoplasma reference strains belonging to the 16SrI-B ribosomal subgroup as defined by Lee *et al*., (1998a; 1998b). Sequence analyses also indicated that the four strains were distinguishable for a SNP that is also a differential *Tru1*I restriction site distinguishing between these phytoplasmas (Table 2.5; Figure 2.15).

Table 2.4. Estimation of evolutionary divergence between 16S rDNA sequences obtained from phytoplasma strains associated with CFSD and CWB from Costa Rica, Paraguay and Vietnam. The number of base differences per sequence between sequences is shown. The analysis involved nine cassava samples and fourteen 16SrI-B reference sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1,097 positions in the final dataset. The evolutionary analyses were conducted in MEGA5.

Strains[a]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1.'*Ca.* P. asteris' OY-M																							
2. CCFSD_16-6	5.00																						
3.CCFSD_31-1	4.00	7.00																					
4.CCFSD_9-4	2.00	5.00	4.00																				
5.CPFSD_B2(1)	2.00	7.00	6.00	3.00																			
6.CCFSD_23-4 *rrnA*	1.00	6.00	5.00	3.00	1.00																		
7.CCFSD_23-4 *rrnB*	4.00	7.00	8.00	6.00	4.00	3.00																	
8.CVWB_12BNMix	2.00	7.00	6.00	4.00	0.00	1.00	4.00																
9.CVWB_3-6	5.00	10.00	9.00	7.00	7.00	6.00	9.00	7.00															
10.CPFSD_B1(2)	3.00	8.00	7.00	4.00	5.00	4.00	7.00	5.00	6.00														
11.PhyArg *rrnA*	0.00	5.00	4.00	2.00	2.00	1.00	4.00	2.00	5.00	3.00													
12.PhyArg *rrnB*	3.00	8.00	7.00	5.00	5.00	4.00	7.00	5.00	8.00	6.00	3.00												
13.VCP Kon Tum-17	0.00	5.00	4.00	2.00	2.00	1.00	4.00	2.00	5.00	3.00	0.00	3.00											
14.VCP Dong Nai-34	0.00	5.00	4.00	2.00	2.00	1.00	4.00	2.00	5.00	3.00	0.00	3.00	0.00										
15.AV2192	0.00	5.00	4.00	2.00	2.00	1.00	4.00	2.00	5.00	3.00	0.00	3.00	0.00	0.00									
16.AVUT	0.00	5.00	4.00	2.00	2.00	1.00	4.00	2.00	5.00	3.00	0.00	3.00	0.00	0.00	0.00								
17.MBSCol	0.00	5.00	4.00	2.00	2.00	1.00	4.00	2.00	5.00	3.00	0.00	3.00	0.00	0.00	0.00	0.00							
18.RV	0.00	5.00	4.00	2.00	2.00	1.00	4.00	2.00	5.00	3.00	0.00	3.00	0.00	0.00	0.00	0.00	0.00						
19.ca2006/9	0.00	5.00	4.00	2.00	2.00	1.00	4.00	2.00	5.00	3.00	0.00	3.00	0.00	0.00	0.00	0.00	0.00	0.00					
20.PRIVA	0.00	5.00	4.00	2.00	2.00	1.00	4.00	2.00	5.00	3,00	0,00	3,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00				
21.Si	0,00	5,00	4,00	2,00	2,00	1,00	4,00	2,00	5,00	3,00	0,00	3,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00			
22.LetY-TX10	0,00	5,00	4,00	2,00	2,00	1,00	4,00	2,00	5,00	3,00	0,00	3,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00		
23.LetY-TX7	1,00	6,00	5,00	3,00	1,00	0,00	3,00	1,00	6,00	4,00	1,00	4,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	
24.LindLL2	1,00	6,00	5,00	3,00	1,00	0,00	3,00	1,00	6,00	4,00	1,00	4,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	0,00

[a]Strains Si (KF826901), LetY-TX10 (KF573456), LetY-TX7 (KF573449), and LindLL2 (JQ772016) sequences showing high significant score using BLAST® tool. Acronyms are as indicated in Table 2.3.

The results obtained show that phytoplasmas detected in Vietnamese and Thailand cassava witches' broom and frog skin diseased plants belong to 16SrI group, in particular to subgroups 16SrI-B and to a new subgroup or lineage differentiable on 16S rDNA. This is the first report of the association of phytoplasmas belonging to the 16SrI group with witches' broom disease symptoms in cassava in Vietnam, Thailand and with frog skin disease in Costa Rica and Paraguay. Cassava witches' broom have been associated in other parts of the world with aster yellows phytoplasma in Wallis and Fotune Islands (CWFWB, 16SrI-A) and Cuba (CCubWB, 16SrI-C (Figure 2.13), with X-disease phytoplasmas in Brazil (CaWB-Br01, 16SrIII-B) (Flores *et al*., 2013) and with peanut witches' broom phytoplasmas (16SrII) in Uganda (Arocha *et al*., 2008). However cassava frog skin disease have been only associated with X-disease phytoplasmas in Colombia (CFSDY15, 16SrIII-L) and in Brazil (CFSD_Br1, 16SrIII-A) (Alvarez *et al*., 2009, Souza *et al*., 2014). The results of this work confirm that

differentiable phytoplasmas are associated with the same disease as already widely reported (Bertaccini, 2007).

The wide host range of 16SrI group-related phytoplasmas includes many unrelated plant species. This lack of host specificity probably indicates that there is activity of effective insect vectors across various plant families; however, those vectoring witches' broom and frog skin in cassava were not identified yet, indicating a research area to be developed in the future in order to obtain the correct disease management.

Table 2.5. Differential SNP positions in 16Sr DNA sequences (from 200 to 1,297 bp), of nineteen '*Ca*. P. asteris' strains belonging to subgroup 16SrI-B, from Argentina (PhyArg), Lithuania (LindLL2), India (Si), from central (VCP Kong Tum-17) and south (VCP Dong Nai-34) Vietnam, from USA (LetY-TX7, LetY-TX10) compared with cassava strains detected in Costa Rica (CCFSD), Paraguay (CPFSD) and Vietnam (CVWB) samples.

	Single nucleotide positions in 16S ribosomal RNA gene (position 200 to 1297)																							
Strains	**210**	**211**	**270***	**273***	**354**	**408**	**428***	**433**	**483***	**485***	**491**	**512**	**719**	**736**	**741***	**834***	**860***	**966**	**970**	**1004**	**1023**	**1026**	**1155**	**1261**
'*Ca.* P. asteris'OY-M	A	G	C	C	C	G	A	A	A	C	A	T	A	G	T	A	A	A	C	A	A	T	A	T
CCFSD 16-6	T	A	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	G	.
CCFSD 31-1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	G	.	.	.	.	C	G	.
CCFSD 9-4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	A
CPFSD B2(1)	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	-
CCFSD_23-4 *rrnA*	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CCFSD_23-4 *rrnB*	-	-	.	A	.	A	.	.	.	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.
PhyArg *rrnA*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
PhyArg *rrnB*	.	.	G	.	.	.	G	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
CVWB_12BNMix	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.
CVWB_3-6	.	.	.	.	T	.	.	.	.	.	.	C	.	.	.	T	.	G	T	.	.	.	.	.
VCP Kon Tum-17	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
VCP Dong Nai-34	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CPFSD_B1(2)	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	T	.	G	.	.	-
AV2192	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AVUT	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MBSCol	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
RV	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ca2006/9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
PRIVA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Si	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
LetY-TX10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
LetY-TX7	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
LindLL2	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

[a]Strains Si (KF826901), LetY-TX10 (KF573456), LetY-TX7 (KF573449), and LindLL2 (JQ772016) sequences showed high significant score using BLAST® tool. Strain OY-M (NC_005303) was used to calculated the SNPs positions. *SNPs making differential restriction sites RFLP for potential new 16SrI strain characterization and for *rrnA* and *rrnB* differentiation: 270, 273, 428, 483, 485 (*Rsa*I); 485, 834, 860 (*Tru1*I), 741 (*Sau96*I). Dots represent nucleotides identical to the '*Ca.* P. asteris' OY-M consensus sequence and dashes are gaps positions.

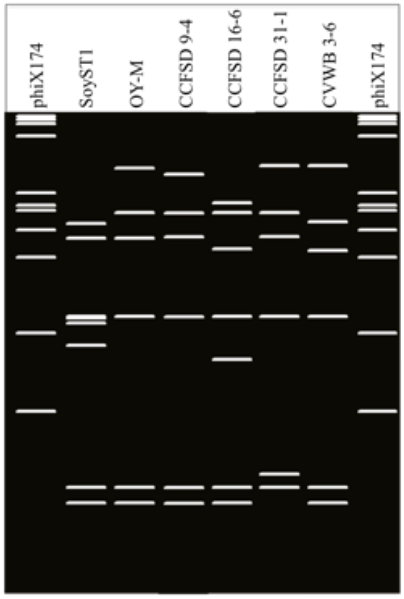


Figure 2.15. *In silico* RFLP analyses of 16S rDNA amplified with R16(I)F1/R16(I)R1 primers using the endonuclease *Tru1*I. Four different 16SrI patterns were obtained from CFSD-associated phytoplasmas from Costa Rica (CCFSD_9-4, CCFSD_16-6 and CCFSD_31-1) and one strain from Vietnam (CVWB_3-6). References strains SoyST1, '*Ca*. P. costaricanum'; OY-M, '*Ca.* P. asteris'. phiX174, marker ΦX174 *Hae*III digested, fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

2.4.2.2 X-disease-related strains, 16SrIII

RFLP and phylogenetic analyses on 16Sr DNA and *tuf* genes using the restriction enzymes *Tru1*I and *Tsp509*I in nested PCR amplicons obtained with the generic phytoplasma primers B5/P7A, M1/M2 and Tuf400/Tuf835, allowed identifying, only in 16Sr DNA, in the sample CVWB_13L from Vietnam, a phytoplasma belonging to the X-disease or 16SrIII- group (Figure 2.18A and B). Nested PCR assays with primers specific for 16SrIII phytoplasma group [R16(III)F2/R1] were also carried out and gave no amplification from this cassava sample, bands of the expected lengths were obtained only from corresponding controls. Furthermore, PCR with the primers *tuf* resulted in amplification of the expected fragment length of about 450 bp in one symptomatic cassava sample from Thailand (CThaiWB_22) and two from Costa Rica (CCFSD_6-1, CCFSD_27-2) associated with witches' broom and frog skin diseases, respectively. Particularly, in Costa Rica two different profiles related to 16SrIII group phytoplasmas in mixed infection with 16SrI and 16SrXII group phytoplasmas were distinguished (Table 2.6, Figure 2.16 and 2.17), after *in silico* and phylogenetic analyses on the elongation factor gene (tuf), both strains were clearly differentiated in 5 SNPs positions and unique restrictions sites with the enzymes *Tsp509*I (position 79), *Hpa*I (position 167) and *Mbo*I (position 370). No differences were obtained between the CCFSD_6-1 and the reference strain CFSDY15 (cassava frog skin disease, strain Y15, 16SrIII-L) from Colombia, while CCFSD_27-2 was closer to 16SrIII-J related strains and showed the overall average of 1.75 varying from 1.0 to 3.0. (Table 2.8, Figure 2.18c).

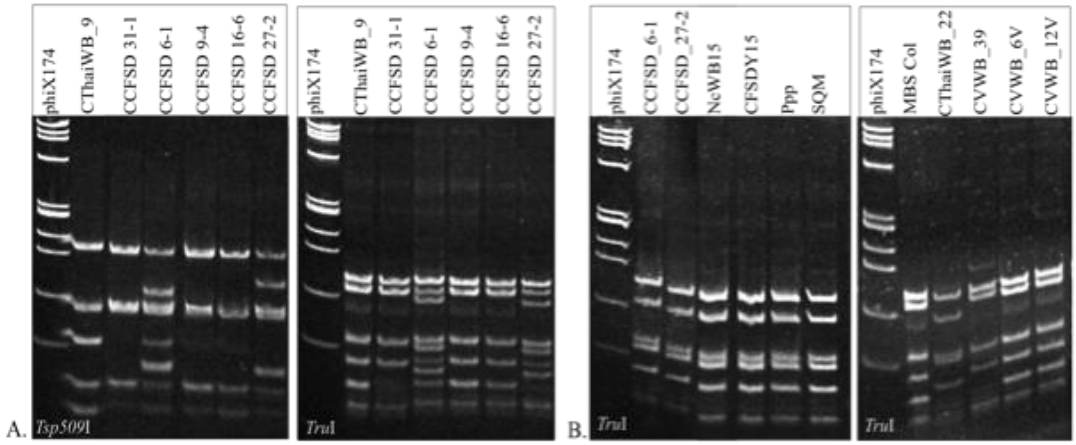


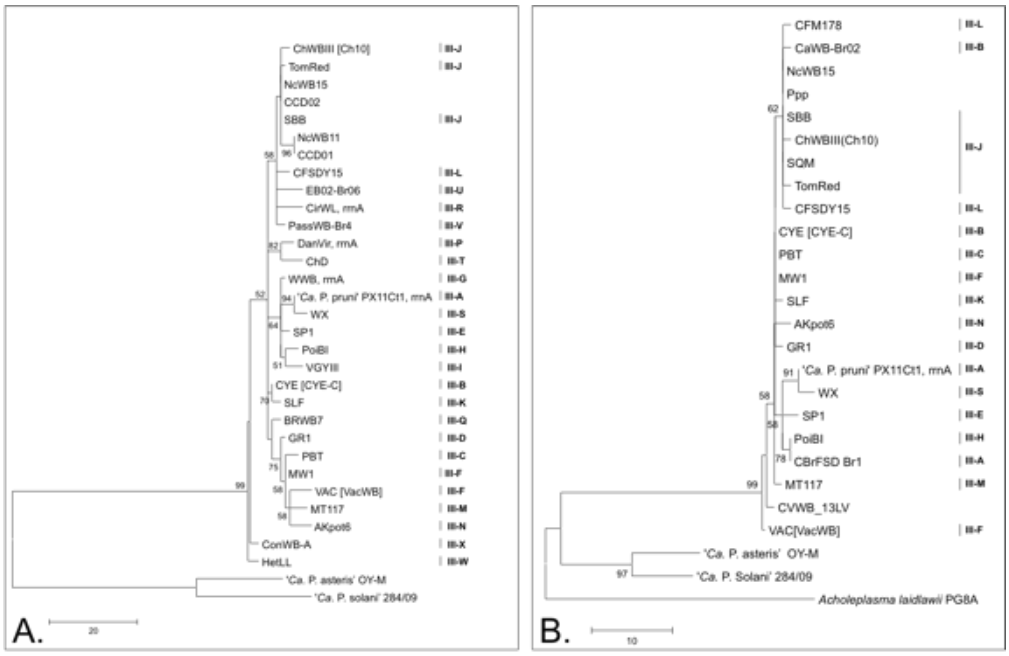
Figure 2.17. Polyacrylamide gel 6.7% showing the *Tsp509*I and *Tru1*I RFLP patterns of *tuf* gene amplicons obtained with Tuf400/Tuf835 primer pair. **A.** CThaiWB_9 (16SrI-C), CCFSD_31-1 (16SrXII); CCFSD_9-4 and CCFSD_16-6 (16SrI). Mixed infection of 16SrIII with CCFSD_6-1 (16SrI) and CCFSD_39 (16SrXII). **B.** Tuf X-disease profile of CCFSD_6-1, CCFSD_27-2 and CThaiWB_22; CVWB_39 (16SrXII); CVWB_6V and CVWB_12V (16SrI). Reference strains used: MBS Col (16SrI-B), maize bushy stunt from Colombia; NcWB15 (16SrIII), Nogal cafetero witches' broom; CFSDY15 (16SrIII-L), Cassava frog skin disease; Ppp (16SrIII), *Physalis peruviana* phytoplasma; SQM (16SrIII-J), *Solanum quitoense* machorreo; phiX174, marker ΦX174 *Hae*III digested, fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

Figure 2.7. X-disease related reference phytoplasma ('*Ca.* P. pruni') strains employed for 16S rDNA and tuf characterization.

Phytoplasma associated disease (acronym)	Geographic origin	GenBank accession numbers		16SrIII subgroup[b]
		16S rDNA	*Tuf*[a]	
Peach X-disease (PX11CT1) '*Ca.* P. pruni'	Canada	JQ044393	JQ824211	A
Cassava frog skin disease (CBrFSD Br1)	Brazil	KF667079	-	A
Western X disease (BF)	USA	-	JQ824233	A
Green valley X (GVX)	USA	-	JQ824252	A
Clover yellow edge (CYE [CYE-C]	Lithuania	AF173558	-	B
Cassava witches' broom (CaWB-Br02)	Brazil	GU193977	-	B
Crepis biennis yellows (CR)	Italy		JQ824258	B
Plum leptonecrosis (LNI)	Italy		JQ824230	B
Pecan bunch (PBT)	USA	GU004371	-	C
Goldenrod yellows (GR1)	USA	GU004372	JQ824232	D
Spirea stunt (SP1)	USA	AF190228	JQ824281	E
Milkweed yellows (MW1)	USA	AF510724	JQ824206	F
Vaccinium witches' broom (VAC [VacWB])	Germany	X76430	JQ824260	F
Walnut witches' broom (WWB rrnA)	USA	JQ044395	-	G
Poinsettia branch inducing (JR-1)	USA	-	JQ824267	H
Poinsettia cv. Lilo - (PoiLilo)	Denmark	-	JQ824286	-
Poinsettia branch-inducing (PoiBI)	USA	AF190223	-	H
Virginia grapevine yellows (VGYIII)	USA	AF060875	-	I
Chayote witches' broom (ChWBIII[Ch10])	Brazil	AF147706	-	J
Solanum marginatum big bud (SBB)	Ecuador	HQ589207	-	J
Delphinium phytoplasma	UK	EF514210	-	J

Phytoplasma associated disease (acronym)	Geographic origin	GenBank accession numbers		16SrIII subgroup[b]
		16S rDNA	*Tuf*[a]	
Solanum quitoense machorreo (SQM)	Colombia	AY731819	Unpublished	J
Nogal cafetero witches broom (NcWB11)	Colombia	Unpublished	Unpublished	-
Nogal cafetero witches broom (NcWB15)	Colombia	Unpublished	Unpublished	-
Physalis peruviana phytoplasma (Ppp)	Colombia	Unpublished	Unpublished	-
Coffee crispiness (CCD01 and CCD02)	Colombia	Unpublished	Unpublished	-
Solanum lycopersicum phytoplasma (TomRed)	Argentina	KC412031	-	J
Strawberry leafy fruit (SLF)	USA	AF274876	-	K
Cassava frog skin disease (CFSDY15)	Colombia	EU346761	Unpublished	L
Cassava frog skin disease CBrFSD [CFM178]	Brazil	KF019184	-	L
Potato purple top (MT117)	USA	FJ226074	-	M
Potato purple top (AKpot6)	USA	GU004365	-	N
Dandelion virescence (DanVir rrnA)	Lithuania	AF370119	-	P
Black raspberry witches' broom (BRWB7)	USA	AF302841	-	Q
Cirsium white leaf (CirWL rrnA)	Lithuania	AF373105	-	R
Western X phytoplasma (WX)	-	L04682	-	S
Sweet and sour cherry (ChD)	Lithuania	FJ231728	-	T
Eggplant Giant Calyx (EB02-Br06)	Brazil	HM589213	-	U
Passion fruit phytoplasma (PassWB-Br4)	Brazil	GU292082	-	V
Heterothalamus alienus phytoplasma (HetLL)	Argentina	KC412029	-	W
Conyza bonariensis phytoplasma (ConWB-A)	Argentina	KC412026	-	X

[a]References sequences for *tuf* from Makarova *et al*., 2012.[b]Different letter represent diverse RFLP subgroups in the X-disease group.



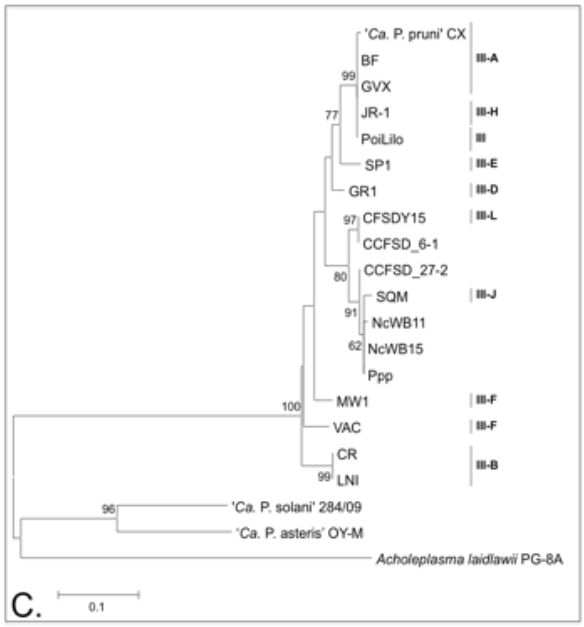


Figure 2.18. Phylogenetic trees from 16Sr and elongation factor genes from X-disease related phytoplasmas from cassava witches' broom from Vietnam (CVWB_13LV) and frog skin disease from Costa Rica (CCFSD_6-1 and CCFSD_27-2). **A.** Phylogenetic tree of partial 16S rRNA gene, R16F2n/R16R2 sequences from reference phytoplasmas of 16SrIII subgroups. **B.** Phylogenetic tree of partial 16S rRNA gene, strain CVWB_13LV and selected reference phytoplasmas within the 16SrIII subgroups. **C.** Conserved region within the *tuf* gene, amplified with primer cocktails Tuf400/Tuf835 of CCFSD_6-1 and CCFSD_27-2 from samples from Costa Rica and from reference phytoplasmas in 16SrIII subgroups. Accession numbers and acronyms are indicated in the Table 2.7. *'Ca.* P. asteris'(NC 005303), '*Ca*. P. solani' (NC 022588) and *A. laidlawii* PG-8A (NC010163) were used as outgroups in A, B and C respectively.

Table 2.8. Estimation of evolutionary divergence between *tuf* gene sequences obtained from two phytoplasma strains associated with CFSD from Costa Rica. The number of base differences per sequence between sequences are shown. The analysis involved also sixteen 16SrIII reference nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 391 positions in the final dataset. The evolutionary analyses were conducted in MEGA5.

Strains[a]	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
1. '*Ca*. P. pruni' CX																	
2. PoiLilo	1.0																
3. BF	1.0	0.0															
4. GVX	1.0	0.0	0.0														
5. CR	16.0	15.0	15.0	15.0													
6. LNI	16.0	15.0	15.0	15.0	0.0												
7. GR1	10.0	9.0	9.0	9.0	13.0	13.0											
8. SP1	10.0	9.0	9.0	9.0	17.0	17.0	10.0										
9. MW1	14.0	13.0	13.0	13.0	15.0	15.0	9.0	14.0									
10. VAC	19.0	18.0	18.0	18.0	14.0	14.0	16.0	17.0	13.0								
11. JR-1	1.0	0.0	0.0	0.0	15.0	15.0	9.0	9.0	13.0	18.0							
12. NcWB15	15.0	14.0	14.0	14.0	13.0	13.0	14.0	18.0	15.0	14.0	14.0						
13. NcWB11	16.0	15.0	15.0	15.0	14.0	14.0	15.0	19.0	15.0	15.0	15.0	1.0					
14. CFSDY15	14.0	13.0	13.0	13.0	13.0	13.0	12.0	16.0	15.0	16.0	13.0	6.0	7.0				
15. CCFSD_6-1	14.0	13.0	13.0	13.0	13.0	13.0	12.0	16.0	15.0	16.0	13.0	6.0	7.0	0.0			
16. CCFSD_27-2	14.0	13.0	13.0	13.0	12.0	12.0	13.0	17.0	14.0	13.0	13.0	1.0	2.0	5.0	5.0		
17. Ppp	15.0	14.0	14.0	14.0	13.0	13.0	14.0	18.0	15.0	14.0	14.0	0.0	1.0	6.0	6.0	1.0	
18. SQM	17.0	16.0	16.0	16.0	15.0	15.0	16.0	20.0	17.0	16.0	16.0	2.0	3.0	8.0	8.0	3.0	2.0

[a]Strains acronyms are as indicated in Table 2.7.

In the 16S rDNA sequence the strain CVWB 13LV is identical to the strain VAC (VacWB) belonging to 16SrIII-F except for a restriction site *Mae*III in position 1,216 and a SNP in position 1,224 that differentiate this strain from the others 16SrIII subgroups phytoplasmas already reported. The tuf gene, encoding the elongation factor Tu, is a conserved gene involved in the process of translation and a highly conserved gene that has been recently used for differentiation of phytoplasmas. In some cases, it was found also to be useful in the differentiation of various ecological strains or strain variants within 16S rRNA subgroups (Schneider *et al*., 1997; Makarova *et al*., 2012). Based on the strong phylogenetic relationships between 16S rDNA based classification and tuf system, the 16SrIII strains cassava frog skin disease (CFSDY15, 16SrIII-L) and *Solanum quitoense* machorreo (SQM, 16SrIII-J) showed to be strongly related on tuf gene with phytoplasmas detected in Vietnamese and Thailand cassava witches' broom and Costa Rican frog skin disease, in particular the strains from Costa Rica result to be related to phytoplasmas described in subgroups 16SrIII-L and 16SrIII-J. This is the first evidence of the presence of phytoplasmas belonging to the 16SrIII group with witches' broom disease symptoms in cassava in Vietnam, Thailand and with frog skin disease in Costa Rica. However, as mentioned above, cassava witches' broom have been associated in other parts of the world with the ribosomal groups 16SrI-A, 16SrI-C, 16SrII and 16SrIII-B, while cassava frog skin disease have been only associated with 16SrIII-A and 16SrIII–L. Since in both diseases phytoplasmas belonging to X-disease group were detected based on analysis of tuf gene sequences, this gene could be useful as additional marker for epidemiologic studies and/or to recognize strain variants within 16SrIII-B and 16SrIII-A, 16SrIII-L associated with cassava wiches' broom and frog sking diseases respectively (Souza *et al*., 2014, Flores *et al*., 2013, Alvarez *et al*., 2009).

2.4.2.3 Elm yellows-related strains, 16SrV

Two positives samples CVWB_29 and CVWB_35 from Vietnam in nested PCR assays with R16(V)F1/R16(V)R1, M1/V1731, B5/P7 and M1/M2 primer pair showed restriction profiles when subjected to RFLP analyses with *Tru*I and *Taq*I restriction enzymes that were identical (Figure 2.19) and referable to the profile jujube witches' broom phytoplasma (JWB, 16SrV-B). Primers R16(CJ)F1/R16(CJ)R1 yielded the expected length bands from these samples and for the phytoplasma control belonging to the 16SrV-B phytoplasma subgroup, but no amplification was obtained for elm yellows (EY, 16SrV-A) and rubus stunt (RuS, 16SrV-E) used as additional controls. The same primer pair, used in direct or in nested PCR with the cycle reported by Zhu *et al*. (1998), showed specificity only for samples CVWB_29,

CVWB_35 and JWB used as positive control (Figure 2.19). Additionally, using the same set of primers also ribosomal group 16SrV was detected in the sample CVWB_2V (Table 2.2b). The molecular characterization on 16S ribosomal DNA by RFLP analyses with *Taq*I restriction enzyme allow to confirm that these positives samples collected in the three areas of Vietnam were infected by 16SrV and 16SrV-B phytoplasmas. Sequencing of phytoplasma strain CVWB_29 allow to obtain 1,036 bp showing the highest homology of 100% with strains JWB-FJP1 (jujube witches' broom) and XJA1 (apricot chlorotic leafroll) from China (KC331046 and KC331046, respectively), but also showed same homology with other phytoplasma strains reported in Japan (‘*Ca.* P. ziziphi', JWB-G1) and Korea (Jujube witches' broom, JWB-Kor1 (Table 2.10). Calculation of the evolutionary divergence values of CVWB_29 16S rDNA sequences plus reference strains showed the overall average of 5.7 varying from 0 to 18.00. The value of comparison between the cassava sequences and members of 16SrV-B ribosomal subgroup varied from 0 to 3.00 showing an overall average of 1.1 (Table 2.4). The phylogenetic tree constructed using near full-length R16F2n/R16R2 sequences allowed to confirm the very close relationship between the cassava strain and the jujube witches’ broom ribosomal subgroup (16SrV-B) (Figure 2.20). The results confirm that this specific molecular test can be useful to evaluate samples showing cassava witches’ broom disease to verify phytoplasma related to 16SrV-B subgroup presence.

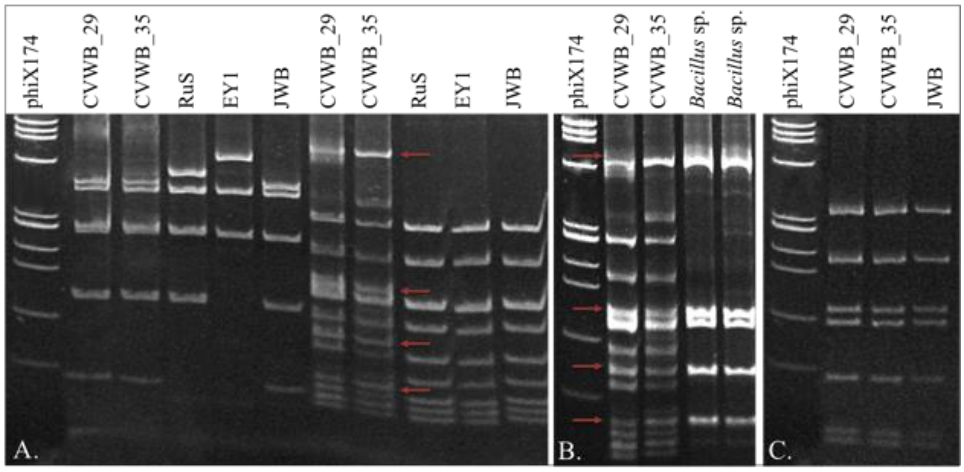


Figure 2.19. RFLP analyses of 16S rDNA amplified in nested PCR with B5/P7 and R16(CJ)F1/R16(CJ)R1 primers using the endonuclease *Taq*I and *Tru*I. **A.** B5/P7 amplicons of cassava witches’ broom samples from Vietnam with restriction enzymes *Taq*I (left) and *Tru*I (right) and from phytoplasma reference strains in periwinkle: RuS (16SrV-E, *‘Ca.* P. rubi'); EY1 (16SrV-A, *‘Ca.* P. ulmi'); JWB (16SrV-B, ‘*Ca*. P. ziziphi). **B.** B5/P7 amplicons digested with *Tru*I restriction enzyme that evidence in cassava samples presence of 16SrV group phytoplasmas and *Bacillus* sp; the red arrows are indicating extra bands of *Bacillus* within phytoplasma profile. **C.** Specific fragments obtained only from cassava witches’ broom samples and the reference control JWB (16SrV-B), with specific primers R16(CJ)F1/R16(CJ)R1 using the restriction enzyme *Taq*I.

Table 2.9. Elm yellows group related reference phytoplasma strains employed for 16S rDNA classification.

Phytoplasma associated disease (acronym)	Geographic origin	GenBank accession numbers, 16S rDNA	16SrV subgroup[a]
'Ca. P. ulmi' (EY1)	USA	AY197655	A
Elm yellows (EY-125)	Italy	AY197656	A
Elm yellows (EY-626)	Italy	AY197657	A
Elm yellows (EY-627)	Italy	AY197658	A
Jujube witches'-broom (JWB-Ky)	Japan	AB052875	B
Jujube witches-broom (JWB)	China	AY197661	B
'Candidatus Phytoplasma ziziphi' (JWB-G1)	Japan	AB052876	B
Jujube witches' broom (JWB-Kor1)	Korea	AB052879	B
Cherry lethal yellows (CLY-5)	China	AY197659	B
Peach yellows (PY-In)	India	AY197660	B
Honeylocust witches' broom (Hon1)	China	FJ457095	B
Cassava witches' broom (CVWB_29)	Vietnam	Unpublished	B
Virginia creeper (VC)	USA	AF305198	C
"Flavescence dorée" (FD-Ca)	Italy	X76560	C
Hemp dogbane (HD1)	USA	AF122912	C
"Flavescence dorée" (FD70)	France	AF176319	C
"Flavescence dorée" (FD-Cb)	Italy	AY197645	C
Solanum phureja phytoplasma (SPCol)	Colombia	Unpublished	C
Alder yellows (ALY882)	Germany	AY197642	C
"Flavescence dorée" (FD-D)	Italy	AY197644	D
"Flavescence dorée" (FD-D) [FD1487]	Spain	AJ548787	D
'Ca. P. rubi' (RuS)	Italy	AY197648	E
Rubus stunt (Rus400)	Italy	AY197649	E
Rubus stunt (RuSR19)	Germany	AY197651	E
Rubus stunt (Rus971)	Switzerland	AY197650	E
'Ca. P. balanitae' (BltWB)	Myanmar	AB689678	F

[a]Different letter represent diverse RFLP subgroups in the 16S rDNA gene of Elm yellows group.

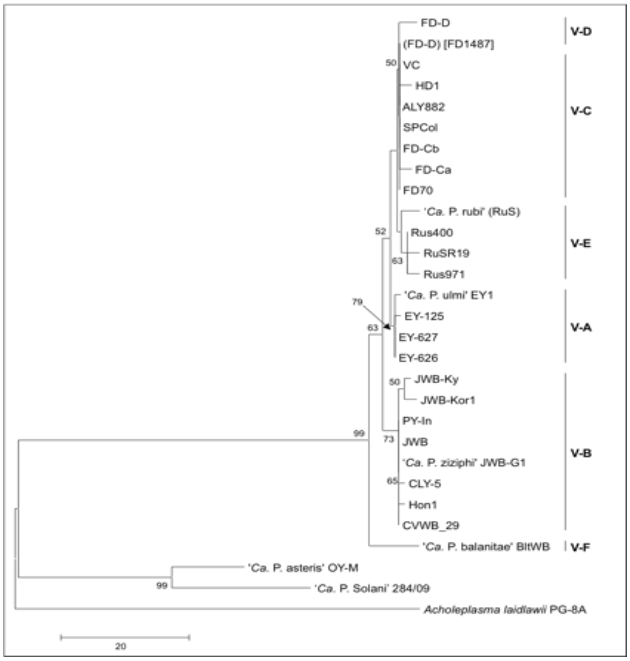


Figure 2.20. Phylogenetic trees constructed by maximum parsimony analysis of 16S rDNA gene sequences with one selected CWB strain. Strains references employed from 16SrV subgroups are described in Table 2.9. '*Ca.* P.

asteris' strain OY-M (NC_005303); '*Ca.* P. solani' strain 284/09 (NC_022588) and *A. laidlawii* PG-8A (NC_010163) were used as outgroups. Numbers on the branches are bootstrap values obtained for 1,000 replicates (only values above 50% are shown). Letters in bars, represent diverse RFLP subgroups in the 16S rDNA gene of elm yellows strains.

Table 2.10. Estimation of evolutionary divergence between 16S rDNA sequences obtained from a phytoplasma strains associated with CWB from Vietnam. The number of base differences per sequence between sequences is shown. The analysis involved one cassava samples and twenty-four 16SrV reference nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1,063 positions in the final dataset. The evolutionary analyses were conducted in MEGA5.

Strains	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**
1. EY1																									
2. EY-125	2.0																								
3. EY-626	1.0	1.0																							
4. EY-627	1.0	1.0	0.0																						
5. JWB-Ky	7.0	7.0	6.0	6.0																					
6. JWB	5.0	5.0	4.0	4.0	2.0																				
7. JWB-G1	5.0	5.0	4.0	4.0	2.0	0.0																			
8. JWB-Kor1	8.0	8.0	7.0	7.0	3.0	3.0	3.0																		
9. CLY-5	6.0	6.0	5.0	5.0	3.0	1.0	1.0	4.0																	
10. PY-In	6.0	6.0	5.0	5.0	3.0	1.0	1.0	4.0	2.0																
11. Hon1	6.0	6.0	5.0	5.0	3.0	1.0	1.0	4.0	2.0	2.0															
12. CVWB_29	5.0	5.0	4.0	4.0	2.0	**0.0**	**0.0**	3.0	**1.0**	**1.0**	**1.0**														
13. VC	3.0	3.0	2.0	2.0	7.0	5.0	5.0	8.0	6.0	6.0	6.0	5.0													
14. FD-C	5.0	5.0	4.0	4.0	9.0	7.0	7.0	10.0	8.0	8.0	8.0	7.0	2.0												
15. HD1	5.0	5.0	4.0	4.0	9.0	7.0	7.0	10.0	8.0	8.0	8.0	7.0	2.0	4.0											
16. FD70	3.0	3.0	2.0	2.0	7.0	5.0	5.0	8.0	6.0	6.0	6.0	5.0	0.0	2.0	2.0										
17. FD-C	3.0	3.0	2.0	2.0	7.0	5.0	5.0	8.0	6.0	6.0	6.0	5.0	0.0	2.0	2.0	0.0									
18. SPCol	3.0	3.0	2.0	2.0	7.0	5.0	5.0	8.0	6.0	6.0	6.0	5.0	0.0	2.0	20	0.0	0.0								
19. ALY882	3.0	3.0	2.0	2.0	7.0	5.0	5.0	8.0	6.0	6.0	6.0	5.0	0.0	2.0	2.0	0.0	0.0	0.0							
20. FD-D	6.0	6.0	5.0	5.0	10.0	8.0	8.0	11.0	9.0	9.0	9.0	8.0	3.0	5.0	5.0	3.0	3.0	3.0	3.0						
21. FD1487	3.0	3.0	2.0	2.0	7.0	5.0	5.0	8.0	6.0	6.0	6.0	5.0	0.0	2.0	2.0	0.0	0.0	0.0	0.0	3.0					
22. RuS	7.0	7.0	6.0	6.0	11.0	9.0	9.0	12.0	10.0	10.0	10.0	9.0	4.0	6.0	6.0	4.0	4.0	4.0	4.0	7.0	4.0				
23. Rus400	5.0	5.0	4.0	4.0	9.0	7.0	7.0	10.0	8.0	8.0	8.0	7.0	2.0	4.0	4.0	2.0	2.0	2.0	2.0	5.0	2.0	4.0			
24. RuSR19	7.0	7.0	6.0	6.0	11.0	9.0	9.0	12.0	10.0	10.0	10.0	9.0	4.0	6.0	6.0	4.0	4.0	4.0	4.0	7.0	4.0	6.0	2.0		
25. Rus971	7.0	7.0	6.0	6.0	11.0	9.0	9.0	12.0	10.0	10.0	10.0	9.0	4.0	6.0	6.0	4.0	4.0	4.0	4.0	7.0	4.0	6.0	2.0	4.0	
26. BltWB	15.0	15.0	14.0	14.0	17.0	15.0	15.0	18.0	16.0	14.0	16.0	15.0	14.0	16.0	16.0	14.0	14.0	14.0	14.0	17.0	14.0	16.0	12.0	14.0	14.0

[a]Strain acronyms are as indicated in Table 2.9.

2.4.2.4 Clover proliferation-related strains, 16SrVI

Nested PCR results with universal phytoplasma primers R16F2n/R16R2 and B5/P7 provided positive results from some of the cassava plants affected with CWB in Vietnam and Thailand. RFLP analyses on R16F2n/R16R2 and B5/P7 amplicons with *Tru*I allow to identify the

possible presence of phytoplasmas belonging to 16SrV, -VI or -VII groups in the samples CThaiWB_1, CThaiWB_3, CThaiWB_9, CThaiWB_13 and CThaiWB_22 in Thailand and CVWB_5V, CVWB_33, CVWB_39, CVWB_43, CVWB_46 in Vietnam (Figure 2.20a). Further analyses were carried out to achieve better identification and to verify the possible presence of mixed infection. RFLP analyses with *Hha*I excluded the presence of 16SrVII group phytoplasmas; while *Alu*I suggested the presence of groups 16SrV or 16SrVI phytoplasmas. A nested PCR with 16SrV group specific primers provided negative results indicating the possible presence of 16SrVI phytoplasmas. Additionally, RFLP was carried out using M1/M2 amplicons with the restriction enzymes *Tru*I and *Tsp509*I, on the different strains described above and allowed different profiles to be distinguished; one of them was similar to the one of the reference strains clover proliferation (CP) and vinca virescence (VR) which belongs to ribosomal group 16SrVI, while the sample CThaiWB_1 was not identical to any of the employed reference strain. Also with this primers combination, it was possible to identify mixed phytoplasma profiles with the one of 16SrI group in the samples CThaiWB_3, CThaiWB_13 and CVWB_39 and with the one of 16SrXV group in CThaiWB_3 and CThaiWB_13 (Figure 2.20a).

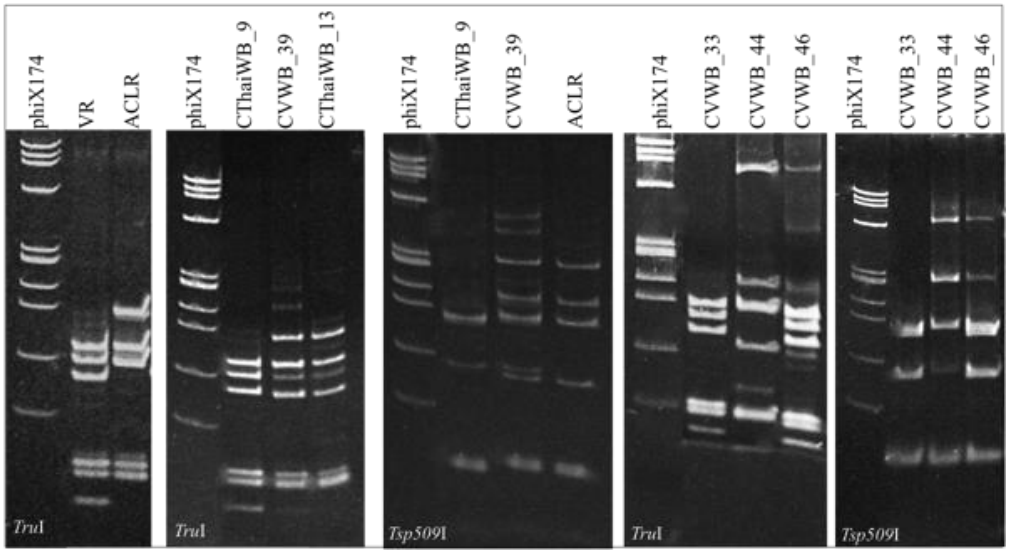


Figure 2.20a. Polyacrylamide gel 6.7% showing the *Tru1*I and *Tsp509*I RFLP patterns of 16Sr DNA gene obtained with M1/M2 primer pair in nested PCR from cassava samples. CThaiWB_9 and CVWB_33 showed single infection with a specific clover proliferation profile (16SrVI). Mixed infection of aster yellows (16SrI) with 16SrVI in samples CVWB_39 and CThaiWB_13 is shown with both enzymes compared to the reference controls. In sample CVWB_46 mixed phytoplasma/*Bacillus* sp profile is visible. CVWB_44, show presence of only *Bacillus* sp profile. ACLR (A-AY) (16SrI-F), aster yellows apricot and VR (16SrVI-A), vinca virescence; phiX174, marker ΦX174 *Hae*III digested, fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

Table 2.11. Clover proliferation related reference phytoplasma strains employed for 16S rDNA classification.

Phytoplasma associated disease (acronym)	Geographic origin	GenBank accession numbers, 16S rDNA	16SrV subgroup
'*Ca.* P. trifolii' (CP)	Canada	AY390261	A
Clover proliferation (CP)a	USA	L33761	A
Potato witches' broom (PWB)	Canada	AY500818	A
Potato purple top (CN-151)	China	HQ609490	A
Lucerne virescence (LUM)	France	EF186821	A
Dry bean phyllody (DBPh2)	USA	AY496002	A
Potato purple top (AKpot1)	USA	GU004366	A
Potato purple top (AKpot2)	USA	GU004367	A
Potato purple top (AKpot4)	USA	GU004368	A
Potato purple top (AKpot5)	USA	GU004369	A
Vinca virescence (VR)	USA	AY500817	A
Strawberry multiplier disease (MC)	Canada	AF190224	B
Illinois elm yellows (EY-IL1)	USA	AF409070	C
Periwinkle little leaf (PLL-Bd)	Bangladesh	AF228053	D
Brinjal little leaf (BLL)	India	EF186820	D
Centarurea solstitialis virescence (CSVI)	Italy	AY270156	E
Catharanthus phyllody phytoplasma (CPS)	Sudan	EF186819	F
Datura inoxia (DatLL-In)	India	EU573925	H
Portulaca little leaf phytoplasma (PLL-Ind)	India	EF651786	H
'*Ca*. P. sudamericanum' (PassWB-Br3)	Brazil	GU292081	I
Tomato big bud (ZYT-3)	China	JQ807736	-

Since some of the differential SNPs positions in the cassava samples were identified out site of R16F2n/R16R2 amplicon, estimation of evolutionary divergence was carried out based on position 87 to 1,481 (1,394 bp) on the 16Sr DNA gene instead then on positions 150 to 1,395 (1,245 bp) that are those conventionally employed.

Cassava strains were closely to reference strain CPS that belongs to subgroup 16SrVI-F (Wei *et al*., 2007a) from Sudan (Table 2.11) and it can be distinguished from the others members of clover proliferation subgroups in the R16F2n/R16R2 amplicon for the presence of an *Rsa*I restriction site in the position 984 and a *Hae*III restriction site at position 1,262. The four cassava samples analyzed (CThaiWB_1, CThaiWB_9, CThaiWB_22, CVWB_39) share the *Hae*III restriction site but not the *Rsa*I restriction site; key enzymes reported to differentiate some subgroups in the clover proliferation phytoplasma group (Wei *et al*., 2007a; Hiruki and Wang, 2004). Additionally, a SNP site at position 1,423 out of R16F2n/R16R2 amplicon also distinguish CPS and cassava samples to each other with the restriction enzyme *HpyCH4*III; moreover in position 1,469 a single SNPs was also identified in cassava samples and not in the CPS strain and vice versa in the position 1,397.

Table 2.12. Estimation of evolutionary divergence between 16S rDNA sequences obtained from three different phytoplasma strains associated with CWB from Vietnam and Thailand. The number of base differences per sequence between sequences is shown. The analysis involved three cassava samples and eigthteen 16SrVI reference nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1,396 positions in the final dataset. The evolutionary analyses were conducted in MEGA5.

Strains	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
1. CThaiWB_22																				
2. CVWB_39	3.0																			
3. CThaiWB_9	2.0	1.0																		
4. CPS	7.0	6.0	5.0																	
5. ZYT-3	6.0	5.0	4.0	90																
6. VR	8.0	7.0	6,0	9.0	6.0															
7. AKpot1	13.0	12.0	11.0	14.0	11.0	7.0														
8. AKpot2	12.0	11.0	10.0	13.0	10.0	6.0	3.0													
9. AKpot4	11.0	10.0	9.0	12.0	9.0	5.0	2.0	1.0												
10. AKpot5	13.0	12.0	11.0	14.0	11.0	7.0	4.0	3.0	2.0											
11. BLL	15.0	14.0	13.0	16.0	13.0	9.0	6.0	5.0	4.0	6.0										
12. DBPh2	9.0	8.0	7.0	10.0	7.0	3.0	8.0	7.0	6.0	8.0	10.0									
13. LUM	10.0	9.0	8.0	11.0	8.0	6.0	3.0	2.0	1.0	3.0	5.0	7.0								
14. CN-151	13.0	14.0	13.0	16.0	13.0	9.0	6.0	5.0	4.0	6.0	8.0	10.0	5.0							
15. PWB	14.0	13.0	12.0	15.0	12.0	8.0	1.0	4.0	3.0	5.0	7.0	9.0	4,0	7.0						
16. CP	11.0	10.0	9.0	12.0	9.0	5.0	2.0	1.0	0.0	2.0	4.0	6.0	1.0	4.0	3.0					
17. MC	16.0	15.0	14.0	17.0	14.0	10.0	7.0	6.0	5.0	7.0	9.0	11.0	6.0	9.0	8.0	5.0				
18. EY-IL1	15.0	14.0	13.0	16.0	13.0	9.0	6.0	5.0	4.0	6.0	6.0	10.0	5.0	8.0	7.0	4.0	9.0			
19. PLL-Bd	16.0	15.0	14.0	17.0	14.0	10.0	7.0	6.0	5.0	7.0	1.0	11.0	6.0	9.0	8.0	5.0	10.0	7.0		
20. CSVI	21.0	20.0	19.0	22.0	19.0	15.0	12.0	11.0	10.0	12.0	6.0	16.0	11.0	14.0	13.0	10.0	15.0	12.0	7.0	
21. PassWB-Br3	40.0	37.0	38.0	41.0	40.0	38.0	39.0	40.0	39.0	41.0	39.0	39.0	38.0	43.0	38.0	39.0	44.0	41.0	40.0	45.0

[a]Strain acronyms are indicated in Table 2.11.

A further SNP (position 1,233) that represents a specific restriction site with the enzyme *Hpy166*II, in the strain CThaiWB_1 was identified and allows the specific differentiation of the cassava strains directly in the R16F2n/R16R2 amplicon. With this strain all reference sequences showed 99% homology and no differential *Mse*I restriction site was identifed after *in silico* analyses instead of actual RFLP on M1/M2 amplicon (Figure 2.20b). The comparison between real and virtual RFLP analyses showed differences between real and virtual RFLP profiles confirming the possible presence of interoperon heterogeneity and/or of mixed phytoplasma population this sample.

Specific differential SNPs positions were also identified between the cassava strains obtained from Thailand and Vietnam. In particular the strain CThaiWB_22 have a differential nucleotide at position 630 and another at position 643, the second one shared with the strain CN-151 (HQ609490) from potato in China; meanwhile the cassava strain CVWB_39 has one SNP in position 641 that is shared with the strain PassWB-Br3 (GU292081) from passion fruit in Brazil.

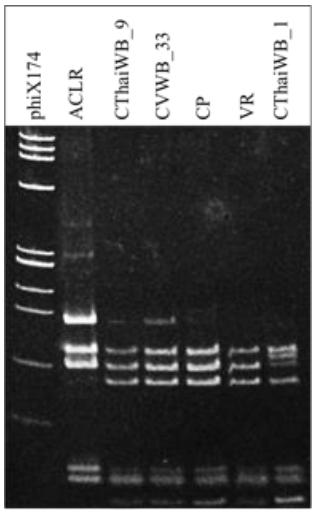


Figure 2.20b. Polyacrylamide gel 6.7% showing *Tru1*I RFLP patterns of 16Sr DNA gene obtained with M1/M2 primer pair in nested PCR from cassava showing witches' broom symptoms in which strains associated with clover proliferation group were detected in Thailand CThaiWB_1, CThaiWB_9 and in Vietnam CVWB_33. Differential profile obtained in sample CThaiWB_1 belonging to clover proliferation group. ACLR [A-AY] (16SrI-F), aster yellows from apricot; CP (16SrVI-A), clover proliferation and VR (16SrVI-A), vinca virescence; phiX174, marker ΦX174 *Hae*III digested, fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

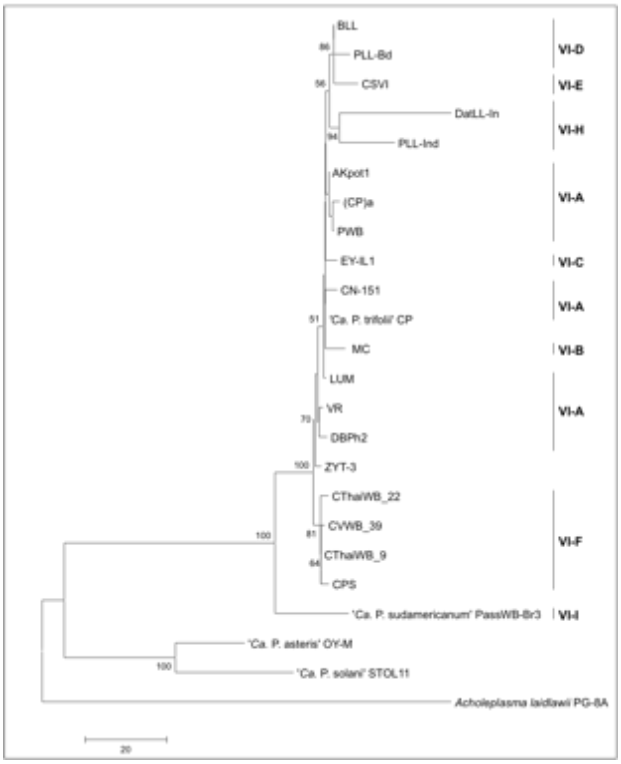


Figure 2.20. Phylogenetic trees constructed by maximum likelihood analysis of 16S rDNA gene sequences with three selected CWB strain from Vietnam and Thailand associated with clover proliferation ribosomal group. Strains references employed of 16SrVI subgroups are described in Table 2.11. '*Ca*. P. asteris' strain OY-M (NC_005303); '*Ca.* P. solani' strain STOL11 (AF248959) and *A. laidlawii* PG-8A (NC_010163) were used as outgroups. Numbers on the branches are bootstrap values obtained for 1,000 replicates (only values above 50% are shown). Letters in bars, represent diverse RFLP subgroups in the 16S rDNA group of clover proliferation-related strains.

2.4.2.5 Apple proliferation-related strains, 16SrX

Nested PCR amplification with R16(X)F1/R16(X)R1 and M1/M2 primers followed by RFLP analyses with *Rsa*I, *Ssp*I, *Bfa*I and *Mbo*II allow to confirm that in three samples from Costa Rica (CCFSD_6-1, CCFSD_16-6 and CCFSD_23-2), and in one from Paraguay [CPFSD_B2(2)] and Vietnam (CVWB_6-3) phytoplasmas related to apple proliferation (AP, 16SrX-A) and European stone fruit yellows (ESFY, 16SrX-B) were present. In particular, 16SrX-B was detected in the sample CCFSD_16-6, while in the others 16SrX-A was identified. Since as was mentioned above, in the cassava samples CCFSD_6-1 and 16-6 were also detected 16SrI phytoplasma and this was shown also with RFLP on M1/M2 amplicons

using the restriction enzyme *Mbo*II, only in Costa Rica samples a mixed infection was present. According with amplicon employed in these analyses RFLP showed different profiles in several of the examined samples; overall restriction profiles indicate the possible presence of interoperon heterogeneity and/or of mixed phytoplasma population in some of the samples (Figure 2.21a).

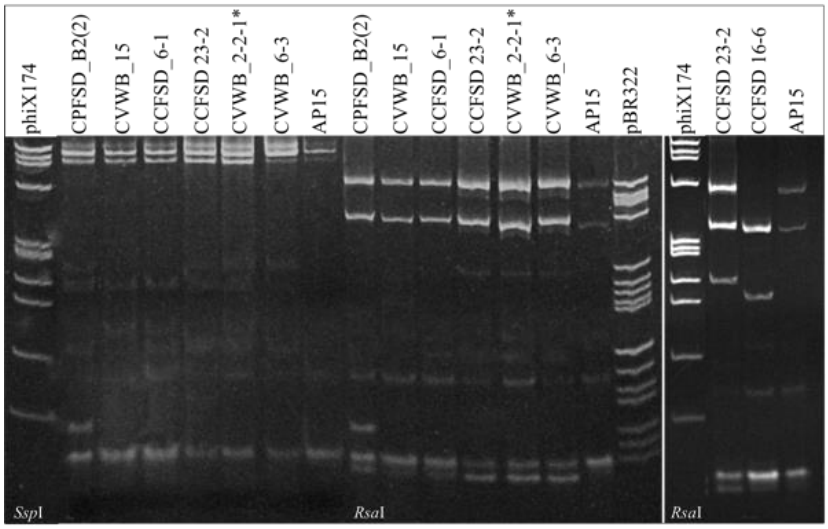


Figure 2.21a. RFLP analyses of 16S rDNA amplicons obtained in nested PCR with R16(X)F1/R16(X)R1 primers using the endonucleases *Ssp*I and *Rsa*I. Different 16SrX patterns obtained from CFSD-associated phytoplasmas from Costa Rica CCFSD_6-1, CCFSD_23-2 and CCFSD_16-6 belonging to subgroups 16SrX-A and –B, respectively. 16SrX-A subgroup identified in samples from Paraguay [CPFSD_B2(2)] and Vietnam (CVWB_15, CVWB_2-2-1* andCVWB_6-3). Reference strain AP15 (16SrX-A), '*Ca.* P. mali'; phiX174, marker ΦX174 *Hae*III digested, fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

Table 2.13. Apple proliferation related reference phytoplasma strains employed for 16S rDNA classification.

Phytoplasma associated disease (acronym)	**Geographic origin**	**GenBank accession numbers, 16S rDNA**	**16SrX subgroup**
'Candidatus Phytoplasma mali' (AP15)	Italy	AJ542541	A
'*Ca.* P. mali' (Przel)	Poland	HG423141	A
'*Ca.* P. mali' (Oz)	Poland	HG423140_	A
'*Ca*. P. prunorum' (ESFY-G1)	Germany	AJ542544	B
'*Ca*. P. prunorum' (P167)	Iran	KJ001833	B
Iranian pear (SP1S)	Iran	KC902808	B
'*Ca*. P. prunorum' (T164-F2n)	Iran	KF739404	B
'*Ca*. P. pyri' (PD1)	Germany	AJ542543	C
'*Ca*. P. spartii'	Italy	X92869	D
Black alder (BAWB[BWB])	Germany	X76431	E

Among the sequences of phytoplasma strains obtained from Costa Rica, Paraguay and Vietnam after GenBank comparison using the BLAST® tool, the cassava strain CCFSD_16-6 showed 100% (1000/1000 bp) of homology with the strains P167 (KJ001833) and SP1S (KC902808) reported in plum and pear respectively from Iran and related to '*Ca.* P. prunorum' (ESFY, 16SrX-B); one SNP between this sequence and ESFY reference strain (AJ542544) was found in the position 628. While the strain CCFSD_23-2 showed 99% (980/982 bp) with the strain "Przel" (HG423141) from Poland of *Malus domestica* belonging to '*Ca*. P. mali' (AP, 16SrX-A). Additionally, two SNPs were found, one in position 1,095 that is also present in the reference strain BAWB (BWB) belonging to 16SrX-E subgroup and an additional one in position 795 only present in the CCFSD23-2 (Table 2.14a and 2.15). Furthermore, the strain CPFSD_B2(2) from Paraguay of which the M1/M2 amplicon was sequenced showed also 99% (496/500) with '*Ca.* P. mali', but three specific SNPs differentiate this strain from all representative phytoplasmas belonging to apple proliferation already reported. The specific SNPs were identified in positions 807(specific restriction site for *Sau96*I), 919 (that eliminate an *Mbo*I restriction site), and 933 (that eliminate a *Tsp509*I restriction site) (Table 2.14 and 2.15). The strain obtained from Vietnam CVWB_6-3 was 99% (1050/1053) homologous with '*Ca.* P. mali', but three SNPs were present in positions 426, 403 and 569. In particular the SNP at 403 bp eliminates the *Ssp*I restriction site and the one at 569 bp is a specific restriction site for *Fnu4HI*; position 403 is the restriction for *Ssp*I that distinguish it from strains belonging to 16SrX-A (Table 2.14a and 2.15).

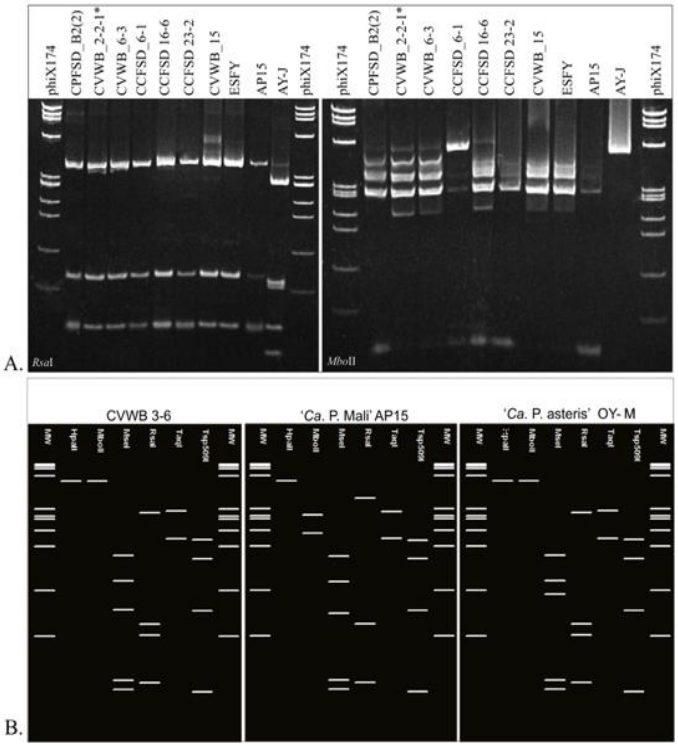


Figure 2.21b. Polyacrylamide gel 6.7% showing the *Rsa*I and *Mbo*II RFLP patterns of 16Sr DNA gene obtained with M1/M2 assay in nested PCR from cassava samples. **A.** CPFSD_B2(2), CVWB_2-2-1*, CVWB_6-3, CCFSD_16-6, CCFSD_23-2 and CVWB_15 showed single infection with specific apple proliferation group phytoplasmas (16SrX) using both enzymes. Mixed infection of aster yellows (16SrI) and 16SrX in samples CCFSD_6-1 is evident with both enzymes compared to the reference controls. **B.** *In silico* RFLP showed aster yellows strain obtained from Vietnam that is undistinguishable using only the endomuclease *Tru*I. ESFY (16SrX-B), '*Ca.* P. prunorum'; AP15 (16SrX-A), '*Ca.* P. mali' (HG423140); AY-J (16SrI-B), aster yellows and OY-M (16SrI-B) '*Ca.* P. asteris' strain (NC_005303); phiX174, marker ΦX174 *Hae*III digested, fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

Table 2.14. Estimation of evolutionary divergence between 16S rDNA sequences obtained from four different phytoplasma strains associated with CFSD and CWB from Costa Rica, Paraguay and Vietnam. The number of base differences per sequence between sequences is shown. The analysis involved also ten 16SrX reference nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 484 positions in the final dataset. The evolutionary analyses were conducted in MEGA5.

Strains	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
1. CCFSD_16-6													
2. CCFSD_23-2	3.0												
3. CPFSD_B2(2)	4.0	5.0											
4. CVWB_6-3	1.0	2.0	3.0										
5. AP15	1.0	2.0	3.0	0.0									
6. Przel	1.0	2.0	30	0.0	0.0								
7. Oz	1.0	2.0	3.0	0.0	0.0	0.0							
8. ESFY-G1	0.0	3.0	4.0	1.0	1.0	1.0	1.0						
9. P167	0.0	3.0	4.0	1.0	1.0	1.0	1.0	0.0					
10. SP1S	0.0	3.0	4.0	1.0	1.0	1.0	1.0	0.0	0.0				
11. T164-F2n	0.0	3.0	4.0	1.0	1.0	1.0	1.0	0.0	0.0	0.0			
12. PD1	2.0	3.0	4.0	1.0	1.0	1.0	1.0	2.0	2.0	2.0	2.0		
13. '*Ca.* P. spartii'	11.0	12.0	13.0	10.0	10.0	10.0	10.0	11.0	11.0	11.0	11.0	11.0	
14. BAWB (BWB)	11.0	12.0	15.0	12.0	12.0	12.0	12.0	11.0	11.0	11.0	11.0	13.0	22.0

[a]Strain acronyms are indicated in Table 2.13.

RFLP using the amplicon obtained with primers M1/M2 in nested PCR assays and with the restriction enzyme *Rsa*I and *Mbo*II, showed the presence of mixed infection with aster yellows group in the sample CCFSD_6-1. In particular, using the restriction enzyme *Tru*I, one aster yellows profile was undistinguishable for 16SrX profiles (i.e CVWB_3-3, Figure 2.12b).

Table 2.14a. Estimation of evolutionary divergence between 16S rDNA sequences obtained only from the strains associated with CFSD and CWB from Costa Rica and Vietnam. There were a total of 967 positions in the final dataset.

Strains	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1. CCFSD_16-6												
2. CCFSD_23-2	10.0											
3. CVWB_6-3	11.0	5.0										
4. AP15	8.0	2.0	3.0									
5. Przel	8.0	2.0	3.0	0.0								
6. Oz	8.0	2.0	3.0	0.0	0.0							
7. ESFY-G1	1.0	11.0	12.0	9.0	9.0	9.0						
8. P167	0.0	10.0	11.0	8.0	8.0	8.0	1.0					
9. SP1S	0.0	10.0	11.0	8.0	8.0	8.0	1.0	0.0				
10. T164-F2n	0.0	10.0	11.0	8.0	8.0	8.0	1.0	0.0	0.0			
11. PD1	8.0	10.0	11.0	8.0	8.0	8.0	9.0	8.0	8.0	8.0		
12. 'Ca. P. spartii'	25.0	26.0	27.0	24.0	24.0	24.0	26.0	25.0	25.0	25.0	23.0	
13. BAWB (BWB)	28.0	34.0	37.0	34.0	34.0	34.0	27.0	28.0	28.0	28.0	31.0	41.0

[a]Strain acronyms are indicated in Table 2.13.

Table 2.15. Differential SNP positions in 16Sr DNA sequences (position about 210 to 1,230 bp), of ten representative strains for all apple proliferation ribosomal subgroups 16SrX, from Italy (AP15 and '*Ca.* P. spartii'), Poland (Przel and Oz), Germany (ESFY-G1 and PD1) and Iran (P167, SP1S and T164-F2n) compared with cassava phytoplasma strains detected in Costa Rica (CCFSD), Paraguay (CPFSD) and Vietnam (CVWB) samples.

	Single nucleotide positions in 16S ribosomal RNA gene (position about 210 to 1230)																		
Strains[a]	**250**	**308**	**402**	**403***	**413***	**426**	**560**	**569***	**571**	**617**	**628**	**795**	**807**	**919***	**933***	**1088**	**1095**	**1221**	**1231**
AP15	C	T	A	T	T	T	T	T	T	G	G	A	A	T	T	G	A	A	G
CCFSD_16-6	T	C	G	.	C	.	C	.	A	A	.	.	.	.	.	A	.	-	-
CCFSD_23-2	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	G	.	-
CPFSD_B2(2)	-	-	-	-	-	-	-	-	-	-	.	.	T	C	A	.	.	G	-
CVWB_6-3	.	.	.	C	.	C	.	C	.	.	.	.	.	.	.	.	.	.	.
Przel	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Oz	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ESFY-G1	T	C	G	.	C	.	C	.	A	A	A	.	.	.	.	A	.	.	A
P167	T	C	G	.	C	.	C	.	A	A	.	.	.	.	.	A	.	.	A
SP1S	T	C	G	.	C	.	C	.	A	A	.	.	.	.	.	A	.	.	A
T164-F2n	T	C	G	.	C	.	C	.	A	A	.	.	.	.	.	A	.	.	A
PD1	.	C	G	.	.	.	C	.	.	A	.	.	.	.	.	.	.	.	.
'*Ca.* P. spartii'	A	C	G	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	A
BAWB (BWB)	T	C	G	.	C	.	C	.	.	A	.	.	.	.	.	A	G	.	.

[a]Strains Przel, Oz, P167, SP1S and T164-F2n sequences showed high significant score using BLAST® tool. Strain AP15 (AJ542541) was used to calculated the SNPs positions. *SNPs making differential restriction sites RFLP for potential new 16SrX strain differentiation: 403 (*Ssp*I); 413 (*Rsa*I); 569 (*Fnu4H*I); 919 (*Sau96*I or *Mbo*I); 933 (*Tsp509*I). Dots represent nucleotides identical to the '*Ca.* P. mali' AP15 consensus sequence and dashes are gaps positions.

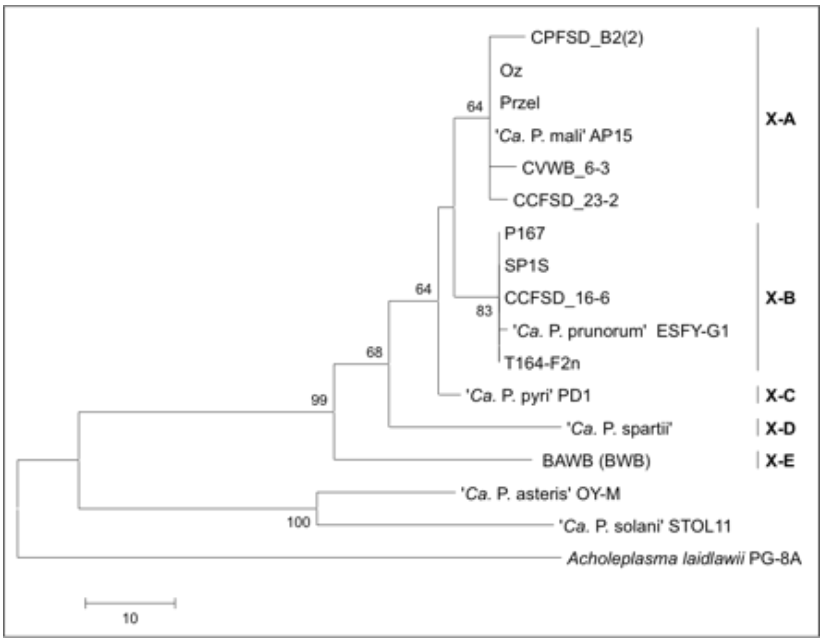


Figure 2.23. Phylogenetic trees constructed using the Maximum Parsimony method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (only values above 60% are shown). The tree is drawn to scale; with

branch lengths calculated using the average pathway method and are in the units of the number of changes over the whole sequence. All ambiguous positions were removed for each sequence pair. There were a total of 1,061 positions in the final dataset. The analysis involved 17 nucleotide sequences including four-cassava strains from Costa Rica, Paraguay, Vietnam and ten reference strain belonging to apple proliferation group. Strains references employed of 16SrX subgroups are described in Table 2.13. '*Ca*. P. asteris' strain OY-M (NC_005303); '*Ca.* P. solani' strain STOL11 (AF248959) and *A. laidlawii* PG-8A (NC_010163) were used as outgroups. Letters in bars, represent diverse RFLP subgroups in the 16SrX group.

2.4.2.6 "Stolbur" related-strains, 16SrXII

After direct amplification of 16Sr DNA gene with P1/P7 and/or R16mF1/mR1 primer pair and nested PCR reactions with R16(I)F1/R16(I)R1 primers expected length fragments for phytoplasmas (1,100 bp) were obtained from cassava symptomatic samples tested; samples CCFSD_1-2, CCFSD_19-4, CCFSD_21-4 and CCFSD_22-2 from Costa Rica showing frog skin disease and CVWB_24 from Vietnam resulted positive. All the cassava samples positive in nested PCR showed restriction profiles with *Tru*I and *Taq*I restriction enzymes that were identical (Figure 2.24a) and referable to the profile of "stolbur" phytoplasma, strain STOL, belonging to 16SrXII-A ribosomal group.

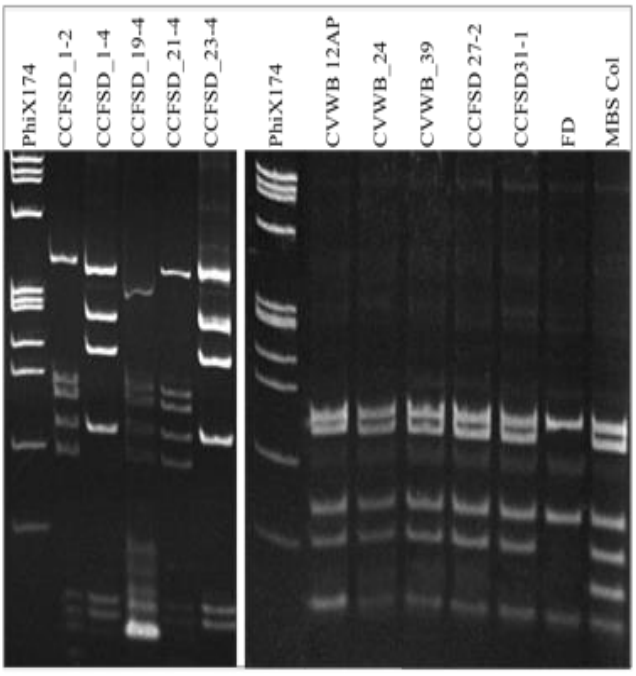


Figure 2.24a. Polyacrylamide gel 6.7% showing the *Tru1*I RFLP patterns of 16Sr DNA and *tuf* gene obtained with R16(I)F1/R1 (left) and Tuf400/Tuf835 (rigth) assays, respectively. *Left*, different profiles obtained of stolbur (16SrXII) and aster yellows (16SrI) in samples from Costa Rica associated with cassava frog skin disease: CCFSD_1-2 (16SrXII), CCFSD_1-4 (16SrI), CCFSD_19-4 (16SrXII), CCFSD_21-4 (16SrXII) and CCFSD_23-4 (16SrI). *Right*, unique and specific profile obtained to stolbur in differnt cassava samples associated with cassava witches'-broom and frog skin disease in Vietnam and Costa Rica, respectively. Elm yellows and aster yellows reference strains used in RFLP analyses: FD (16SrV), Flavescence dorèe and MBS Col (16SrI-B), *Maiz bushy stunt* ; phiX174, marker ΦX174 *Hae*III digested, fragment

Table 2.16. "Stolbur" related reference phytoplasma strains employed for 16S rDNA classification.

Phytoplasma associated disease (acronym)	Geographic origin	GenBank accession numbers, 16 rDNA	16Sr subgroup
'*Ca*. P. solani' (STOL11)		AF248959	A
Phytoplasma sp. (Insect [BA])	Italy	JQ868436	A
"Bois noir" (BN-FC13)	Italy	EU836649	A
Ca. P. solani' (284/09)	Serbia	JQ730740	A
Solanum phureja' yellows (SPCol)	Colombia	Unpublished	A
'*Ca*. P. australiense' (AUSGY)	Australia	L76865	B
Strawberry lethal yellows (StrawLY)	Australia	AJ243045	C
'*Ca*. P. japonicum' (JHp)	Japan	AB010425	D
'*Ca*. P. fragariae' (StrawY)	Lithuania	DQ086423	E
"Bois noir" (BN-Op121)	Italy	EU836651	F
"Bois noir" (BN-Op125)	Italy	EU836646	G
'*Ca*. P. convolvuli' (BY-S57/11)	Serbia	JN833705	H
"Bois noir" (400_05)	France	EU010007	H
"Bois noir" (409_05)	France	EU010008	I
"Bois noir" (92_04)	Italy	EU014777	J
Corn-reddening (2005/2)	Serbia	DQ222972	K
Cocos nucifera' stunt (PorrocaC5)	Panama	EU131021	L
Rhododendron' stunt (RHOD-CZ)	Czech Republic	DQ160244	M
Gravevine yellows (2642BN)	Spain	AJ964960	N
Grapevine yellows (VK)	Italy	X76428	O
"Boir noir" (06PS085)	Canada	EU086529	P
Grapevine decline (GrIRAN08)	Iran	GQ403235	Q

SNPs, *in silico* and Blast analyses showed four specific different positions between the strains CCFSD_1-2 and CCFSD_21-4 and respect to all reference strains of 16SrXII subgroups. In particular both strains can be distinguished between them using the restriction enzymes *BstU*I, *Aci*I and *Alu*I at position 671; with the enzymes *BstU*I and *Aci*I to CCFSD_1-2 and at position 334; with the enzyme *Alu*I to the strain CCFSD_21-4, each one have a unique restriction profile respected to the "stolbur" subgroups reported. Additional specifics SNPs sites were also identified in CCFSD_21-4 at positions 531 and 654. The virtual RFLP pattern derived from the query 16S rDNA R16F2n/R16R2 and R16F1(I)/R16(I)R1 fragment is different from the reference patterns of all previously established 16Sr groups/subgroups. The most similar is the reference pattern of the 16SrXII-A strain STOL11 (AF248959), with a similarity coefficient of 0.97 to CCFSD_1-2 and 0.95 to CCFSD_21-4, which is less than or equal to 0.97. Those strains may represent new subgroups within the 16Sr group XII (Table 2.17, Figure 2.24b) and are distributed in a new geographic area and a new host plant species. On

the other hand, using also the endoglucanase *Aci*I the strain SPCol (sample obtained from *Solanum phureja*, diploid cultivate herbaceous and tuberous species of potato from Colombia) showed a unique profile with this enzyme, that was distinguishable to the other strains and subgroups already reported. This confirms the potentially of this molecular tool to be used for "stolbur" differentiation in tropical roots and tuber species such as *M. esculenta* and *S. phureja.*

Using the Blast® comparison the strains CCFSD_1-2 and CCFSD_21-4 showed 99% similarity (1057/1058 and 988/991, respectively) with strains from *Prunus persica* 119T-PLR (KF739408) and *Sophora alopecuroides* S135 (KF923878) from Iran and also resulted to have four SNPs difference with all reference strains in reported subgroups. Phylogenetic comparison of the 16S rRNA gene of those strains with 14 representative strains of the "stolbur" subgroup confirmed that the phytoplasmas detected in cassava are very closely related to the "stolbur" phytoplasmas in the 16SrXII-A subgroup (Figure. 2.25) and to other members of the 16SrXII group as defined previously (Lee et al., 1998a; 1998b).

Tuf gene. In nested PCR assays with Tuf400/Tuf835 cocktail primer, expected length amplicons (about 500 bp) were obtained from four additional symptomatic samples (CCFSD_27-2, CVWB_12AP, CVWB_39, and CCFSD_31-1) that confirmed results obtained in 16Sr DNA detection (CVWB_24 and CCFSD_21-4). Positive samples subjected to RFLP analyses with *Tru*I, and *Tsp509*I restriction enzymes showed all restriction profiles of cassava samples and of control strains used as belonging to "stolbur" group and were identical to each other; the only different profiles were those from control strains FD (16SrV) and MBSCol (16SrI) (Figure 2.24a), as reported by Makarova *et al*., 2012. In particular in the samples CCFSD_27-2 and CVWB_12 "stolbur" phytoplasmas were detected in mixed infection with X-disease in the Costa Rica sample and with aster yellows groups in the Vietnam sample, respectively. These molecular tools could be useful to detect single and mixed infection with "stolbur" but not for strains differentiation and characterization.

Table 2.17. Estimation of evolutionary divergence between 16S rDNA sequences obtained from two phytoplasma strains associated with CFSD from Costa Rica. The number of base differences per sequence between sequences is shown. The analysis involved also 22 16SrXII reference nucleotide sequences. All ambiguous positions were removed for each sequence pair. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were not allowed. There were a total of 996 positions (about 212 to 1,270) in the final dataset.

Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1. STOL11																							
2. CCFSD_1-2	5.0																						
3. CCFSD_21-4	7.0	4.0																					
4. SPCol	7.0	4.0	6.0																				
5. 284/09	4.0	1.0	3.0	3.0																			
6. Insect (BA)	4.0	1.0	3.0	3.0	0.0																		
7. BN-FC13	4.0	1.0	3.0	3.0	0.0	0.0																	
8. AUSGY	21.0	18.0	20.0	16.0	17.0	17.0	17.0																
9. StrawLY	19.0	16.0	18.0	14.0	15.0	15.0	15.0	2.0															
10. JHp	34.0	32.0	34.0	30.0	31.0	31.0	31.0	32.0	30.0														
11. StrawY	24.0	21.0	23.0	18.0	20.0	20.0	20.0	21.0	19.0	23.0													
12. BN-Op121	5.0	2.0	4.0	4.0	1.0	1.0	1.0	18.0	16.0	32.0	21.0												
13. BN-Op125	5.0	2.0	4.0	3.0	1.0	1.0	1.0	18.0	16.0	32.0	21.0	2.0											
14. BY-S57/11	20.0	17.0	19.0	17.0	16.0	16.0	16.0	18.0	16.0	32.0	21.0	17.0	17.0										
15. 400_05	7.0	4.0	6.0	6.0	3.0	3.0	3.0	20.0	18.0	34.0	23.0	4.0	4.0	19.0									
16. 409_05	6.0	3.0	5.0	5.0	2.0	2.0	2.0	19.0	17.0	33.0	22.0	3.0	3.0	18.0	5.0								
17. 92_04	6.0	3.0	5.0	5.0	2.0	2.0	2.0	19.0	17.0	33.0	20.0	3.0	3.0	18.0	5.0	4.0							
18. 2005/2	7.0	4.0	6.0	6.0	3.0	3.0	3.0	20.0	18.0	34.0	23.0	4.0	4.0	19.0	6.0	5.0	5.0						
19. PorrocaC5	8.0	5.0	7.0	7.0	4.0	4.0	4.0	21.0	19.0	35.0	24.0	5.0	5.0	20.0	7.0	6.0	6.0	7.0					
20. RHOD-CZ	8.0	5.0	7.0	7.0	4.0	4.0	4.0	21.0	19.0	34.0	23.0	5.0	5.0	20.0	7.0	6.0	6.0	7.0	8.0				
21. 2642BN	5.0	2.0	4.0	4.0	1.0	1.0	1.0	18.0	16.0	32.0	19.0	2.0	2.0	17.0	4.0	3.0	1.0	4.0	5.0	5.0			
22. 06PS085	5.0	2.0	4.0	4.0	1.0	1.0	1.0	18.0	16.0	32.0	19.0	2.0	2.0	17.0	4.0	3.0	1.0	4.0	5.0	5.0	0.0		
23. VK	5.0	2.0	4.0	4.0	1.0	1.0	1.0	18.0	16.0	32.0	21.0	2.0	2.0	17.0	4.0	3.0	3.0	4.0	5.0	5.0	2.0	2.0	
24. GrIRAN08	7.0	4.0	6.0	6.0	3.0	3.0	3.0	20.0	18.0	34.0	23.0	4.0	4.0	19.0	6.0	5.0	5.0	6.0	7.0	7.0	4.0	4.0	4.0

[a]Strain acronyms are indicated in Table 2.16.

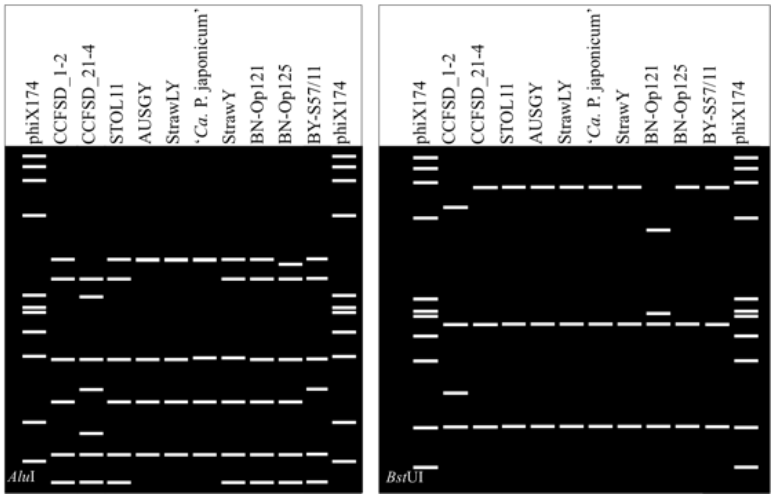


Figure 2.24b. *In silico* RFLP analysis on virtual R16F2n/R16R2 amplicons using the endoglucanase *Alu*I and *BstU*I showed the specific restriction profile to the strains CCFSD_1-2 and CCFSD_21-4 associated with cassava frog skin disease in Costa Rica. Strain references used for in silico analyses are described in Table 2.16; phiX174, marker ΦX174 *Hae*III digested, fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

Further research is necessary, on higher number of strains from cassava, on other host plants and possibly on other specific genes (Davis *et al*., 1993; Cimermann *et al*., 2006), to further verify the epidemiological relevance and the geographic distribution of these strains, considering that the two strains originated from same province but, from different locations, and from diverse cassava genotypes and tissues this finding demonstrates that pathogen populations are present.

In the tropical area near to Costa Rica also species such as *Cocos nucifera* affected with "Porroca" disease and *Solanum phureja* infected with elm yellows and stolbur has been reported in Colombia and Panama (Gilbert and Parker, 2008). In the present study, in particular the strain obtained from *S. phureja* Colombia used as a control also have a specific restriction site and profile with de endoglucanase *Aci*l that is distinguishable to the others 16SrXII subgroups already reported.

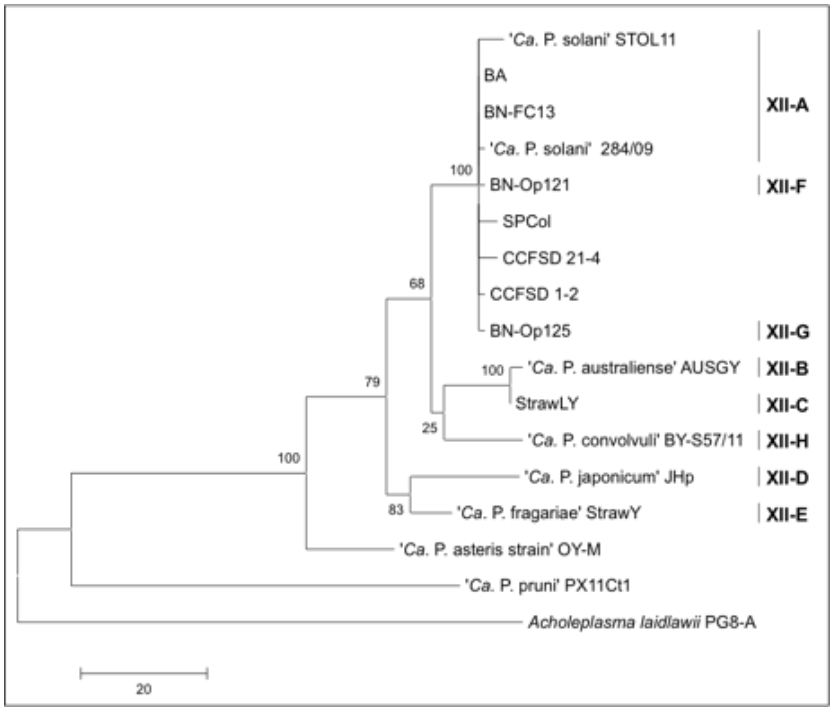


Figure 2.25. Phylogenetic tree constructed using the Maximum Parsimony method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. There were a total of 1,112 positions in the final dataset (about 212 to 1,270). The analysis involved 17 nucleotide sequences including two-cassava strains from Costa Rica and 15 reference strain belonging to "stolbur" group. Strains references employed of 16SrXII subgroups are described in Table 2.16. All ambiguous positions were removed for each sequence pair. '*Ca*. P. asteris' strain OY-M (NC_005303); '*Ca*. P. pruni' strain PX11CT1 (JQ044393) and *A. laidlawii* PG-8A (NC_010163) were used as outgroups. Letters in bars, represent diverse RFLP subgroups in the 16S rDNA gene of "stolbur" strains.

2.4.2.7 Hibiscus witches' broom-related strains, 16SrXV

Nested PCR results with universal and specific phytoplasma primers R16F2n/R16R2, B5/P7, M1/M2 and R16(I)F1/R16(I)R1 provided positive results for hibiscus witches' broom related strains from cassava plants affected with CWB in Vietnam and Thailand. In the sample CThaiWB_3 RFLP analyses on R16F2n/R16R2 and B5/P7 amplicons with *Tru*I allow to identify mixed phytoplasma infection with the ribosomal group 16SrVI. With R16F2n/R16R2 a typical restriction profile belonging to 16SrV, -VI or -VII (data not shown) was obtained and in the same sample with the specific primer pair R16(I)F1/R16(I)R1, also group 16SrXV and aster yellows phytoplasmas were amplified (Figure 2.26A and B). The restriction profiles obtained with amplicons M1/M2 using *Hpa*II and *Tru*I restriction enzymes in the strains CThaiWB_4, CThaiWB_12, CVWB_38 and CVWB_42, allowed three different groups of profiles to be distinguished; two of them in the strains CThaiWB_4 and CVWB_42 indistinguishable from the reference strain SuV, which is related to the ribosomal subgroup 16SrXV-A, while the samples CThaiWB_12 and CVWB_38 were not identical to any employed reference strain. (Figure 26A and B).

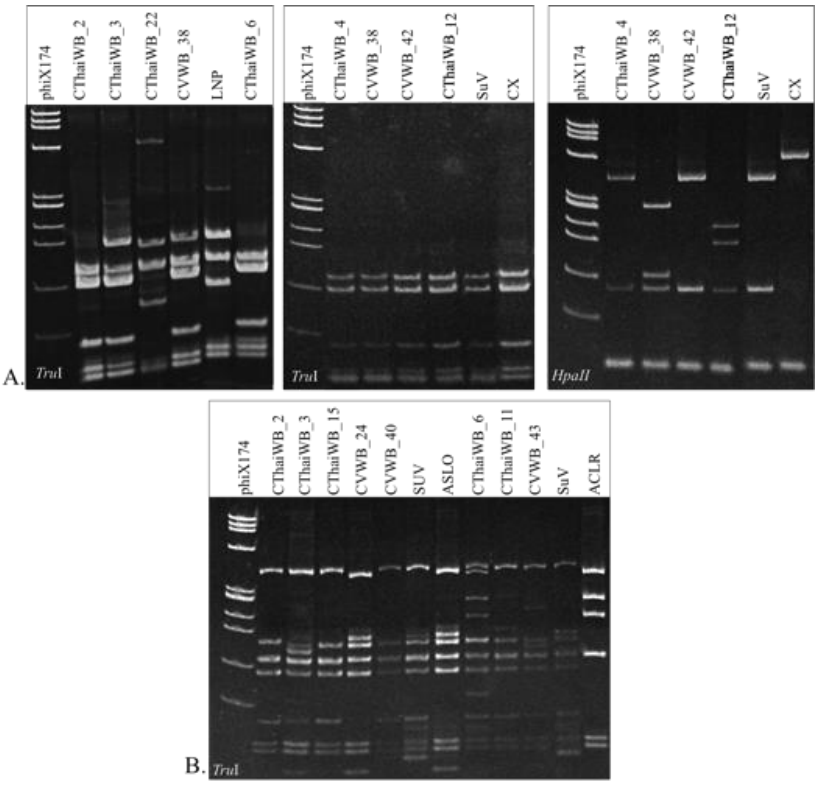


Figure 2.26. Different restriction profiles obtained with M1/M2 and R16(I)F1/R16(I)R1 assays using *Tru*I and *Hpa*II restriction enzymes in samples affected with cassava witches' broom disease from Thailand (CThaiWB) and Vietnam (CVWB). **A.** Nested PCR amplification with M1/M2 assay using the restriction enzyme *Tru*I showed infection with hibiscus witches' broom related phytoplasma (16SrXV) in the samples CThaiWB_2, CThaiWB_4, CThaiWB_6, CThaiWB_12, CVWB_38,

CVWB_42 and mixed infection with aster yellows phytoplasmas (16SrI) in the samples CThaiWB_3 and CVWB_38. Three different profiles obtained with the restriction enzyme *Hpa*II in the strains CThaiWB_4, CThaiWB_12, CVWB_38 and CVWB_42 are related to ribosomal subgroup 16SrXV. **B.** RFLP analyses on R16(I)F1/R16(I)R1 amplicons using the restriction enzyme *Tru*I showed differential 16SrXV profiles in samples CThaiWB_2, CThaiWB_3, CThaiWB_11, CThaiWB_15, CVWB_40 and CVWB_43. Sample CThaiWB_6 showed mixed infection of 16SrXV and 16SrI. CVWB_24, typical profile of "stolbur" related strains. LNp (16SrX-B), plum leptonecrosis; SuV (16SrXV), Surinam virescence; CX (16SrIII-A), peach X-disease; A-SLO (16SrXII), "stolbur"; (ACLR [A-AY] (16SrI-F), aster yellows from apricot; phiX174, marker ΦX174 *Hae*III digested, fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

Since 16SrII was also reported associated with cassava diseases and is phylogenetically related with 16SrXV ribosomal group, representative strains of the subgroup 16SrII were also included in the sequence analyses. Four cassava strains were selected for sequencing analyses.

Table 2.16a. Hibiscus and peanut witches' broom-related reference phytoplasma strains employed for 16S rDNA classification.

Phytoplasma associated disease (acronym)	**Geographic origin**	**GenBank accession numbers, 16 rDNA***	**16Sr subgroup**
'*Ca.* P. brasiliensis' (HibWB26)	Brazil	AF147708	XV-A
Crotalaria juncea' (L32)	Brazil	KF878382	XV-A
Yellow peach (peach19)	Azerbaijan	FR717540	XV-A
Sida rhombifolia' witches'-broom (SiWB-Br1)	Brazil	HQ230579	XV-A
Hibiscus witches'-broom (WBAZ3)	Egypt	KF716175	XV-A
Cauliflower stunt (Cfs-Br07)	Brazil	JN818845	XV-A
Suriname virescence (SuV)	Suriname	Q-Bank	XV
Guazuma ulmifolia' (GWB#10)	Costa Rica	HQ258883	XV-B
Guazuma ulmifolia' (GWB#4)	Costa Rica	HQ258882	XV-B
Peanut witches' broom (PnWB)	Taiwan	L33765	II-A
'*Ca*. P. aurantifolia' (WBDL)	Oman	U15442	II-B
Faba bean phyllody (FBP)	Sudan	X83432	II-C
'*Ca*. P. australasia' (PpM)	Australia	Y10096	II-D
Pichris echioides phyllody (PEY)	Italy	Y16393	II-E
Cotton phyllody (CoP)	Burkina Faso	EF186827	II-F
Australian lucerne yellows (LYSP-D8)	Australia	JX861234	II

*The sequence of the strain SuV was retrieved from the comprehensive databases on quarantine plant pest and diseases (Q-bank, [http://www.q-bank.eu/Phytoplasmas/]).

The strains selected for further sequences analyses were CThaiWB_3, CThaiWB_12, CThaiWB_15 and CVWB_38, they showed 99% (1052/1058), (508/512), (1234/1244) and (509/513) homology with '*Ca.* P. brasiliensis' HibWB26, *Crotalaria juncea* (strain L32) and

Sida rhombifolia (strain SiWB-Br1) from Brazil and in particular the strains CThaiWB_12, CThaiWB_38 also with *Prunus persica* (strain peach19) from Azerbaijan and CThaiWB_15 with a strain from Brazil GWB#10 from *Guazuma ulmifolia* belonging to the 16SrXV-B (Table 2.16).

In silico RFLP, SNPs calculation and phylogenetic analyses from the near full-length 16S rDNA gene (217 to 1,461) revealed that the strains CThaiWB_15 and CThaiWB_3 are closely related to 16SrXV-A ribosomal subgroup. The overall estimation of evolutionary divergence in those strains compared with the other representative strain belonging to 16SrXV and 16SrII ribosomal groups is 4.8 (Table 2.17a). The virtual RFLP pattern derived from the query 16S rDNA R16F2n/R16R2 and/or R16F1(I)/R16(I)R1 fragment is different from the reference patterns of all previously reported 16Sr groups/subgroups and strains. The most similar is the reference pattern of the 16SrXV-A (GenBank accession: AF147708), with a similarity coefficient of 0.94 to CThaiWB_15 and 0.97 to CThaiWB_3, which is less than or equal to 0.97. This strain may represent a new subgroup within the 16Sr group XV. In some cases also some SNPs positions that are also present in 16SrII group were present in cassava strain but, not very significant to the restriction and phylogenetic analysis for classification and differentiation (Tables 2.17 and 2.18).

The strains CThaiWB_12 and CVWB_38 from Thailand and Vietnam, respectively showed differential profile with the enzyme *Hpa*II to each one and also specific SNPs positions. These results were also confirmed after SNPs calculation results. To demonstrate and confirm the highly variability presented in both strains, actual and *in silico* RFLP of R16F2n/R16R2 and/or R16(I)F1/R16(I)R1 amplicons was obtained with this enzyme and it assigned two new 16SrXV ribosomal subgroups associated with the same disease, plant species but from different countries. The enzyme *Hpa*II (position 490) distinguished CThaiWB_12 and CVWB_38 phytoplasma from the closely related *'Ca.* P. brasiliense' (Table 2.17 and 2.18). The actual laboratory restriction digestion with the key enzyme *Hpa*II confirmed the new subgroup pattern (Figure 2.26). Comparison with the Guazuma witches' broom phytoplasma showed that the phytoplasmas strains from Thailand and Vietnam resulted closely related to the 16SrVX-B subgroup but can eventually be classified as a new subgroup. This is the first report of the natural occurrence of phytoplasma strains closely related to '*Ca.* P. brasiliense' in Thailand and Vietnam. Moreover Villalobos *et al*. (2011) report that 16SrXV-B (strains GWB#10 and GWB#4) subgroup can be differentiated with the enzymes *Hae*III (position 258) and *Hpa*II (position 490) from 16SrXV-A; in the strains CThaiWB_15, CThaiWB_3 and

in the reference strain SuV that are closely related to the subgroup 16SrXV-A, the restriction site *Hae*III is also present in the same position, but no *Hpa*II restriction site is present (Table 2.18, Figure 2.29). However, several others SNPs variability is present in those strains and also in GWB#10 and GWB#4 (Table 2.18), confirmed the high variability that are present among the member of this ribosomal group and the potential new 16SrXV ribosomal subgroups from *M. esculenta*.

Table 2.17. Estimation of evolutionary divergence between 16S rDNA sequences obtained from four different phytoplasma strains associated with CWB from Vietnam and Thailand. The number of base differences per sequence between sequences is shown. The analysis involved also sixteen 16SrXV reference nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 513 positions (M1/M2 amplicon) in the final dataset. The evolutionary analyses were conducted in MEGA5.

Strains	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
1. '*Ca.* P. brasiliensis' HibWB26																			
2. CThaiWB_15	2.0																		
3. CThaiWB_3	0.0	2.0																	
4. CThaiWB_12	5.0	5.0	5.0																
5. CVWB_38	4.0	4.0	4.0	2.0															
6. SuV	1.0	1.0	1.0	4.0	3.0														
7. peach19	0.0	2.0	0.0	5.0	4.0	1.0													
8. CfS-Br07	3.0	5.0	3.0	8.0	7.0	4.0	3.0												
9. WBAZ3	0.0	2.0	0.0	5.0	4.0	1.0	0.0	3.0											
10. L32	0.0	2.0	0.0	5.0	4.0	1.0	0.0	3.0	0.0										
11. SiWB-Br1	0.0	2.0	0.0	5.0	4.0	1.0	0.0	3.0	0.0	0.0									
12. GWB#10	3.0	3.0	3.0	6.0	5.0	2.0	3.0	6.0	3.0	3.0	3.0								
13. GWB#4	3.0	3.0	3.0	6.0	5.0	2.0	3.0	6.0	3.0	3.0	3.0	0.0							
14. PnWB	8.0	8.0	8.0	9.0	8.0	7.0	8.0	11.0	8.0	8.0	8.0	7.0	7.0						
15. LYSP-D8	5.0	7.0	5.0	8.0	7.0	6.0	5.0	8.0	5.0	5.0	5.0	8.0	8.0	7.0					
16. '*Ca.* P. aurantifolia' WBDL	5.0	7.0	5.0	8.0	7.0	6.0	5.0	8.0	5.0	5.0	5.0	8.0	8.0	5.0	2.0				
17. FBP	7.0	9.0	7.0	10.0	9.0	8.0	7.0	10.0	7.0	7.0	7.0	10.0	10.0	7.0	2.0	2.0			
18. '*Ca.* P. australasia' PpM	8.0	8.0	8.0	9.0	8.0	7.0	8.0	11.0	8.0	8.0	8.0	7.0	7.0	0.0	7.0	5.0	7.0		
19. PEY	6.0	8.0	6.0	9.0	8.0	7.0	6.0	9.0	6.0	6.0	6.0	9.0	9.0	6.0	5.0	3.0	5.0	6.0	
20. CoP	7.0	9.0	7.0	10.0	9.0	8.0	7.0	10.0	7.0	7.0	7.0	10.0	10.0	7.0	2.0	2.0	2.0	7.0	5.0

[a]Strain acronyms are indicated in Table 2.16.

Phylogenetic, divergence and *in silico* RFLP analyses derived from the query 16S rDNA fragment demonstrated that the most similar is the reference pattern of the 16SrXV group, subgroup A (AF147708), with a similarity coefficient of 0.95, suggesting that this strain may represent a new subgroup within the 16SrXVgroup.

Since 16SrXV-B have been only reported in Costa Rica and now a new subgroup in a new species is detected the information could be useful for epidemiological studies to prevent also the possible presence or spreading of the disease in Costa Rica.

Table 2.17a. Estimation of evolutionary divergence between 16S rDNA sequences obtained from two different phytoplasma strains associated with CWB from Vietnam and Thailand. There were a total of 1,023 positions in the final dataset. 16S ribosomal RNA gene position about 217 to 1,461. The evolutionary analyses were conducted in MEGA5.

Strains	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
1. '*Ca*. P. brasiliensis' HibWB26																	
2. CThaiWB_15	5.0																
3. CThaiWB_3	3.0	2.0															
4. SuV	6.0	3.0	3.0														
5. peach19	0.0	5.0	3.0	6.0													
6. CfS-Br07	4.0	9.0	7.0	10.0	4.0												
7. WBAZ3	0.0	5.0	3.0	6.0	0.0	4.0											
8. L32	0.0	5.0	3.0	6.0	0.0	4.0	0.0										
9. SiWB-Br1	0.0	5.0	3.0	6.0	0.0	4.0	0.0	0.0									
10. GWB#10	7.0	8.0	8.0	9.0	7.0	11.0	7.0	7.0	7.0								
11. GWB#4	7.0	8.0	8.0	9.0	7.0	11.0	7.0	7.0	7.0	0.0							
12. PnWB	27.0	28.0	28.0	29.0	27.0	31.0	27.0	27.0	27.0	26.0	26.0						
13. LYSP-D8	24.0	27.0	25.0	28.0	24.0	28.0	24.0	24.0	24.0	27.0	27.0	11.0					
14. '*Ca*. P. aurantifolia' WBDL	25.0	28.0	26.0	29.0	25.0	29.0	25.0	25.0	25.0	28.0	28.0	12.0	5.0				
15. FBP	26.0	29.0	27.0	30.0	26.0	30.0	26.0	26.0	26.0	31.0	31.0	15.0	6.0	5.0			
16. '*Ca*. P. australasia' PpM	26.0	27.0	27.0	28.0	26.0	30.0	26.0	26.0	26.0	25.0	25.0	1.0	10.0	11.0	14.0		
17. PEY	30.0	31.0	29.0	32.0	30.0	34.0	30.0	30.0	30.0	33.0	33.0	16.0	15.0	14.0	17.0	15.0	
18. CoP	27.0	30.0	28.0	31.0	27.0	31.0	27.0	27.0	27.0	30.0	30.0	14.0	5.0	2.0	5.0	13.0	16.0

[a]Strain acronyms are indicated in Table 2.16.

Table 2.18. Differential SNP positions in 16Sr DNA sequences (position about 217 to 1,461), of sixteen representative strains for hibiscus witches' broom-related strains, from Brazil (HibWB26, L32, SiWB-Br1 and Cfs-Br07), Azerbaijan (peach19), Egypt (WBAZ3), Surinam (SuV) and Costa Rica (GWB#10 and GWB#4) and representative subgroups from peanut witches' broom, compared with cassava phytoplasma strains detected in Vietnam (CVWB) and Thailand (CThaiWB) samples.

	Single nucleotide positions in 16S ribosomal RNA gene (position about 217 to 1461)																
Strains[a]	**248**	**254**	**258***	**402**	**490**	**519**	**523***	**599**	**630**	**733**	**951***	**1012**	**1014***	**1028**	**1223**	**1302**	**1461**
'*Ca*. P. brasiliensis' HibWB26	A	C	T	C	C	A	A	C	A	A	C	T	A	T	C	C	T
CThaiWB_15	?	T	C	T	.	.	.	T	G	.	.	C	.	C	.	T	C
CThaiWB_3	G	T	C	T	.	.	.	T	G	.	.	.	.	.	.	-	-
CThaiWB_12M1M2	-	-	-	-	-	-	-	-	-	T	.	C	G	.	T	-	-
CVWB_38M1M2	-	-	-	-	-	-	-	-	-	T	G	C	.	.	T	-	-
SuV	G	T	C	T	.	T	C	T	G	.	.	C	.	.	.	.	C
peach19	-	-	-	.	.	.	.	.	.	.	.	.	.	.	.	.	-
CfS-Br07	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-
WBAZ3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-
L32	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
SiWB-Br1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-
GWB10	G	T	C	.	T	.	.	.	G	.	.	C	.	.	.	.	C
GWB4	G	T	C	.	T	.	.	.	G	.	.	C	.	.	.	.	C
PnWB	G	T	C	.	.	.	.	.	G	.	.	C	.	.	T	.	C
LYSP-D8	G	T	C	.	.	.	.	.	G	.	.	.	.	.	T	.	C
'Ca. P. aurantifolia' WBDL	G	T	C	.	.	.	.	.	G	.	.	.	.	.	T	.	C
FBP	G	T	C	.	.	.	.	.	G	.	.	.	.	.	T	.	C
'Ca. P australasia' PpM	G	T	C	.	.	.	.	.	G	.	.	C	.	.	T	.	C
PEY	G	.	C	T	.	.	.	.	G	.	.	.	.	.	T	.	-
CoP	G	T	C	.	.	.	.	.	G	.	.	.	.	.	T	.	C

[a]Strains peach19, CfS-Br07, WBAZ3, L32 and SiWB-Br1 sequences showed high significant score using BLAST® tool. Strain HibWB26 was used to calculated the SNPs positions. *SNPs making differential restriction sites RFLP for potential new 16SrXV strain differentiation: 258 (*Hae*III); 490, 951 and 1014 (*Hpa*II); 523 (*Dde*I).

Dots represent nucleotides identical to the '*Ca.* P. brasiliensis' HibWB26 consensus sequence and dashes are gaps positions. Strains acronyms are indicated in Table 2.16.

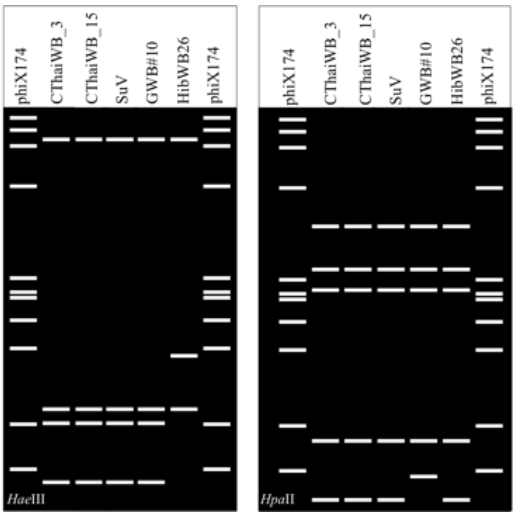


Figure 2.29. *In silico* RFLP analysis on virtual R16F2n/R16R2 amplicons using the endoclucanase *Hae*III and *Hpa*II showed the specific restriction profile to the strains CThaiWB_3 and CThaiWB_15 associated with cassava witches' broom disease in Thailand. Strain references used to *in silico* analyses are described in Table 2.16a; phiX174, marker ΦX174 *Hae*III digested, fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

'*Ca.* P. aurantifolia', is the agent associated with witches' broom disease in Mexican lime trees (*Citrus aurantifolia* L.), and is responsible for major losses of Mexican lime trees in Southern Iran and Oman. The pathogen is strictly biotrophic, and thus is completely dependent on living host cells for its survival. Hibiscus witches' broom is the closer phytoplasma ribosomal group that recently was detected in new host and outside of America.

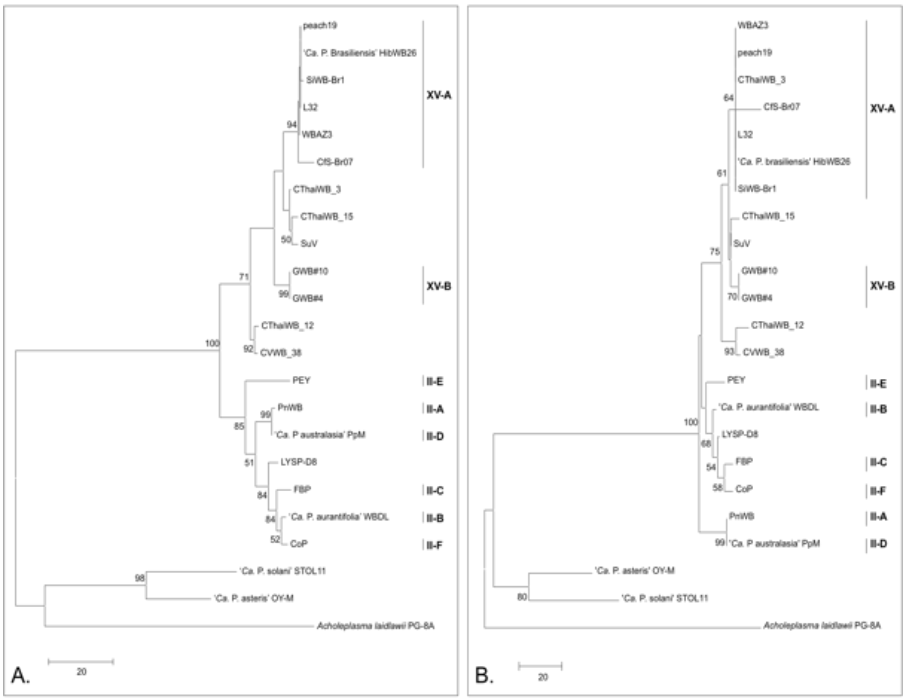


Figure 2.30. Phylogenetic trees from 16Sr DNA gene from hibiscus witches' broom (16SrXV), peanut witches' broom (16SrII) and cassava witches' broom strains related from Thailand (CThaiWB_3, CThaiWB_12,

CThaiWB_15) and Vietnam (CVWB_38). Strains references employed of 16SrXV and 16SrII subgroups are described in Table 2.16a. '*Ca*. P. asteris' strain OY-M (NC_005303); '*Ca.* P. solani' strain STOL11 (AF248959) and *A. laidlawii* PG-8A (NC_010163) were used as outgroups. Letters in bars, represent diverse RFLP subgroups in the 16S rDNA gene of Hibiscus and peanut witches' broom strains. **A.** Phylogenetic tree constructed using the Maximum Parsimony (MP) method. There were a total of 1,251 positions in the final dataset (position about 217 to 1,461). **B.** Phylogenetic tree constructed using the Neighbour-Joining method. There were a total of 511 positions (M1/M2 amplicon) in the final dataset.

2.4.3 Transmission assay by dodder (*Cuscuta* sp.)

To elucidate the pathogenicity role of phytoplasmas in CWB disease, using a '*Ca*. P. asteris' infected potted cassava from the genotype KM2112, as source of inoculum, and the ectoparasite *Cuscuta* spp. (dodder) as bridge to healthy periwinkle plants [*Catharanthus roseus* (L.) G. Don], the pathogen's transmission was obtained. The infection was achieved after 6 months of connection, when kept at 30°C during the day and 26°C at night from infected cassava (CVWB_12B) to healthy periwinkle plants (treatment 2). Two of the six plants evaluated, showed typical symptoms of phytoplasma infection such as yellowing, small leaves on shoots with short internodes (Table 2.19 and Figure 2.31). PCR/RFLP assays for phytoplasma identification confirmed the presence of '*Ca.* P. asteris' related-strains using nested-PCR assays with R16F2n/R16R2 primers followed by nested PCR with primers R16(I)F1/R16(I)R1 and/or M1/M2 and RFLP analyses with *Tru*I in both symptomatic periwinkle plants and in two additional plants that no showed symptoms (treatment 2). No amplification was obtained in the asymptomatic plants for the treatments 1 and 4 corresponding to infected cassava (CVWB_12A) and healthy periwinkle. Periwinkle plants infected with AY, included as positive control in the experiments, were also successfully transmitted to healthy periwinkle plants developing particular symptoms such as very small leaves, yellowing and leave discoloration in the 80% of the plants connected.

Table 2.19. Results obtained with *Cuscuta* spp. as bridge for phytoplasma transmission from cassava infected with cassava witches' broom to periwinkle.

Treatment		Nested PCR[a]	
No	Description	R16(I)F1/R1	M1M2
1	Infected cassava (CVWB_12A) to healthy periwinkle	0/6	0/6
2	Infected cassava (CVWB_12B) to healthy periwinkle	2/6	4/6
3	Infected periwinkle AY to healthy periwinkle	5/6	6/6
4	Healthy periwinkle to healthy periwinkle	0/6	0/6

[a]Values refer to number of replications out of 6, where detection was successful.

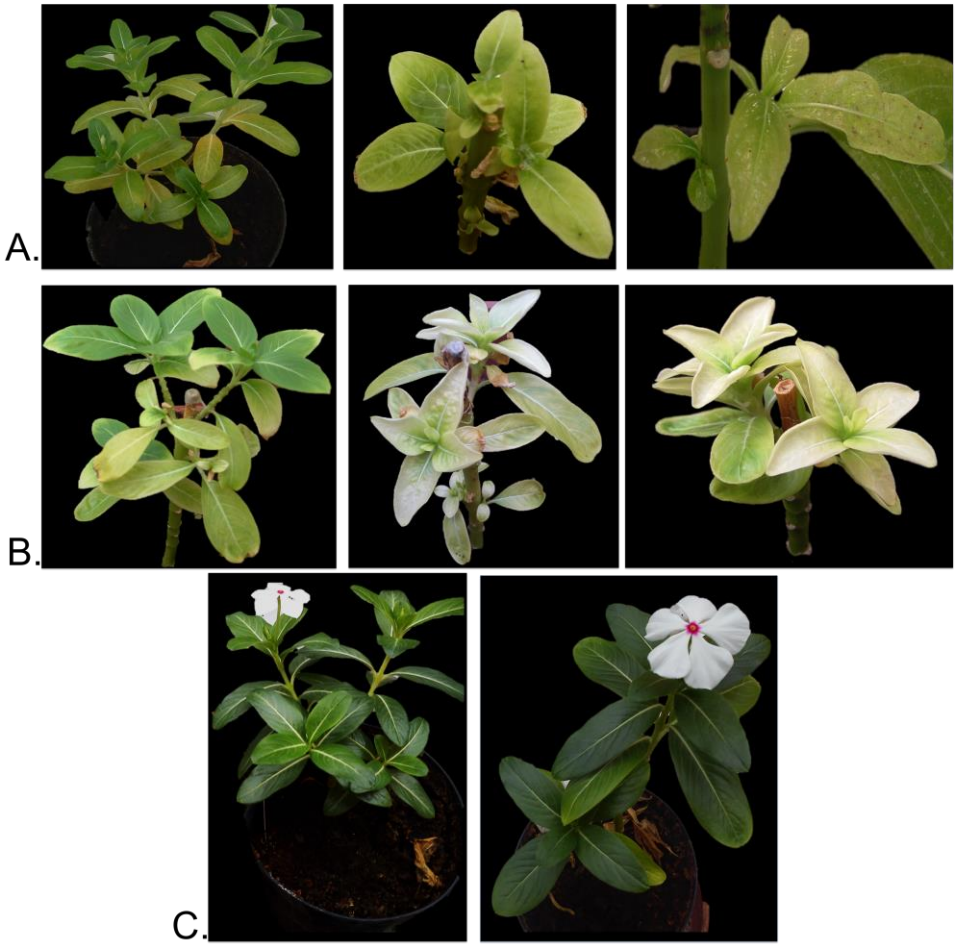


Figure 2.31. Symptoms expression in periwinkle after phytoplasma dodder transmission. **A.** Treatment 2, infected cassava (CVWB_12B) to healthy periwinkle. **B.** Treatment 3, infected periwinkle plants with AY to healthy periwinkle. **C.** Treatment 1 (right), asymptomatic plant negative to PCR test; treatment 4 (left), healthy periwinkle to healthy periwinkle plants.

So far, we have not obtained 100% of phytoplasma symptoms expression in periwinkle dodder connected with cassava, even though the presence of phytoplasmas with the nested-PCR technique was detected in infected cassava plants and later in periwinkle plants infected through dodder. A major cause of the lack of phytoplasma symptoms was probably their low concentration in the host plant, compared to the reference strain used as positive control, because it had been maintained in periwinkle by a long incubation period under the same environmental conditions (2 years). The periods of incubation and optimal greenhouse conditions are fundamental for the expression of symptoms characteristic of phytoplasmas in periwinkle plants. The plants evaluated as positive by PCR will be exposed to different periods and greenhouse conditions to seek the optimal for reproducing symptoms. Additionally the positive periwinkle plants obtained will be again connected to healthy cassava plants, to confirm aetiology of this cassava disease by fulfilling Koch's postulates.

2.4.4 *'Candidatus* Phytoplasma brasiliense' partial genome annotation

The genome draft of '*Ca.* P. brasiliense' strain SuV (16SrXV) was sequenced and 245 small contigs representing 361,4 kbp were obtained. The semi-automatic annotation was carried out using the platform iANT. Out of 434 chromosomal coding sequences (CDS, Coding DNA Sequence), 152 CDS (35%) were involved in information transfer (protein translation, DNA replication and modification, protein production, RNA modification and regulation), 86 CDS (20%) were encoding metabolic enzymes (protein translation, glycolisis and energy production, nucleic acid, lipids and phospholipids, cofactors, amino acids and others), 54 CDS (12%) corresponded to transporters (ABC transporters, protein secretion, cation dependent p-ATPase and others), 4 CDS (1%) corresponded to cellular processes, whereas 133 CDS (30%) remained cryptic functions (i.e. bacterial conserved hypothetical proteins, conserved hypothetical proteins specific to the phytoplasma genes or hypothetical proteins). Repeated sequences were underestimated and transposon and phage-related CDS (2%) could not yet be precisely evaluated.

The partial SuV chromosome possesses 2 rRNA operons, 29 tRNAs as well as 434 coding sequences (CDS) (Figure 2.32, Table 2.20). Most of the sequenced CDS (35%) participate to the information transfer, *i.e.* replication, transcription and production of the proteins. Regarding to DNA rearrangement, SuV should be capable of homologous recombination as it has a complete *rec*A gene encoding DNA helicases, RuvX and RecU, a putative holiday junction resolvase. The complement of genes necessary for resolving holiday junction and recombination differs in phytoplasmas, however, genes recA, recU, ruvA, and ruvB, which are absent in '*Ca.* P. asteris' and '*Ca.* P. australiense', do not belong to the essential gene set of *M. genitalium* but may have long-term influence on the chromosome maintenance (Glass *et al*., 2006).

Metabolic genes account for 20% of the sequenced CDS out of which 22% participate to protein degradation. It comprises *Hfl*B/*Fts*H encoding ATP-dependent zinc proteases as well as various other proteases. '*Ca.* P. asteris' strain OY-M possesses 20 copies of *Hfl*B/*Fts*H genes very likely not all functional (Oshima *et al.*, 2004).

Most of the transporters were ABC transporters (57%) aimed to transport maltose, spermidin/putrescine, oligopeptides, methionine, cobalt and manganese/zinc. Other transporters were assigned to protein secretion (sec system, *secA*, *secE*, *secY* and *yidC*), to cation dependent P-ATPase and one 2- hydroxycarboxylate (malate/citrate) cation transporter (*citS*) and *citG,* ATP-dephospho-CoA triphosphoribosyl transferase. The *citG* gene have not

been annotated in the genomes previously reported (Table 2.20).

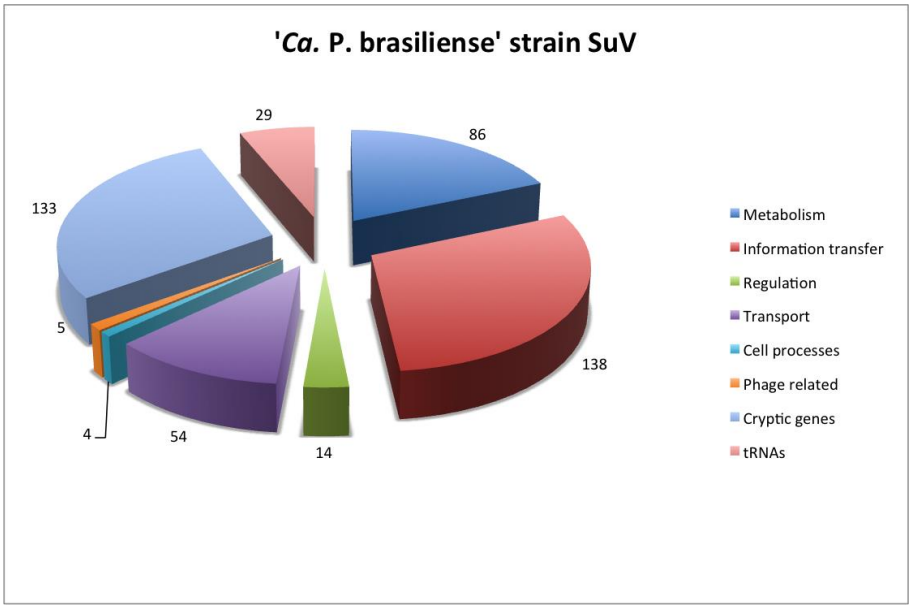


Figure 2.32. Structural DNAs and functional distribution CDS from partial SuV chromosomal sequences.

As long as the remaining 40% repeated part of the genome are not sequenced and annotated, it is too early to state about the presence of complete potential mobiles units homologous to '*Ca*. P. asteris' PMU (Bai *et al.*, 2006). However, *dna*D, *dnaB*, the DNA primase (dnaG), *ins*K (transposase), and others involved in replication such as the single-stranded binding protein (ssb) and *hup* (putative bacterial nucleoid binding protein) and the delta subunit of the DNA polymerase III (polC) were present in multicopy.

Since the eda (Eda, aldolase) have been only annotated in '*Ca.* P. mali', pyruvate might be generated in the same way. Aldolase, serving two possible functions. A 4-hydroxy-2-oxoglutarate aldolase (KHG-aldolase) would catalyze the interconversion of 4-hydroxy-2-oxoglutarate into pyruvate and glyoxylate. Phospho-2-dehydro-3-deoxygluconate aldolase (KDPG-aldolase) would catalyse the interconversion of 6-phospho-2-dehydro-3-deoxy-D-gluconate into pyruvate and glyceraldehyde-3-phosphate. In both cases, pyruvate would be formed independently from glycolysis (Kube *et al*., 2012).

In particular Amp is encoded in the genomes of the two '*Ca.* P. asteris' strains. A putative homolog is also present in '*Ca.* P. australiense', while Suv as '*Ca.* P. mali' encodes the immunodominant protein Imp. However, both *imp* and *amp* genes have been also identified in

'*Ca.* P. asteris' strain OY-W (Kakizawa *et al*., 2009). Similar situation was present with the predicted endoglucanase FrvX (M42 peptidase family) that is present in SuV and in both fully sequenced aster yellows strains, but not in '*Ca*. P. australiense' and '*Ca.* P. mali'. Since the FrvX proteins in *A. thaliana* have been reported associated with xylem development, plant growth elongation and cell wall thickening the phytoplasma endo-1,4-beta-glucanase protein could potentially contribute to their virulence (Kube *et al*., 2012).

Table 2.20. Partial annotation on the gene content of *'Ca.* P. brasiliense" strain SuV according to functional categories and sequence homology. Gene comparison with four of the phytoplasma genome reported ('Ca. P. asteris' strain OY-M; '*Ca*. P. asteris' strain AY-WB; '*Ca.* P. australiense and '*Ca*. P. mali') using iANT program.

Gen[a]	Description	OY-M[b]	AYWB	'*Ca*. P. australiense'	'*Ca.* P. mali'
Genes involved in DNA replication					
dnaA	Replication initiation and membrane attachment protein	+	+	+	+
dnaB	Replicative DNA helicase	+	+	+	+
dnaC	Replicative DNA helicase	+	+	+	+
dnaD	DNA replication protein	+	+	+	+
dnaE	DNA polymerase III alpha subunit	+	+	+	+
dnaG	DNA primase	+	+	+	+
dnaI	probable DNA replication protein dnaI	-	-	-	-
dnaX	DNA polymerase III subunit gamma/tau	+	+	+	+
gyrA	DNA gyrase (type II topoisomerase), subunit A	+	+	+	+
gyrB	DNA gyrase (type II topoisomerase), subunit B	+	+	+	+
holA	DNA polymerase III delta subunit	+	+	+	+
holB	DNA polymerase III, delta prime subunit	+	+	+	+
ligA	DNA ligase, NAD(+)-dependent	+	+	+	+
polC	DNA polymerase III alpha subunit	+	+	+	+
priA	hypothetical primosomal protein N'	+	+	+	+
rnc[rnhC]	RNase III	+	+	+	+
ssb	Single-stranded DNA-binding protein	+	+	+	+
-	Prokaryotic chromosome segregation/condensation protein (like MukB, N-terminal)	-	-	-	-
-	YbaB/EbfC DNA-binding family; pfam02575	-	-	-	-
-	Hypotheticla protein DNA related	-	-	-	-
pmbA	Modulator of DNA gyrase	+	+	+	+
DNA repair and degradation					
polA	DNA polymerase I - 3'-5' exonuclease and polymerase domain	+	+	+	+
srmB	Superfamily II DNA and RNA helicase	+	+	+	+
deaD	ATP-dependent RNA helicase	-	-	-	-
mutM	Probable DNA glycosylase	+	+	+	+
uvrC	excinuclease UvrABC, endonuclease subunit	-	-	-	-
nfo	endonuclease IV	+	+	+	+
Genes involved in modification and structure					
hup[himA]	DNA-binding protein HU	+	+	+	+
topA	DNA topoisomerase I	+	+	+	+
Recombination-related functions					
-	Holliday junction resolvase (*ruvX*-like)	+	+	+	+
recA	Protein RecA	-	-	-	+
recU	Holliday junction resolvase recU	-	-	-	+
Transcriptional regulators					
hrcA	Probable Heat-inducible transcription repressor hrcA	+	+	+	+
Factors affecting the RNA polymerase					
greA	transcription elongation factor	+	+	+	+
nusA	transcription termination/antitermination L factor	+	+	+	+
RNA polymerase and sigma factors					
rpoA	RNA polymerase, alpha subunit	+	+	+	+

Gen[a]	Description	OY-M[b]	AYWB	'*Ca.* P. australiense'	'*Ca.* P. mali'
rpoB	RNA polymerase, beta subunit	+	+	+	+
rpoC	RNA polymerase, beta prime subunit	+	+	+	+
rpoD	RNA polymerase sigma factor	+	+	+	+
rpoE	Putative DNA-directed RNA polymerase delta subunit	+	+	+	+
rpoZ	Probable DNA-directed RNA polymerase omega chain	+	+	+	+
Miscellaneous					
gcp	tRNA N6-adenosine threonylcarbamoyltransferase(RNA modification)	+	+	+	+
frvX	Putative aminopeptidase of the M42 family/endo-1,4-beta-glucanase	+	+	-	-
nfnB	putative NAD(P)H nitroreductase (Nitrogen metabolism)	+	+	+	+
tlyC	Probable transmembrane hemolysin/Virulence associated	+	+	+	+
degV	Probable DegV family protein	+	+	+	+
Factors affecting the RNA polymerase					
miaA	tRNA delta(2)-isopentenylpyrophosphate transferase	+	+	+	+
mnmA	tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase	+	+	+	+
mnmE[trmE]	tRNA modification GTPase	+	+	+	+
mnmG[*gidA*]	tRNA uridine 5-carboxymethylaminomethyl modification enzyme mnmG	+	+	+	+
rluA	23S rRNA pseudouridine synthase	+	+	+	+
yacO	23S rRNA (Gm2251)-methyltransferase	+	-	+	-
rsmA[ksgA]	S-adenosylmethionine-6-N',N'-adenosyl (rRNA) dimethyltransferase	+	+	+	+
rsmD	16S rRNA m(2)G966 methyltransferase, SAM-dependent	+	+	+	+
rsmG	Ribosomal RNA small subunit methyltransferase G	+	+	+	-
rsmI	Ribosomal RNA small subunit methyltransferase I	+	+	+	+
spoU	rRNA methyltransferase	+	+	-	+
trmD	tRNA (guanine-N(1)-) methyl transferase	+	+	+	+
truB	tRNA pseuroudine synthase	+	+	+	+
pth	probable peptidyl-tRNA hydrolase	+	+	+	+
Other proteins involved in translation					
rnpA	probable ribonuclease P protein component	+	+	+	+
pcnB	Polynucleotide adenyltransferase	+	+	+	+
smpB	SsrA-binding protein	+	+	+	+
map	methionine aminopeptidase	+	+	+	+
pnp	Polyribonucleotide nucleotidyltransferase	+	+	+	+
tRNA synthetases					
alaS	alanyl-tRNA synthetase	+	+	+	+
argS	Arginyl-tRNA synthetase	+	+	+	+
asnS[asnC]	asparaginyl tRNA synthetase	+	+	+	+
aspS	aspartyl-tRNA synthetase	+	+	+	+
cysS	cysteinyl-tRNA synthetase	+	+	+	+
glnS	glutamyl-tRNA synthetase	+	+	+	+
gltX	glutamyl-tRNA synthetase	+	+	+	+
glyS	Glycyl-tRNA synthetase	-	-	+	-
hisS	histidyl-tRNA synthetase	+	+	+	+
ileS	isoleucyl-tRNA synthetase	+	+	+	+
leuS	leucyl-tRNA synthetase	+	+	+	+
lysU	lysine tRNA synthetase, inducible	+	+	+	+
metG	methionyl-tRNA synthetase	+	+	+	+
pheS	phenylalanine tRNA synthetase, alpha subunit	+	+	+	+
pheT	Phenylalanine tRNA synthetase, beta subunit	+	+	+	+
proS	Prolyl-tRNA synthetase	+	+	+	+
serS	Seryl-tRNA synthetase	+	+	+	+
tilS[lysU]	Lysidine-tRNA(Ile) synthetase	+	+	+	+
trpS	Tryptophanyl-tRNA synthetase	+	+	+	+
valS	Valyl-tRNA synthetase	+	+	+	+
Nucleotide metabolism					
tadA	tRNA-specific adenosine deaminase	+	+	+	+
cmk	cytidylate kinase	+	+	+	+
nrdE	ribonucleotide-diphosphate reductase subunit alpha (nrdA-like)	+	+	+	+

Gen[a]	Description	OY-M[b]	AYWB	'*Ca.* P. australiense'	'*Ca.* P. mali'
nrdI	protein that stimulates ribonucleotide reduction	-	-	-	-
tmk	Thymidylate kinase	+	+	+	+
dut	deoxyuridinetriphosphatase	+	+	+	+
adk	adenylate kinase	+	+	+	+
gmk	guanylate kinase	+	+	+	+
pyrG	CTP synthetase	+	+	+	+
pyrH	uridylate kinase	+	+	+	+
thyA	thymidylate synthetase	+	+	+	+
tdk	thymidine kinase	+	+	+	+
Translation factors					
efp	Elongation factor EF-P	+	+	+	+
frr	ribosome recycling factor	+	+	+	+
infA	translation initiation factor IF-1	+	+	+	+
prfA	peptide chain release factor RF-1	+	+	+	+
prfB	peptide chain release factor RF-2	+	+	+	+
sua5	Translation factor	+	+	+	+
tsf	Probable Elongation factor Ts	+	+	+	+
Modifying and accessory factors					
engA	GTP-binding protein	+	+	+	+
engB	GTP-binding protein	+	+	+	+
engD	Predicted GTP-dependent nucleic acid-binding protein	+	+	+	+
obg	GTP binding protein	+	+	+	+
rsgA	Putative ribosome biogenesis GTPase EngC (engC)	+	+	+	+
Heat shock proteins and chaperons					
dnaJ	chaperone Hsp40, co-chaperone with DnaK	+	+	+	+
dnaK	Molecular chaperone DnaK	+	+	+	+
groEL	molecular chaperone GroEL/Chaperonin Cpn60	+	+	+	+
grpE	probable Protein grpE	+	+	+	+
tig	putative trigger factor	+	+	+	+
lon	ATP-dependent Lon protease	+	+	+	+
Ribosomal proteins					
rpsB	30S ribosomal protein S2	+	+	+	+
rpsD	30S ribosomal protein S4	+	+	+	+
rpsJ	30S ribosomal subunit protein S10	+	+	+	+
rpsK	30S ribosomal subunit protein S11	+	+	+	+
rpsL	30S ribosomal subunit protein S12	+	+	+	+
rpsM	30S ribosomal subunit protein S13	+	+	+	+
rpsO	30S ribosomal subunit protein S15	+	+	+	+
rpsR	30S ribosomal subunit protein S18	+	+	+	+
rpsS	30S ribosomal subunit protein S19	+	+	+	+
rpsT	30S ribosomal subunit protein S20	+	+	+	+
rpsU	30S ribosomal subunit protein S21	+	+	+	+
rpsC	30S ribosomal subunit protein S3	+	+	+	+
rpsE	30S ribosomal subunit protein S5	+	+	+	+
rpsF	30S ribosomal subunit protein S6	+	+	+	+
rplO	50S ribosomal protein L15	+	+	+	+
rplQ	50S ribosomal protein L17	+	+	+	+
rplT	50S ribosomal protein L20	+	+	+	+
rpmB	50S ribosomal protein L28	+	+	+	+
rpmF	50S ribosomal protein L32	+	+	+	+
rpmG	50S ribosomal protein L33	+	+	+	+
rplP	50S ribosomal subunit protein L16	+	+	+	+
rplS	50S ribosomal subunit protein L19	+	+	+	+
rplV	50S ribosomal subunit protein L22	+	+	+	+
rplC	50S ribosomal subunit protein L3	+	+	+	+
rpmE	50S ribosomal subunit protein L31	+	+	+	+
rpmH	50S ribosomal subunit protein L34	+	+	+	+
rplL	50S ribosomal subunit protein L7/L12	+	+	+	+
rplI	50S ribosomal subunit protein L9	+	+	+	+
rpmJ	50S subunit ribosomal protein L36	+	+	+	+
Acyl carrier protein metabolism					

Gen[a]	Description	OY-M[b]	AYWB	'*Ca.* P. australiense'	'*Ca.* P. mali'
citE	citrate lyase subunit beta				
acpP	acyl carrier protein (ACP)	+	+	+	+
citD	citrate lyase, acyl carrier (gamma) subunit	-	-	-	-
acpS	Probable Holo-[acyl-carrier-protein] synthase	+	+	+	+
Proteins involved in iron-sulfur cluster biosynthesis					
iscU	Iron sulfur cluster assembly protein NifU	+	+	+	+
cdsB	putative cysteine desulfurase	+	+	+	+
Amino acid synthesis and modification					
metK	S-adenosylmethionine synthetase	-	-	-	+
smtA	SAM-dependent methyltransferase	+	+	+	-
Glycolysis and related genes					
cof	Putative hydrolases of the HAD superfamily	+	+	+	+
pykF	pyruvate kinase I	+	+	+	-
Cell envelelope reated- proteins					
mraW	S-adenosyl-dependent methyltransferase	+	+	+	+
Deduced proteins involved in the formation of acetate from malate					
ackA	Acetate kinase, ATP-binding component	+	+	+	+
pduL	Propanediol utilisation protein	+	+	+	+
pdhA	Pyruvate dehydrogenase E1 component alpha subunit	+	+	+	+
dgoA[eda]	2-oxo-3-deoxygalactonate 6-phosphate aldolase	-	-	-	+
pdhB	Pyruvate dehydrogenase E1 component beta subunit				
pdhC	pyruvate dehydrogenase E2 component N-terminal truncated				
hcaD	pyridine nucleotide-disulphide oxidoreductase				
HflB and TldD proteins grouped by BLASTClust					
hflB/ftsH	conserved hypothetical protein; ATP-dependent Zn metalloprotease	+	+	+	+
tldD	Zn-dependent protease tldD protein	+	+	+	+
Peptidases					
pepP	Probable Xaa-Pro aminopeptidase	+	-	+	+
Lipoyl-protein ligases					
lplA	lipoate-protein ligase A	+	+	+	+
NAD synthesis					
qns/nadE	Glutamine-dependent NAD(+) synthetase	+	+	+	+
Genes involved in lipid synthesis					
pgsA	Putative Phosphatidylglycerophosphate synthase	+	+	+	+
plsX	Probable fatty acid/phospholipid synthesis protein	+	+	+	+
plsY	Glycerol-3-phosphate acyltransferase	+	+	+	+
pssA	Putative CDP-diacylglycerol-serine O-phosphatidyltransferase	+	+	+	+
gpsA	glycerol-3-phosphate dehydrogenase	+	+	+	+
Psd	phosphatidylserine decarboxylase	+	+	+	+
Phosphorus metabolic processes					
ppa	Inorganic pyrophosphatase	+	+	+	+
cbf[hd]	Cmp binding factor, probable Metal-dependent phosphohydrolase	+	+	+	+
Proteins related to chromosome segregation					
spoVG	Putative septation protein spoVG	+	+	+	+
Proteins involved in cell division					
ffh	Signal Recognition Particle (SRP) component with 4.5S RNA (ffs)	+	+	+	+
ftsY	Signal recognition particle-docking protein	+	+	+	+
Extrachromosomal					
-	Putative phage-related recombinase				
insK	Putative transposase				
Symporters					
citS	Putative malate/citrate symporter	+	+	+	+
citG	ATP:dephospho-CoA triphosphoribosyl transferase.	-	-	-	-
Large-conductance mechanosensitive channel proteins					
mscL	putative large-conductance mechanosensitive channel	+	+	+	+
Putative complete ABC transporter subunits with assignment					
dppA	ABC-type peptide/nickel transport system, substrate-binding protein	+	+	+	+

Gen[a]	Description	OY-M[b]	AYWB	'*Ca.* P. australiense'	'*Ca.* P. mali'
dppB	ABC-type dipeptide/oligopeptide transport system, permease component	+	+	+	+
dppC	ABC-type peptide/nickel transport system, permease component	+	+	+	+
dppF	ABC-type dipeptide/oligopeptide transport system, ATPase component	+	+	+	+
glnQ	ABC-type glutamine transport system, ATP-binding protein glnQ	+	+	+	-
mntD[ZnuA]	Mn/Zn ABC transporter permease component	+	+	+	+
CbiO1	Cobalt ABC transporter ATP-binding component	+	+	+	+
CbiO2	Cobalt ABC transporter ATP-binding component	+	+	+	+
malK	Sugar ABC transporter ATP binding component	+	+	+	+
malF	probable sugar ABC transporter, permease component	+	+	+	+
malG	probable ABC transporter, permease component	+	+	+	+
cbiQ	ABC-type cobalt transport system, permease component	+	+	+	+
malE	Putative ABC transporter maltose/maltodextrin-binding component	+	+	+	+
mntB[ZnuB]	Mn/Zn ABC transporter ATP binding component	+	+	+	+
mntC[ZnuC]	Mn/Zn ABC transporter permease component	+	+	+	+
mntA	Mn/Zn ABC transporter solute binding component	+	+	+	+
msbA	fused lipid transporter subunits of ABC superfamily: membrane component/ATP-binding component	+	+	+	+
zntA	Cation uptake P-type ATPase	+	+	+	+
Secretion apparatus					
secA	Protein translocase subunit secA	+	+	+	+
secE	Protein translocase subunit SecE	+	+	+	+
secY	preprotein translocase membrane subunit	+	+	+	+
yidC	preprotein translocase subunit YidC	+	+	+	+
P-type ATPases					
mgtA	Probable P-type ATPase	+	+	+	+
Immunodominant membrane proteins					
imp	Conserved hypothetical protein IMP	-	-	-	+

[a]'*Ca.* P. Brasiliense' gene annotation;
[b]Genes (+) presence and (-) abscense.

The objective of this part of work was to produce a comparative survey of this genome (G+C content, codon and amino acid usage, organization of the larger genetic loci annotated, presence of AMP, IMP, VMP orthologs). Future work will be conducted to select house-keeping genes useful to carry out a preliminary survey of the genetic diversity for this phytoplasma. In the phytoplasma collections of INRA, Bordeaux, France and of Bologna University currently six strains are maintained in periwinkle from Azerbaijan, Lebanon, Surinam, Brazil, they will be used together with the DNA from the two new 16SrXV subgroups identified in cassava in the present work for molecular comparison to verify presence of variability possibly linked to the 16SrXV phytoplasma role in the cassava disease in south east Asia.

2.5 General discussion

The main results of this research was the finding of the presence and/or of genetic variability in selected new strains from CWB and CFSD phytoplasmas detected in heavily symptomatic samples. This work has greatly improved the knowledge about phytoplasmas detected in Asia and in Costa Rica and Paraguay in cassava, since phytoplasmas not reported previously associated with these diseases were identified. Since phytoplasmas related to aster yellows, X-disease, elm yellows, clover/apple proliferation, "stolbur" and hibiscus witches' broom were detected only in samples from symptomatic cassava plants, it can be suggested that these phytoplasmas are involved in both the cassava frog skin and the witches' broom diseases, however, more biological data must be obtained to confirm aetiology of these cassava diseases by fulfilling Koch's postulates. More research is needed to verify epidemiological relevance of the unique restriction profiles detected in cassava strains belonging to ribosomal groups 16SrI, 16SrIII, 16SrVI, 16SrX, 16SrXII and 16SrXV using them as a tool for differentiation of those subgroups find in cassava from phytoplasmas detected in other plant species or insect vectors. Variability was found between strains belonging to the same ribosomal group but with different geographic origin and associated with different symptomatology. The detected genetic variability was not always related to geographic strain distribution but probably also to ability of these phytoplasma strains to rapidly modify inducing severe epidemic outbreaks within cyclic periods (Bertaccini, 2007)

Diseases associated with phytoplasma presence occur worldwide in many crops, although individual phytoplasmas may be limited in their host range or distribution. There are more than 300 distinct plant diseases attributed to phytoplasmas, affecting hundreds of plant genera (Hoshi *et al*., 2007). Many of the economically important diseases are those of cereals, tuber crops and woody plants, including maize bushy stunt, potato purple top wilt/potato witches' broom, peach X-disease, grapevine yellows ("bois noir") and apple proliferation. The causal agents and/or associated pathogens of these diseases have been respectively described as '*Ca.* P. asteris', '*Ca.* P. americanum', '*Ca.* P. pruni, , '*Ca.* P. solani' and '*Ca.* P. prunorum'/'*Ca.* P. pyri'/'*Ca.* P. mali' (Bedendo *et al*., 2000, Gomes *et al*., 2004, González *et al*., 2002, Lee *et al*., 2004a, Quaglino *et al*., 2013, Davis *et al*., 2013 , Seemüller and Schneider, 2004).

Cereals and tuber crops are seriously infected by molecularly differentiable phytoplasmas worldwide; however the symptomatology is mainly the same for the same species. For example potato witches' broom/purple top and maize bushy stunt are among the most widespread diseases in herbaceous hosts causing severe yield losses. Maize bushy stunt phytoplasma (MBS) has been reported in several countries from the southern of United State s

of America to Argentina. The pathogen is transmitted by the leafhoppers *Dalbulus maydis* (De Long & Wolcott, 1923) for most of the vector life's, after it feeds on an infected plant. The mollicutes '*Ca.* P. asteris', 16SrI-B (Lee *et al*., 2004) and *Spiroplasma kunkelii* sp. nov. (Whitcomb *et al*., 1986) are reported as causal agents of maize bushy stunt (MBS) and corn stunt (CS) that are the most economically relevant prokaryotic diseases to maize (*Zea mays* L.), whose incidence has recently increased in the Caribbean, Central and Southern American countries (Bedendo *et al*., 2000, Gomes *et al*., 2004, González *et al*., 2002, Ebbert *et al.,* 2001; Harrison *et al.,* 1996; Mejia *et al*., 2013). Corn resulted to be consistently infected in America by a molecularly distinguishable strain of aster yellows phytoplasmas, while similar symptoms of corn in Europe allow the detection of "stolbur" phytoplasmas (Duduk and Bertaccini, 2006). Potato purple top wilt (PPT) is a devastating disease that occurs in various regions of North America and Mexico. While maize bushy stunt and reddening disease are associated with two different phytoplasmas, symptomatic potatoes are associated with the presence of phytoplasmas belonging to different groups mainly according to different growing areas (Eroglu *et al*., 2010; Girsova *et* al., 2008; Hosseini *et al.,* 2011; Liefting *et al*., 2009; Mejia *et al*., 2011); at least nine distinct phytoplasma strains belonging to seven different phytoplasma groups [16SrI, 16SrII, 16SIII, 16SrVI, 16SrXII, 16SrXIII and 16SrXVIII (most closely related to 16SrXII)] have been associated with potato phytoplasma diseases (Santos-Cervantes *et al*., 2010). As an example, in 2004 and 2005, an outbreak of a new disease of potato occurred in Texas and Nebraska, USA, which caused darkening of potato chips (potato chips) produced from infected tubers. This chip defect has resulted in a considerable economic loss in the local potato industry. Infected potato plants exhibited symptoms similar to those of the potato purple top disease which is associated in USA with a subgroup 16SrVI-A phytoplasma (Lee *et al*., 2004a). In 2010 in potato in Mexico four different phytoplasmas (16SrI, 16SrII, 16SrIII and 16SrXIII), sometimes in mixed infections, were detected in potato growing areas (Santos-Cervantes *et al*., 2010). As found in the present research cassava showing frog skin and witches' broom diseases symptoms in Costa Rica - Paraguay and in Vietnam – Thailand, respectively, were also associated with several phytoplasma ribosomal subgroup; variability was found between strains belonging to the same ribosomal group but having different geographic origin and associated with different type of disease.

The "bois noir" (BN) disease is associated with phytoplasmas belonging to ribosomal subgroup 16SrXII-A and has symptoms undistinguishable from "flavescence dorée" (FD) associated with phytoplasmas belonging to ribosomal subgroups 16SrV-C and 16SrV-D, it is

widespread in all viticultural areas worldwide. BN phytoplasmas are transmitted to grapevine by *H. obsoletus* from *C. arvensis* (Maixner, 1994; Sforza *et al.*, 1998) and *U. dioica* (Alma *et al*., 2002) as recognized sources of inoculum. However, these phytoplasmas were also detected in other plants and auchenorrhyncha species that could be also involved in BN epidemiology. The usefulness of tuf gene polymorphism for such epidemiological studies has been clearly demonstrated (Langer and Maixner, 2004; Mori *et al*., 2008). Recent findings indicate that molecular variability is also present inside the 16S gene of BN and of other related phytoplasmas confirming that also in this case phytoplasma strains, possibly relevant to BN epidemic outbreaks are present. In other viticulture areas of the world different phytoplasmas were detected as associated with symptomatology indistinguishable from FD or BN such as aster yellows (16SrI) in Italy and South Africa (Alma *et al.*, 1996; Engelbrecht *et al*., 2010), ash yellows (16SrVII) in Chile (Gajardo *et al*., 2009) and Australian grapevine yellows in Australia (16SrXII-B) (Padovan *et al*., 1995).

'*Ca.* P. mali', 16SrX-A, the agent of apple proliferation, affects only European and Turkish apple orchards, reducing the size and weight of the fruit of infected apple trees by half, which are therefore unmarketable. The disease is absent in North and South America as well as Asia and rarely reported in other plant species. In Europe, young trees are more susceptible to the disease, but no tree mortality has been reported to date. The highest economic impact seems to occur in Germany and northern Italy. Economic damage to *Prunus* species is very common in Europe and middle East because of European stone fruit yellows presence ('*Ca*. P. prunorum', 16SrX-B), which kills, about 5% of apricot trees per year in southern France where the disease is named apricot chlorotic leaf roll. '*Ca.* P. prunorum' also induces plum leptonecrosis on *Prunus salicina* (Japanese plum) and yellows on most of the peach accessions in southern Europe. Severe epidemic diseases associated with the presence of different phytoplasmas were also reported in peach (*Prunus persica*) in USA and Canada where '*Ca.* P. pruni' was mainly identified (Davis *et al*., 2013), in almond and stone fruit in the middle East mainly in Lebanon and Iran almond and stone fruit witches' broom are associated with '*Ca*. P. phoenicium' presence (Molino-Lova *et al*., 2011; Salehi *et al*., 2006; Zirak *et al*., 2009; 2010), and in China and India in cherry and peach sever decline are associated with the presence of '*Ca.* P. zyziphi' (Zhu *et al*., 2011). This also demonstrated that ribosomal classification are not 100% correlated to host, disease and geographic origin and is also a confirmation that also the different phytoplasma detected in symptomatic cassava plants showing frog skin and witches' broom are associated with these diseases.

The results of these findings could contribute to the start of cassava frog skin and witches' broom control. Knowledge about genetic relationship of these phytoplasmas with others strains described previously only on different plant species can help in further epidemiological work to identify insect vectors and possible weed or woody plants as reservoir hosts of these prokaryotes in the regions where cassava is grown. Witches' broom disease has been recently reported in cassava in China, Cambodia, Philippines and Indian countries. Knowing that cassava is an additional host for several phytoplasmas in agent of severe diseases in other agronomically relevant crops can also help to devise control measures and correct management procedures to reduce frog skin and witches' broom phytoplasma spreading to other crops in Latin America and Asia.

Chapter 3

3 Phytoplasmas associated with oil palm lethal wilt in Colombia

3.1 Introduction

Colombia is the major producer of oil palm (*Elaeis guineensis* Jacq) in Latin America, and the fifth largest grower worldwide that plays an important role in biodiesel markets (Castiblanco *et al*., 2013). Oil palm has become a paramount export crop and source of employment for the national economy. However, its production declined by 7.1% since 2002, with a dramatic drop of 10% in northeastern Colombia (FEDEPALMA, 2010). The cause of this decrease has been the lethal wilt disease ("marchitez letal" in Spanish). Lethal wilt is present in the oil-palm plantations (Perez and Cayón, 2010) of the Upía River area, Palmar del Oriente (July 1994), Palmas del Casanare (1999), Palmeras Santana (2000), and Palmeras del Upía (2002). Recently, the disease has been observed in Palmas Sicarare SAS oil palm plantations in northern Colombia (E. Alvarez, personal communication) (Figures 1C and 1D). The disease had decimated around 690 hectares and a total of 97,619 oil palms by 2010 (FEDEPALMA, 2010). Symptoms of lethal wilt commonly first appear as vascular discoloration and leaf yellowing in seven years old palms (i.e., flowering and fruiting). These symptoms are followed by leaf drying, wilt, and necrosis of infected tissues, and eventual plant collapse (Figure 3.1). Root necrosis often accompanies leaf discoloration. Internal discoloration of trunk tissue may also occur, but does not represent a distinctive symptom. Lethal wilt spreads rapidly and causes plant death within 4 to 6 months after symptoms onset (Perez and Cayón, 2010).

The causal agent was believed to be a phytoplasma, possibly related to that associated with lethal yellowing-like diseases (Alvarez and Claroz, 2003), which affects coconut (*Cocos nucifera* L.) and other 35 palm species including *Phoenix dactylifera* (date palm), *Veitchia merrilli* (manila palm), *Caryota rumphiana* (fishtail palm), *Phoenix canariensis* (Canary Island date palm), and *Elaeis guineensis* (African oil palm) (Nejat *et al*., 2009; 2013). Phytoplasmas have been associated with diseases in several hundred crop and ornamental plant species (Bertaccini and Duduk, 2009). They are phloem-restricted bacteria, transmitted by phloem-feeding Hemiptera vectors. These include planthoppers and leafhoppers of the genera *Macrosteles*, *Euscelis*, *Euscelidius*, *Scaphoideus*, and *Cacopsylla* (Weintraub and Beanland, 2006).

Lethal yellowing-like diseases have killed over 40 million of coconut palms in the Caribbean, Florida, Mexico, Central America and Africa (Harrison *et al*., 2002; 2009; Oropeza *et al*., 2011; Ntushelo *et al*., 2012). Phytoplasma identification was also obtained in Papua New Guinea in symptomatic oil palms and in West New Britain (Turner, 1981) in coconut palms (Kelly *et al.*, 2011). The group 16SrIV lethal yellowing phytoplasma has been shown to be only vectored by *Myndus crudus* (American palm cixiid). Other Hemiptera species has been identified as potential vectors like the derbid planthopper *Cedusa* sp. (Brown *et al*., 2006). Phytoplasmas closely related to the 16SrIV group have also been reported in date palm and other palm species in USA (Harrison *et al*., 2008; 2009). They were also detected in Jamaican Asteraceae weeds such as *Emilia fosbergii*, *Synedrella nodiflora*, and *Vernonia cinerea* (Brown *et al*., 2008a; 2008b) collected near lethal yellowing-affected coconut palms.

Other 16Sr phytoplasma groups have been associated with diseases in palms in Africa and Asia. In Saudi Arabia, a 16SrI group, '*Ca.* P. asteris' was associated with the Al-Wijam disease of date palm (Alhudaib *et al*., 2008). A 16SrXIV group, '*Ca.* P. cynodontis' was identified from date palms showing slow decline symptoms in North Sudan (Cronjé *et al*., 2000) and recently from coconut palms affected with yellow decline and in oil palm seedlings in Malaysia (Nejat *et al*., 2009). Similarly, the Weligama wilt disease in Sri Lanka (Perera *et al.* 2012) and Kalimantan wilt in Indonesia (Warokka, 2005) were associated with phytoplasmas of the 16SrXI group '*Ca.* P. oryzae'.

Lethal wilt-like symptoms were also observed in oil palms in Brazil affected by a disease known as "fatal yellowing" (Brioso *et al*., 2006; Montano *et al*., 2007). Although the cause of this disease is still unknown, its symptoms and distribution in both Colombian and Brazilian plantations suggest that plant pathogens are involved (Tovar and Torres, 2004). A preliminary study detected phytoplasmas in symptomatic plants in commercial crops of the susceptible oil-palm hybrid (*Elaeis guineensis* × *Elaeis oleifera*) (Alvarez and Claroz, 2003) in Colombia. Phytoplasma identification and classification rely on the 16S ribosomal gene analysis to identify '*Candidatus* Phytoplasma' species and discriminate among 16Sr groups and subgroups (Bertaccini and Duduk, 2009). For instance, '*Ca.* P. asteris' is classified in the 16SrI group, in which at least 18 subgroups are recognized (Lee *et al*., 2004).

Figure 3.1. Oil palms in Colombia showing mild (A, severity score 2) and severe (B, severity score 4) lethal wilt symptoms. Lethal wilt symptoms reported in oil palm in the North of Colombia in young plants with yellowish lines parallel to veins (C), and leaf drying and leaf discoloration (D). Figures C and D, *personal communication* Alvarez and Pardo, 2013.

Finer phytoplasma differentiation can be obtained by studying polymorphisms in other gene sequences (Martini *et al.*, 2007; Mitrović *et al.*, 2011a). The large survey carried out in this work allowed to verify the phytoplasma presence in diverse plant tissues from diseased oil palms collected in four lethal wilt-affected areas of Colombia. Phytoplasma strains of the 16SrI group were detected and characterized by multigene typing analysis based on four

phytoplasma genes and distinguished from reference strains (Bertaccini *et al*., 2000) and from a strain infecting corn in Colombia.

3.2 Materials and Methods

3.2.1 Plant samples

Samples from 44 symptomatic and seven asymptomatic oil palms were collected between 2003 and 2011 from four plantations in two sites: Villanueva (Department of Casanare) and Barranca de Upía (Department of Meta), Colombia. The four plantations belonged to (A) Palmar del Oriente S.A. (located at 4°30′15″ N and 72°56′20″ W), (B) Palmas del Casanare S.A. (4°35′58.33″ N and 72°50′58.74″ W), (C) Palmeras Santana Ltda. (4°32′24.18″ N and 72°52′51.38″ W), and (D) Palmeras del Upía Ltda. (4°26′8.13″ N and 72°56′29.39″ W).

Entire meristems were collected from each symptomatic and asymptomatic plant and about 50 to 100 g from chlorotic leaves, spears, and inflorescences. Three 10×10cm segments were also excised from the base of the trunk, along with ten 25-cm-long root segments from the root ball of each palm at 50 cm from the collar. Eighty five samples from different tissues were tested from 44 symptomatic trees. About half of these samples were collected from palms with severe symptoms (see below). Asymptomatic plants were collected at the same time from all four plantations surveyed (three plants from plantation A, two from B, and one plant each from plantations C and D). A total of 44 samples were tested as negative controls.

A symptom severity scale was used to rate each symptomatic plant, where 1 represented a dead inflorescence and fruit rot; 2, chlorosis or necrosis of the oldest leaves; 3, leaf chlorosis in the upper canopy; and 4, a dead spear leaf and apical meristem rot. Plants receiving a score of 1 or 2 were characterized as having mild symptoms, 3 as having moderate symptoms, and 4 as having severe symptoms. The ability of detecting phytoplasmas from infected tissues was then compared between plants with mild symptoms and those exhibiting severe symptoms (Figure 3.2).

3.2.2 Phytoplasma detection and identification

Total DNA was extracted from 0.4 to 1.0 g of tissues from each plant sample according to previously described protocols (Gilbertson and Dellaporta, 1983; Prince *et al*., 1993), see appendix A. To amplify the phytoplasma 16Sr region and the beginning of the 23S rDNA genes, and the internal spacer region, direct and nested PCR assays were performed following

the conditions described in appendix B and C. Positive controls used for the molecular analyses included DNA from phytoplasma reference strains that represented different ribosomal 16S rDNA subgroups. These strains had been either maintained in periwinkle [*Catharanthus roseus* (L.) G. Don.] or total DNA was extracted from the original host plant, as for MBS from Colombia (Table 3.1). Non-DNA template samples and DNA from asymptomatic oil palms were added as negative controls for the PCR reactions.

The 98 amplicons obtained with the R16F2n/R16R2 primers (1.2 kb) were subjected to restriction fragment length polymorphism (RFLP) with restriction enzymes, *Tru1*I and *Hha*I (Fermentas, Vilnius, Lithuania), following the manufacturer's instructions. RFLP profiles were separated in polyacrylamide gels (see Appendix C and D). Additionally, using reference strains (Table 3.1) also SNPs calculation and in silico analyses were conducted as are described in appendix E.

Direct bi-directional sequencing using primers P1 and F1 (Deng and Hiruki, 1991; Davis and Lee, 1993) as forward primers and P7 as reverse primer was performed on the P1/P7 amplicons after purification with a QIAquick PCR Purification Kit (QIAGEN, Valencia, CA). The sequences were assembled using Sequencher 4.1 software. Sequences were compared with selected nucleotide sequences in the GenBank database using BLAST (version BLASTN 2.2.18) (National Center for Biotechnology Information, Bethesda, MD). Sequence alignments and phylogenetic trees were performed as described in Appendix E. Before constructing phylogenetic trees all sequences were trimmed to contain only 16S rDNA (1,245 bp). Phylogenetic analyses were carried out on 16S rDNA sequences from oil palm and from several '*Ca.* Phytoplasma' strains using *A. laidlawii* as the outgroup. GenBank accession numbers and other sources of 16S rRNA gene sequences used in phylogenetic analyses are provided in Table 3.1.

Table 3.1. Aster yellows-related reference phytoplasma ('*Ca.* P. asteris') strains employed.

Phytoplasma associated disease (acronym)[a]	Geographic origin	GenBank accession numbers			RFLP classification[b]		
		16S rDNA	*groEL*	*rp*	16SrI[c]	*groEL*I[d]	rpI[c]
New Jersey aster yellows (NJ-AY)	NJ, USA	HM590622	AB599703	-	A	I	A
Plantago virescence (PVM)	Germany	AY265216	AB599706	AY264867	A	I	A
Carrot yellows (ca2006/1)	Serbia	EU215424	AB599708	EU215428	A	I	A
Grey dogwood stunt (GD1)	NY, USA	DQ112021	AB599694	AY264864	A	II	M
Periwinkle virescence (NA)	Italy	HM590621	AB599702	-	B	III	-
Primula green yellows (PrG)	UK	HM590623	AB599696	-	B	III	-

Phytoplasma associated disease (acronym)[a]	Geographic origin	GenBank accession numbers			RFLP classification[b]		
		16S rDNA	*groEL*	*rp*	16SrI[c]	*groEL*I[d]	rpI[c]
Oilseed rape virescence (RV)	France	HM590625	AB599698	-	B	III	-
Carrot yellows (ca2006/9)	Serbia	EU215426	AB599709	EU215430	B	III	B
Primrose virescence (PRIVA)	Germany	AY265210	AB599705	-	B(L)	III	B
Aster yellows (AV2192)	Germany	AY180957	AB599687	AY183708	B(L)	III	B
Aster yellows (AVUT)	Germany	AY265209	AB599686	AY264855	B(M)	III	B
Aster yellows (AY-J)	France	HM590616	AB599689	-	B	IV	-
Carrot yellows (ca2006/5rrnA)	Serbia	EU215425/	AB599711	EU215429	B(?)	IV	-
Carrot yellows (ca2006/5rrnB)	Serbia	GQ175789					
Maize bushy stunt (MBS Col)	Colombia	HQ530152	AB599712	KF434319	B	V	-
Maize bushy stunt (MBS)	Mexico	AY265208	–	AY264858	B	–	L
Oil palm lethal wilt (OP47)	Colombia	JX681021	JX681023	KF434318	B	V	-
Leontodon yellows (LEO)	Italy	HM590620	AB599701	-	C	VI	-
Carrot yellows (CA)	Italy	HM448473	AB599690	-	C	VI	-
Clover phyllody (KVE)	France	AY265217	–	AY264861	C	–	C
Clover phyllody (KVF)	France	HQ530150	AB599695	-	C	VII	-
Potato purple top (PPT)	France	HQ530151	AB599704	-	C	VII	-
Paulownia witches' broom (PaWB)	Taiwan	AY265206	AB124810	AY264857	D	–	D
Blueberry stunt (BBS3)	MI, USA	AY265213	–	AY264863	E	–	E
Aster yellows apricot (A-AY)	Spain	AY265211	AB599699	AY264866	F	VIII	N
Strawberry multiplier (STRAWB2)	FL, USA	U96616	–	U96617	K	–	J
Ipomea obscura witches' broom (IOWB)	Taiwan	AY265205	–	AY264859	N	–	F
Populus decline (PopD)	Serbia	HM590626	AB599710	-	P	IX	-

[a]Strains in bold were used as references for PCR-RFLP analysis. [b](?) refers to a strain with interoperon heterogeneity that is tentatively classified in this subgroup; – refers to a sequence not available in the GenBank. [c]Different letter represent diverse RFLP subgroups in the 16S rDNA gene of aster yellows strains. [d]Different Roman number represent diverse RFLP groups in the *groEL* gene. [e] Different letter represent diverse RFLP groups in the *rp* gene. OP47: oil palm lethal wilt phytoplasma strain 47.

3.2.3 Strain characterization based on *groEL*, *rp,* and *amp* genes

These gene regions were chosen based on their ability to distinguish among phytoplasma strains in several studies (Kakizawa *et al*., 2006; Martini *et al*., 2007; Mitrović *et al*., 2011b). Nested PCR was carried out from 44 oil-palm samples that were positive to previous phytoplasma identification of the 16S rDNA gene.

Nested-PCR with *groEL* primers AYgroelF/AYampR, followed by AYgroelF/AYgroelR amplicon was performed by diluting 1: 30 those first PCR products as described (Mitrović *et al*., 2011a; 2011b). The negative and positive controls were as described in 3.2.2. RFLP analyses were carried out on amplicons using *Alu*I and *Tru1*I restriction enzymes (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. Restriction fragments were separated as described in 3.2.2. Direct sequencing and sequence assembly were performed on

the AYgroelF/AYgroelR amplicon from sample OP47. A phylogenetic tree was produced, using available reference strains (Table 3.1), as described in 3.2.2.

Previous studies indicated that the part of the ribosomal operon that includes the complete *l22* and *s3* genes can be used as a phylogenetic marker. This has finely differentiated among distinct phytoplasma 16S rDNA subgroups (Martini *et al*., 2007). The 44 oil-palm samples were used for direct amplification with the rpF1/rpR1 primer pair (Lim and Sears, 1992), using the reaction mix and the negative and positive controls as described above. Thirty-eight PCR cycles were conducted under the following conditions: 1 min (2 min for the first cycle) for denaturation step at 94°C, 2 min for annealing at 55°C, and 3 min (10 min for the last cycle) for primer extension at 72°C. RFLP analyses of obtained amplicons with *Tru1*I, *Hpy8*I, *Taa*I, and *Alu*I were then performed. The rpF1/rpR1 fragment of OP47 samples was also sequenced as described above and a search for single nucleotide polymorphisms (SNPs) presence in comparison with reference strains was also carried out using Mega version 5 (Tamura *et al*., 2011).

The *amp* gene codes for a surface membrane protein that was recently reported as being involved in insect to phytoplasma transmission. Therefore, it is also suitable for phytoplasma strain differentiation (Barbara *et al*., 2002; Kakizawa *et al*., 2006). Direct PCR assays with Amp-N1/C1 primers, which amplify 702 bp of the amp gene, were carried out (Kakizawa *et al*., 2004).

The 44 oil-palm samples tested and the negative and positive controls were described above. RFLP profiles generated with *Tru*I and *Tsp509*I were compared with those of the reference strains (Table 1). Direct sequencing and sequence assembly were performed from the OP47 amplicon. A phylogenetic tree was produced using available reference strains (Table 1). The full sequence of the *amp* gene was also analyzed with translated nucleotide query, using BLASTP (version BLASTP 2.2.18) (National Center for Biotechnology Information, Bethesda, MD) (Table 3.2).

3.3 Results and discussion

3.3.1 Detecting and identifying phytoplasmas

Nested-PCR assays amplified 1.2-kb DNA fragments of the 16S rDNA in samples from the various tissues tested at different percentages (Figure 3.2). The assays detected phytoplasmas in samples from all 44 symptomatic oil-palm plants from the four plantations surveyed. All samples collected from the seven asymptomatic plants, together with the non-DNA template

controls were negative by nested PCR. Symptoms were correlated to percentages of phytoplasma detection in the diverse oil-palm tissues showing symptoms at different stages in two localities (A and B) (Figure 3.2). Leaves or spears showed 86% to 100% incidence of phytoplasma in 220 samples collected from plants with either mild (scoring 1 or 2) or severe (scoring 3 or 4) symptoms.

Tissues from roots and trunks resulted in only 10% to 60% incidence of phytoplasma detection, regardless of symptom severity. RFLP analysis of the 1.2-kb 16S rDNA amplicons indicated that a phytoplasma belonging to subgroup 16SrI-B ('*Ca.* P. asteris') was present in all symptomatic oil palms. RFLP patterns from the positive samples were indistinguishable from each other and from phytoplasma reference strains belonging to subgroup 16SrI-B (Figure 3.3A). Phytoplasma strain OP47, obtained from a palm hybrid growing in a Palmar del Oriente field, was selected for further molecular characterization. The 1,491-bp 16S rDNA sequence was deposited in GenBank under accession number JX681021 (Table 3.1). This showed 99% of sequence identity with those of the 16SrI, '*Ca.* P. asteris' phytoplasma strains. The sequence of phytoplasma strain OP47 was used for phylogenetic analysis and 20 equally parsimonious trees were constructed, using 27 additional strains of aster yellows (AY) phytoplasmas from different crops (Table 3.1). Results confirmed that phytoplasma strain OP47 belongs to 16SrI group (Figure 3.4A).

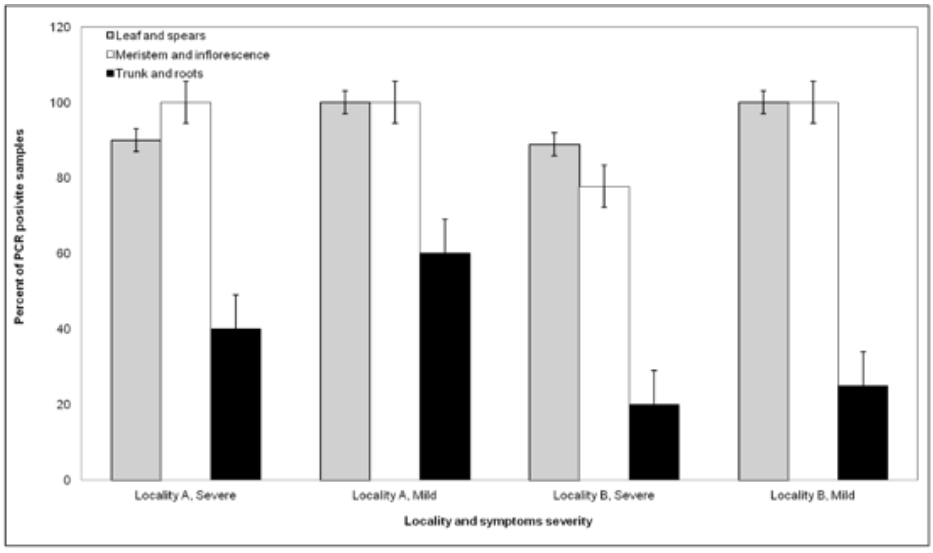
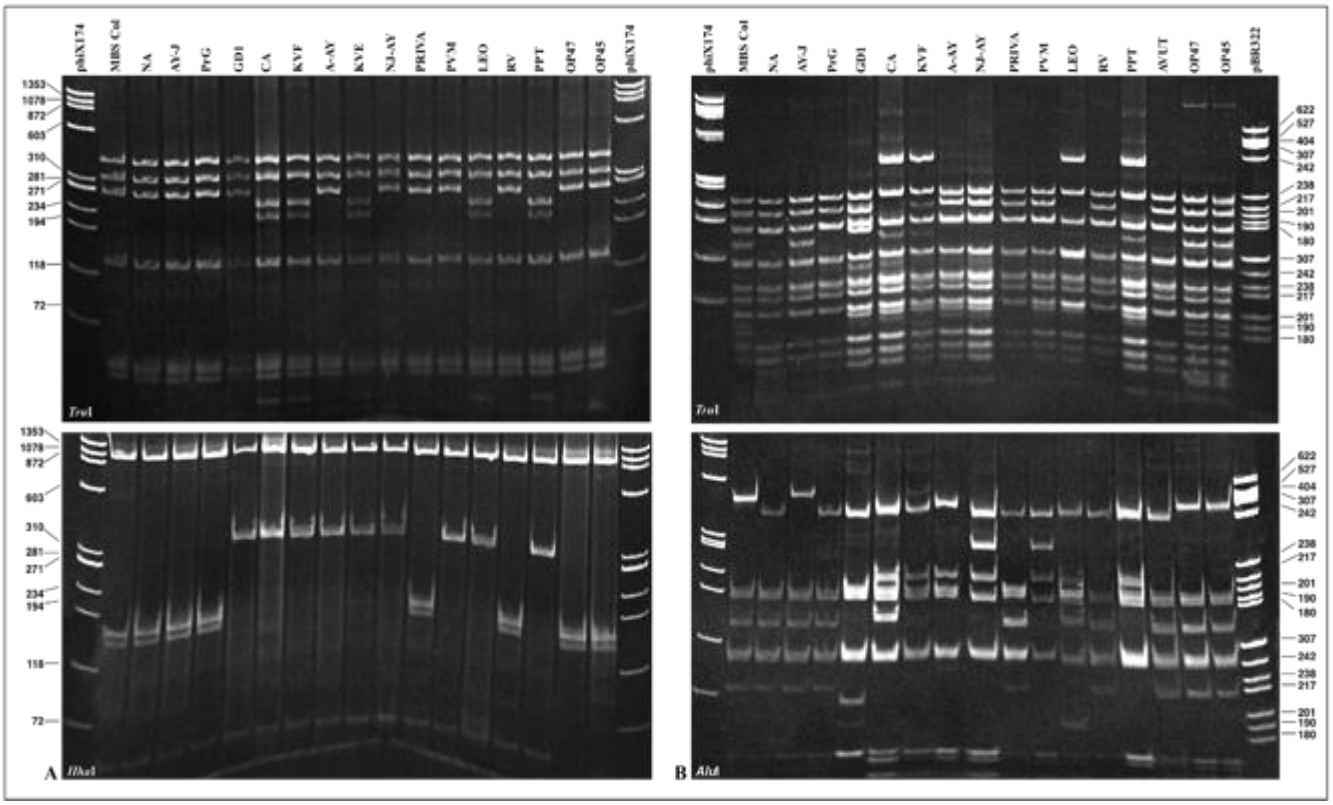


Figure 3.2. Oil palm lethal wilt symptoms observed in relation to percentage of phytoplasma infection in samples from diverse host tissues in localities A and B. Severity scale: mild symptoms with a score of 1 or 2; severe symptoms, 3 or 4. Vertical bars represent standard errors of the means. Two-way analysis of variance for phytoplasma detection related to symptomatic tissues tested indicated significant differences between tissue types sampled ($F = 57.81$ at $P < 0.001$) and no significant differences between phytoplasma detection and symptom severity or geographic locality ($F = 4.90$ at $P = 0.0624$ and $F = 6.45$ at $P = 0.0387$, respectively).

3.3.2 Strain characterization on *groEL*, *rp*, and *amp* genes

Amplicons of expected length (about 1.4 kb) for the *groEL* gene were obtained from 21 out of the 44 oil-palm samples tested. They showed identical RFLP profiles after *Tru1*I and *Alu*I digestion. These profiles were identical to that observed for the MBS phytoplasma strain from Colombia, thus differentiating aster yellows phytoplasmas in oil palm from other AY strains and assigning them to the *groEL*I RFLP subgroup V (Figure 3.3B). The *groEL* sequence from OP47 (1,397 bp) was deposited in GenBank under accession number JX681023. The phylogenetic tree confirmed the differentiation of phytoplasmas from oil palm and maize from Colombia (Figure 3.4B).

PCR assays with the rpF1/rpR1 primer pair amplified the expected fragment length of about 1,200 bp from 18 oil-palm samples. RFLP analyses with four restriction enzymes produced RFLP profiles that were identical to each other. These allowed a clear differentiation of the two oil-palm phytoplasma strains from those AY strains, including the maize bunchy stunt phytoplasma from Colombia (Figure 3.5). The rpF1/rpR1 sequence from OP47 (1,168 bp) was deposited in GenBank under accession number KF434318. The SNPs comparison confirmed the differentiation of phytoplasmas from oil palm and maize bunchy stun phytoplasma from Colombia in the restriction site *Hpy8*I (Table 3.2). However the further differentiation observed after RFLP analyses with *Alu*I and *Taa*I allowed distinguishing oil palm aster yellows phytoplasma from the MBS phytoplasma. These were also differentiated from all other reference strains used not retrieved in SNPs comparison, presumably due to the position of the sequenced fragment outside of the *rp* gene.



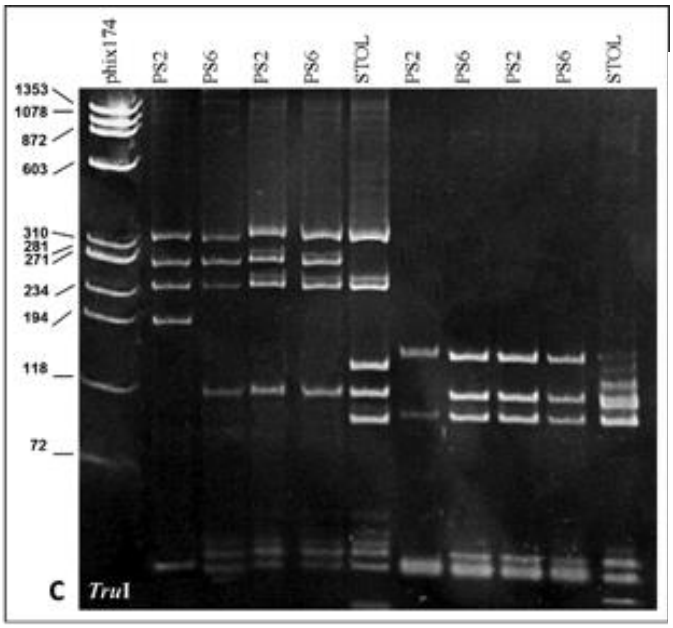


Figure 3.3. RFLP patterns of oil-palm phytoplasma strains OP45, OP47, PS2 and PS6 compared with those of several reference strains from periwinkle. (A) Phytoplasma 16S rDNA amplified in nested-PCR with R16F2n/R16R2 primers and digested with *Tru1*I and *Hha*I restriction enzymes. (B) Phytoplasma *groEL* gene amplified in nested-PCR with AYgroelF/R primers and digested with *Tru1*I and *Alu*I restriction enzymes. (C) Differential patterns obtained with the enzyme *Tru*I from the samples Palma Sicarare2 (PS2) and Palmas Sicarare6 (PS6), STOL (16SrXII group). The acronyms of the strains are described in Table 3.1. Markers: phiX174, phiX174 *Hae*III digested; and pBR322, pBR322 *Hae*I digested.

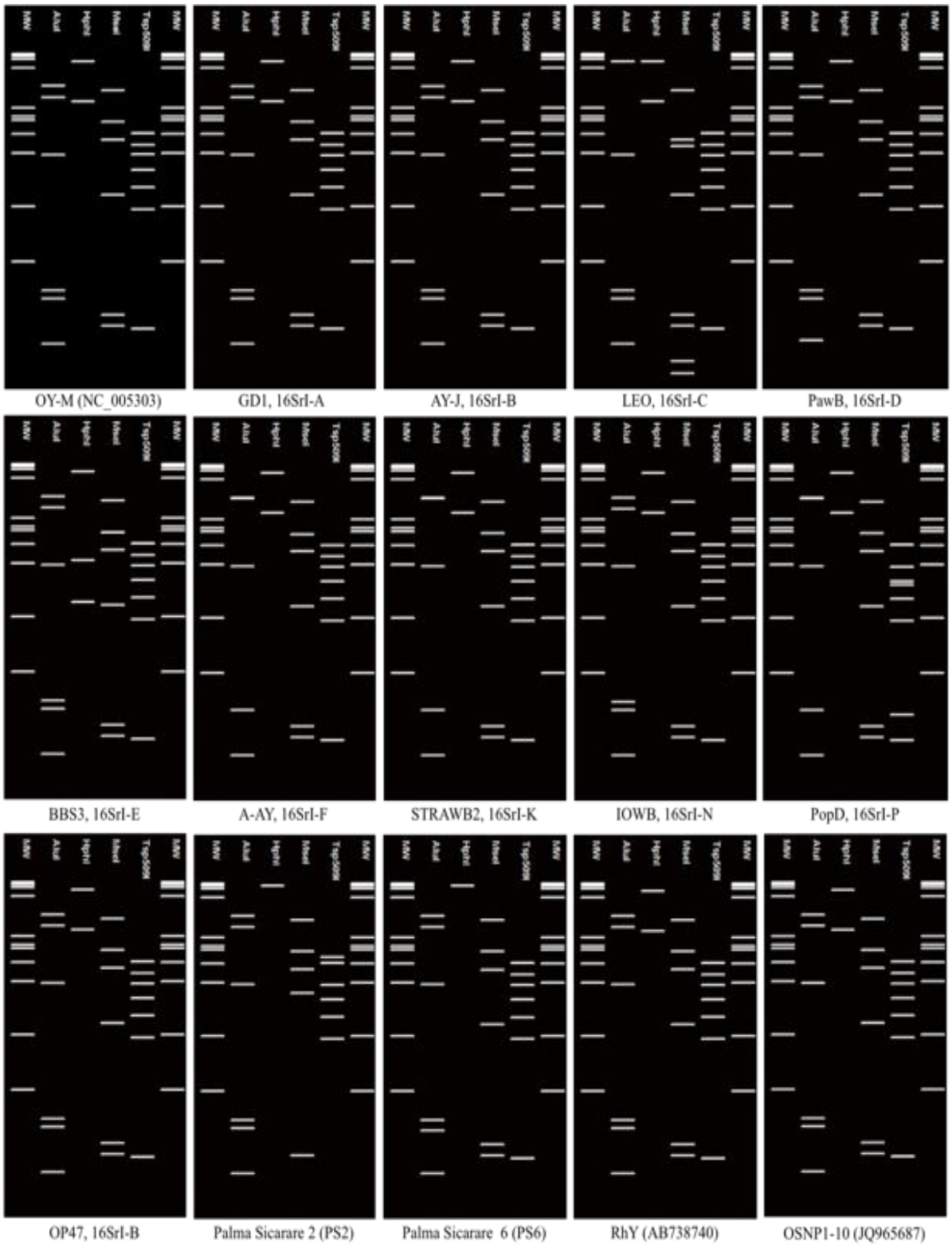


Figure 3.3A. *In silico* RFLP based on the 16S rDNA gene (position 202-1297), using the restriction enzymes *Alu*I, *Hph*I, *Mse*I and *Tsp509*I. Virtual RFLP profiles of the Palma Sicarare 2 (PS2) and Palma Sicarare 6 (PS6) phytoplasma strains, compared with '*Ca.* P. asteris' (16SrI) representatives and the phytoplasma strain OP47 O. The acronyms of the strains are described in Table 1. Markers: phiX174, phiX174 *Hae*III digested.

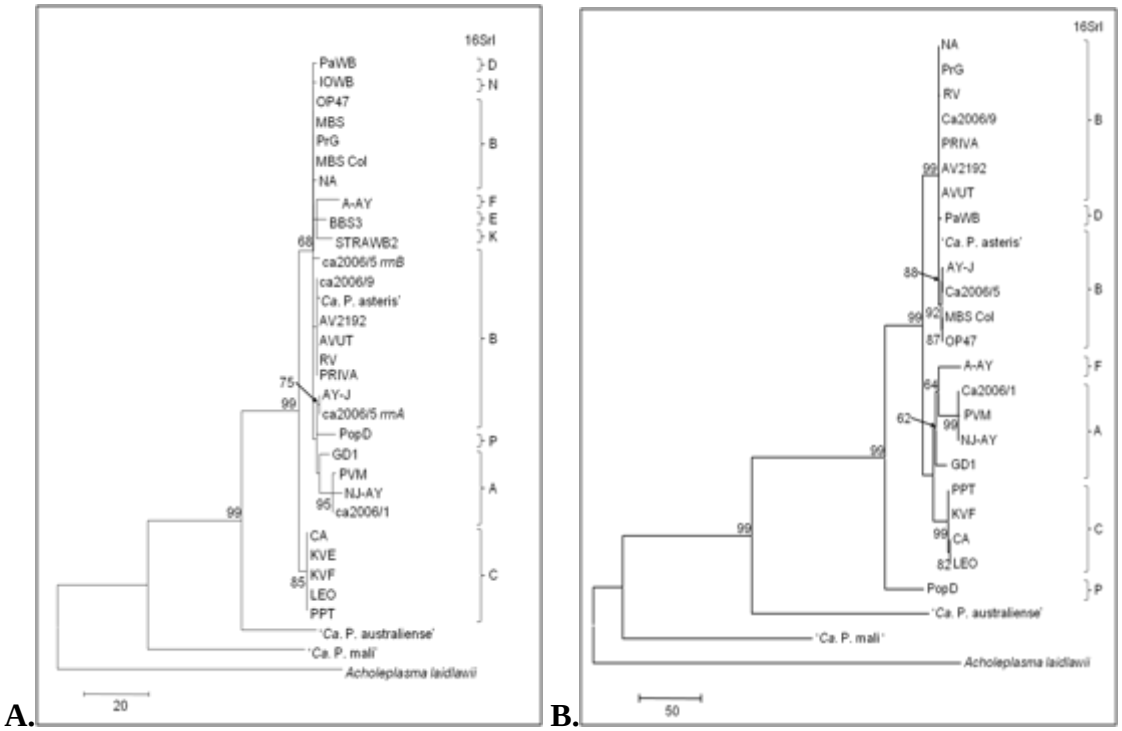


Figure 3.4. Phylogenetic trees constructed by maximum parsimony analysis of (A) 16S rDNA gene sequences and (B) *groEL* gene sequence from selected phytoplasma strains. Strains references employed of 16SrI subgroups are described in Table 1. Oil-palm lethal wilt (OP47); '*Ca.* P. asteris' strain OY-M (NC_005303); '*Ca*. P. australiense' (NC 010544); '*Ca*. P. mali' strain AT (NC 011047) and *A. laidlawii* PG-8A (CP000896). Numbers on the branches are bootstrap values obtained for 1,000 replicates (only values above 60% are shown). Letters in square brackets, represent diverse RFLP subgroups in the 16S rDNA gene of aster yellows strains.

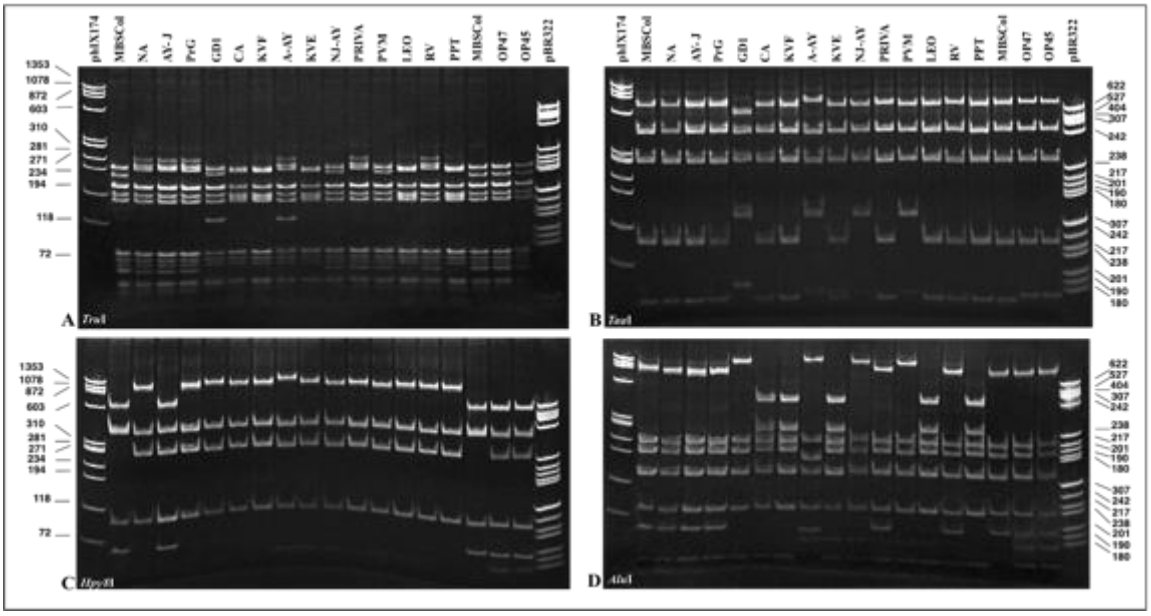


Figure 3.5. Restriction fragment length polymorphism (RFLP) patterns of oil-palm phytoplasma strains OP45 and OP47 compared with several reference strains from periwinkle that were amplified with primers rpF1/rpR1 and digested with restriction enzymes *Tru*I (A); *Taa*I (B); *Hpy8*I (C), and *Alu*I (D). The acronyms of the strains are described in Table 1. Markers: phiX174, phiX174 *Hae*III digested; and pBR322, pBR322 *Hae*I digested.

Amplification of the *amp* gene was obtained for 22 samples. Restriction analysis showed RFLP profiles of all strains from oil palms to be identical to each other and to that of the MBS phytoplasma (data not shown). Sequencing and alignment for OP47 provided a 702-bp sequence. It was deposited in GenBank under accession number JX681022. This sequence encodes 233 amino acids, and its predicted translation showed no significant similarities to any predicted amino acid sequence from aster yellows phytoplasmas available at GenBank (Table 3.3). The phylogenetic tree confirmed the differentiation of phytoplasmas from oil palm and maize in Colombia from other strains tested (data not shown).

The results of this study confirmed the association of a '*Ca.* P. asteris' isolate with oil palm lethal wilt in Colombia. The work carried out expanded knowledge of this oil palm disease since a large geographic area was surveyed and a range of samples from different parts of the plants and from different stages of the disease was examined. Considering the sampling sites from which plants were tested and the presence of phytoplasmas in at least one of the samples from each symptomatic plant, the association of the disease with '*Ca.* P. asteris' phytoplasmas is clear.

The 16S rDNA is a valuable classification tool, but it is not always able to discriminate phytoplasma strains. The finer molecular characterization of the phytoplasma from oil palm indicates that it can be differentiated from all other phytoplasmas in the same ribosomal subgroup enclosing those infecting corn in Colombia. Multigene sequence analysis based on *amp*, *groEL* and *rp* genes indicated that they are useful molecular markers to follow up the Colombian oil palm epidemic.

Only some of the diverse types of samples tested from symptomatic oil palms were negative in PCR assays. This result may be explained by uneven phytoplasma distribution in woody hosts, as recently described (Alvarez *et al*., 2009; EPPO/CABI 1996). The amplification of other genes allowed finer characterization of the phytoplasma strain infecting oil palms in Colombia, and indicated that it can be differentiated from all the other phytoplasma strains in the '*Ca.* P. asteris' group, including a MBS strain from Colombia (Duduk *et al*., 2008). To our knowledge, this is the first study in which the phytoplasma previously associated with lethal wilt of oil palm in Colombia (Alvarez and Claroz, 2003) was characterized. The phytoplasma was assigned to the 16SrI-B '*Ca.* P. asteris' group, which was clearly differentiated from the other reference phytoplasma strains.

Table 3.2. Differential SNP positions in ribosomal protein *s3* sequences, of fifteen '*CA.* P. asteris' strains compared with OP47 strain (oil palm lethal wilt).

	Single nucleotide positions in ribosomal protein *s3*											
Strain	**18**	**124***	**139**	**252**	**277***	**278***	**285***	**444***	**486**	**571**	**667***	**673**
OP47	C	C	A	T	A	A	C	T	C	C	C	A
MBS Col	.	A	.	.	.	.	.	.	.	.	.	.
MBS	T	.	.	.	G	.	.	.	.	.	.	.
PVM	T	.	G	C	G	C	T	A	T	G	T	G
ca2006/1	T	.	G	C	G	C	T	A	T	G	T	G
GD1	T	.	G	C	G	C	T	A	.	G	T	G
ca2006/9	T	.	.	.	G	C	.	.	T	G	.	G
AV2192	T	.	.	.	G	C	.	.	T	G	.	G
AVUT	T	.	.	.	G	C	.	.	T	G	.	G
ca2006/5	T	.	.	.	G	.	.	.	.	.	.	G
KVE	T	.	G	C	G	C	A	A	T	G	.	G
PaWB	T	.	.	.	G	C	.	.	T	G	.	G
BBS3	T	.	G	C	G	T	.	A	T	G	.	G
A-AY	T	.	G	C	G	C	.	A	T	G	T	G
STRAWB2	T	.	G	C	G	T	.	A	T	G	.	G
IOWB	T	.	.	C	G	C	.	.	T	T	.	G

*SNPs in differential restriction sites for RFLP differentiation: 124 and 278 (*Hpy8*I), 277 and 285 (*Tru1*I), 444 (*Alu*I), 667 (*Taa*I). Dots represent nucleotides identical to the OP47 consensus sequence.

Table 3.2a. Differential SNP positions in 16Sr RNA sequences, of fifteen '*Ca.* P. asteris' strains compared with PS2 (Palma Sicare 2), PS6 (Palma Sicarae 6) and OP47 strain (oil palm lethal wilt).

	Single nucleotide positions in 16S ribosomal RNA gene											
Strain[a]	**418***	**616***	**645***	**563**	**798***	**947***	**949***	**970***	**1018**	**1040**	**1089**	**1261**
PS2	G	C	G	A	G	G	A	T	C	G	A	T
PS6	.	.	.	G	.	A	.	.	T	A	G	_
OP47	.	.	.	G	.	A	.	C	T	A	G	.
OSNP1-10	.	.	.	G	.	A	.	C	T	A	G	_
GD1	.	.	.	G	.	A	.	C	T	A	G	.
AY-J	.	.	.	G	.	A	.	C	T	A	G	.
LEO	A	**T**	**A**	G	.	A	.	C	T	A	G	.
PaWB	.	.	.	G	.	A	.	C	T	A	G	.
BBS3	.	.	.	G	.	A	G	C	T	A	G	.
A-AY	.	.	.	G	.	A	.	C	T	A	G	.
STRAWB2	.	.	.	G	A	A	.	C	T	A	G	.
IOWB	.	.	.	G	.	A	.	C	T	A	G	.
PopD	.	.	.	G	A	A	.	C	T	A	G	.
RhY	.	.	.	G	.	A	.	C	T	A	G	.
OY-M	.	.	.	G	.	A	.	C	T	A	G	.

[a]Strains OSNP1-10 (JQ965687) and RhY (AB738740), sequences showing high significant score using using BLAST® tool. Strain OY-M (NC_005303) was used to calculated SNPs positions. *SNPs making differential restriction sites for RFLP differentiation: 616, 645 and 947 (*Tru1*I), 418 and 645 (*Alu*I), 798 and 947 (*Tsp509*I), 970 (*Hph*I). Dots represent nucleotides identical to the PS2 consensus sequence and dashes are gaps positions.

The association of more than one group of phytoplasmas with a specific set of disease symptoms at different locations is not uncommon. Napier grass stunt disease in Kenya was shown to be associated with a phytoplasma from group 16SrXI (Jones *et al*., 2007), while in Ethiopia, it was associated with a phytoplasma from group 16SrIII (Jones *et al*., 2004). Although only the vector for Napier grass stunt phytoplasma in Kenya has been identified (Obura *et al.,* 2009; Koji *et al.,* 2012), these findings support that two different phytoplasmas are being transmitted among plant species at two nearby geographic locations. The epidemiological and etiological significance of the ability of phytoplasmas to move among plant species and into coconut and oil palm is unclear.

Table 3.3. Aster yellows-related reference phytoplasma strains employed for *amp* characterization, and their homology percentages.

Phytoplasma associated disease (acronym)	Geographic origin	GenBank accession number	16SrI RFLP classification[a]	% nucleotide (nt) and amino acid (aa) identity	
				nt	aa
Oil palm lethal wilt (OP47)	Colombia	JX681022	B	100	100
Maize bushy stunt (MBS Col)	Colombia	KF434320	B	100	100
Paulownia witches' broom (PaWB)	Taiwan	AB124810	D	95.3	89.5
Onion yellows (OYW)	Japan	AB124806	B	98.6	95.7
Periwinkle leaf yellowing (PLY)	Taiwan	GQ845122	–	98.3	94.8
Chrysanthemum yellows (CY)	Italy	DQ787852	B	95.3	89.5
Onion yellows (OY-M)	Japan	AB124807	B	97.6	95.2
Onion yellows (OY-NIM)	Japan	AB124808	B	96.3	95.2
Mulberry dwarf (MD)		AB124809	–	91.6	93.8
Rape virescence (RV)	France	AF244540	B	97.6	84.2
Bermudagrass white leaf (AYBG)	Thailand	AB124811	B	97.9	95.2
Iceland poppy yellows (IPY)	Japan	AB242234	B	98.3	95.2
Eggplant dwarf (ED)	Japan	AB242231	B	98.4	94.8
Sumac witches' broom (SWB)	Japan	AB242236	–	90.6	95.3
Porcelain vine witches' broom (PvWB)	Korea	AB242237	–	92.3	80.9
Lettuce yellows (LeY)	Japan	AB242233	B	98.1	83.7
Marguerite yellows (MarY)	Japan	AB242235	B	98.7	94.4
Tomato yellows (TY)	Japan	AB242232	B	98.4	96.1

[a] – means "not described as a ribosomal group". In bold oil palm phytoplasma strain used for similarity comparison. [b] (1), Kakizawa *et al*., 2006, (2) Galetto *et al*., 2008, (3) Barbara *et al*., 2002.

Some phytoplasmas are known to be associated with 'dead-end' hosts, that is, plants to which the vector can transmit pathogens, but from which it cannot acquire them (Weintraub and Beanland, 2006). For example, grapevine is a 'dead-end' host for the stolbur phytoplasma, although this phytoplasma is associated with "bois noir" in grapevine. However,

phytoplasmas are also known to have variable genomes and 'potential mobile units' of DNA within their genomes (Hogenhout *et al*., 2008). Spreading into 'dead-end' hosts is a first step towards these phytoplasmas eventually becoming adapted to these new hosts. Future studies in comparative genomics on more phytoplasma sequences and identification of insect vectors will be key to determine how these organisms are evolving and adapting to old and new plant and insect hosts. To our knowledge, this is the first report of a multigene characterization of conserved genes other than the 16S rRNA gene that distinguished a '*Ca*. P. asteris' strain in a specific host plant.

The close association of a '*Ca*. P. asteris' strain with oil palm lethal wilt disease was also confirmed since all symptomatic plants tested positive for the phytoplasma. Despite the limitations of the data set in number of samples, sampling scheme, number of strains used for molecular characterization, and number of asymptomatic samples, the results of this survey provided important information and tools that can be used to further study the disease. The epidemiology and insect vector identity can be defined for planning disease management strategies and containment of further epidemics.

4 Appendix

4.1 Appendix A.

DNA extractions protocols:

Prince *et al*., 1993 Total nucleic acid was extracted from 1 g of tissue samples (Figure 4.1), which were frozen and ground in liquid nitrogen using a sterilized mortar and pestle with the addition of 8 ml of grinding buffer ($K_2HPO_43H_2O$, 21.7 g/L; KH_2PO_4, 4.1 g/L; Sucrose, 100 g/L; BSA (Fraction V), 1.5 g/L; PVP-10, 20 g/L; L-ascorbic acid, 0.53 g and adjust pH to 7.6 with 2N NaOH). The tissue was transferred in plastic tubes and centrifuged at 13,000 r.p.m for 20 minutes at 4°C. The supernatant was pour off, and 4 ml of extraction buffer (100 mM Tris-HCl pH 8.0, 100 mM EDTA, 250 mM NaCl) and 80 µL of proteinase K (5mg/ml in dH_20) were added. The pellet was gently re-suspended, adding 440 µL of 10% sarkosyl and incubating 1-2 hours at 55°C. After centrifugation of 10 minutes for 8,000 r.p.m at 4°C to pellet debris the supernatant was saved. If necessary, centrifugation at 8,000 r.p.m for 15 minutes to remove remaining debris was done. After, 0.6 volumes isopropanol (~2.5 ml) was added to the supernatant and mixed gently. The tubes were placed at –20°C for 30 minutes (or left overnight at 4°C). After centrifugation at 8,000 r.p.m for 15 minutes the pellet was re-suspended in 3 ml Tris-EDTA (10 mM Tris-Hcl, pH 8.0 and 1 mM EDTA) buffer, adding 75 µl 20% SDS and 60 µl of proteinase K, mixed gently and incubated 1 hour at 37°C. Afterward, 525 µl 5M NaCl and 420 µl CTAB/NaCl (10% CTAB in 0,7 M NaCl) solutions were added, mix thoroughly and incubated 10 minutes at 65°C. An approximately equal volume of chloroform/isoamylic alcohol was added, mixed thoroughly and centrifuged at 8,000 rpm for 10 minutes; this step was repeated until the interface no was visible. The supernatant was transferred to a new tube and an equal volume of chloroform was added, centrifugation at 8,000 r.p.m for 10 minutes was then performed. The supernatant was transferred to a corex tube and added with 2.5 ml of isopropanol alcohol then placed at 4°C overnight. Next day, tubes were centrifuged at 11,000 r.p.m for 30 minutes at 4°C. The supernatant was eliminated and the pellet washed in 1 ml of cold ethanol 70% and then centrifuged 11,000 r.p.m for 10 minutes at 4°C. Pellet was dried and suspended in 50-100 µl of TE buffer and maintained in refrigerator for one month or at –20°C for longer periods.

Gilberston and Dellaporta, 1983 DNA was extracted from 0.2 g of tissue from each plant part. Tissue samples were frozen and macerated with liquid nitrogen using a sterile mortar, the powder obtained, transferred to a microcentrifuge tube of 1.5 ml. Subsequently, 510 µl of extraction buffer (EDTA 0.5 M, 0.4 ml; 5 M NaCl, 0.4 ml; dH_2O, 3.096 ml, ß - mercaptoethanol 14.4 M, 4 µl) was added, the solution was vortexed for 2 minutes. Then 90 µl of 10% SDS was added and vortexed again for 2 minutes, incubating at 65°C for 10 minutes. 150 µl of potassium acetate pH 5.5 was added, this was homogenized turning the tubes up and down and was placed on ice for 10 min. Subsequently centrifuged at 14,000 r.p.m for 10 minutes at 4°C. The supernatant (+ / - 600 µl) was saved and 0.5 volumes of 100% isopropanol (cold) were added. The tubes were placed at –20°C for 30 minutes (or left overnight). After that the tubes were centrifuged at 14,000 r.p.m for 10 minutes and the supernatant was removed, the pellet was washed with 500 µl of 70% ethanol and centrifuged at 10,000 r.p.m for 5 minutes, the supernatant was discarded, the pellet dried and finally resuspended in 30-50 µl of TE buffer.

After the final ethanol precipitation, nucleic acid extracts were resuspended in 30 to 50 µl of buffer and stored in refrigerator for one month or at –20°C for longer periods.

4.2 Appendix B.

Amplification of 16Sr, spacer region, partial 23S rDNA, groEL, rp and amp genes

Each PCR (Polymerase Chain Reaction) reaction was carried out in 0.5-ml tubes in 25-µl reactions, using final concentrations of 20 ng of DNA, 1X buffer, 0.05 U/µl Taq polymerase (Sigma-Aldrich Co., St. Louis, MO, USA), 0.2 mM dNTPs (Invitrogen Life Technologies, Carlsbad, CA, USA) and 0.4 µM of each primer. Nested-PCR assays were performed on amplicons diluted at 1:29 with sterilized high-performance-liquid-chromatography-grade water.

Direct and nested-PCR assays were carried out in a thermal cycler without a heated lid, adding two drops of sterile mineral oil, using the following thermal profile: Thirty-eight PCR cycles were conducted under the following conditions: 1 min (2 min for the first cycle) for denaturation step at 94°C, 2 min for annealing at 55°C or 50°C (see Table 4.1, for specific primer temperature), and 3 min (10 min for the last cycle) for primer extension at 72°C.

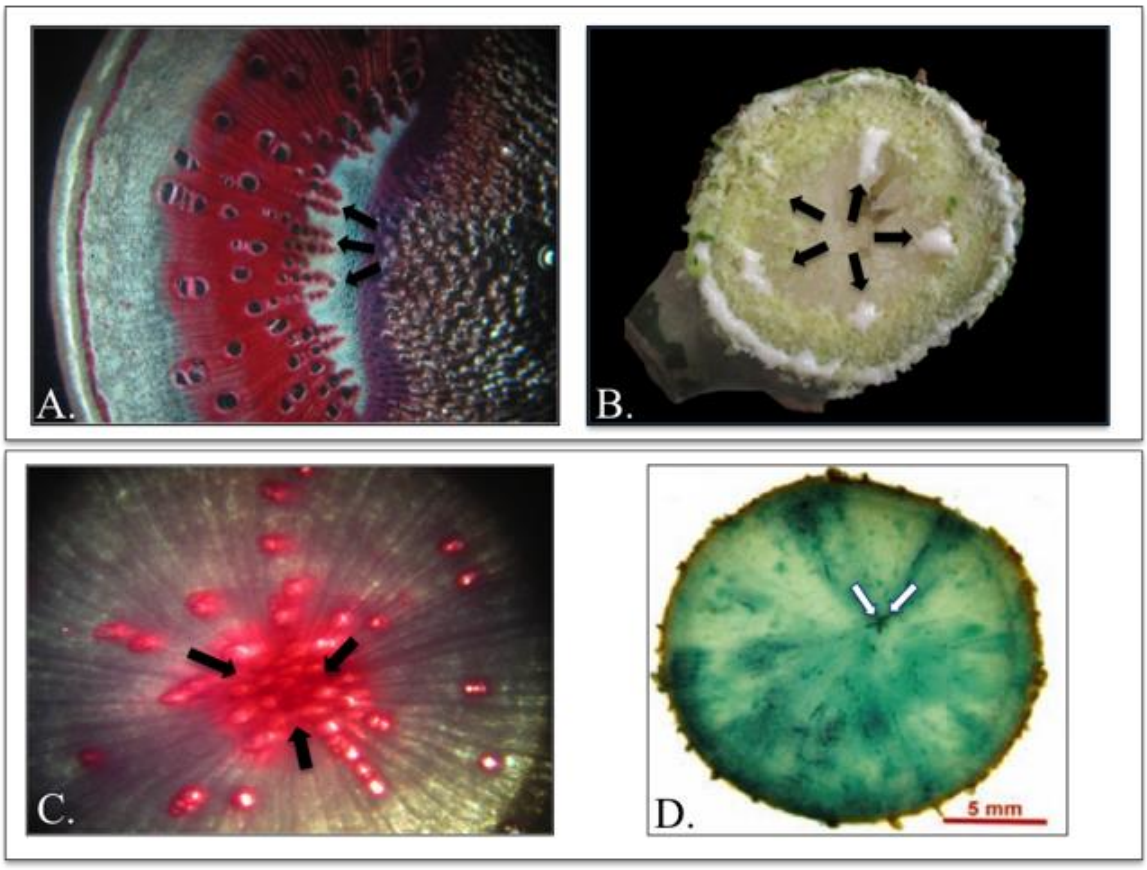


Figure 4.1. Micrograph of young stem **(A)** and field cutting **(B)** of a cassava plant where the secondary phloem is evidenced. Micrograph of a young **(C)** and old **(D)** storage root where the secondary phloem are evidenced. Source: A and C, Cristian Olaya, CIAT 2010; D. Zhang *et al*., 2004.

Table 4.1. Primers used for phytoplasma detection and classification.

Primer Set	**Annealing Temperature (°C)**	**Sequences amplified**	**Reference**
P1A/P7A	55	16Sr—5′ of 23S DNA	Lee *et al*., 2004
P1/P7	55	16Sr—5′ of 23S DNA	Deng and Hiruki, 1991; Schneider *et al*., 1995
B5(16R723f)/P7	50	16Sr—5′ of 23S DNA	Padovan *et al*., 1995
R16mF2/ R16mR1	55	16Sr DNA	Gundersen and Lee, 1996
R16F2/R16R2	55	16Sr DNA	Lee *et al*., 1995
M1(16R758f)/M2(16S1232r)	50	16Sr DNA	Gibb *et al*., 1995
M1/V1731	50	16Sr—5′ of 23S DNA	Martini *et al*., 1999
f01/r01	50	16Sr DNA	Lorenz *et al*.,1995
R16(X)F1/R1	50	16Sr DNA	Lee *et al*., 1994
R16(I)F1/R1	50	16Sr DNA	Lee *et al*., 1994
R16(III)F2/R1	50	16Sr DNA	Lee *et al*., 1994
R16(V)F1/R1	50	16Sr DNA	Lee *et al*., 1994

Primer Set	Annealing Temperature (°C)	Sequences amplified	Reference
R16F1/B6(m23SR)	55	16Sr—*5′* of 23S DNA	Lee *et al*., 1995; Padovan *et al*., 1995
rpF1/R1	55	Ribosomal protein (rp) gene (*rps19*, rplV (*rp22*), rpsC (*rps3*)	Lim and Sears.,1992
AYgroelF/AYampR	55	Molecular chaperonin large subunit groEL	Mitrović *et al*., 2011b
AYgroelF/AYgroelR	55	Molecular chaperonin large subunit groEL	Mitrović *et al*., 2011b
Amp-N1/C1	50	Antigenic membrane protein Amp	Kakizawa *et al*., 2004; Kakizawa et al., 2006
Tuf340/Tuf890	54	Elongation factor Tu	Makarova *et al*., 2012
Tuf400/Tuf835	54	Elongation factor Tu	Makarova *et al*., 2012

Amplification of elongation factor (tuf) gene

PCR and nested-PCR reactions were carried out as described above. Two pairs of primer cocktails were used (Table 1). Each primer cocktail consisted of several variants of the same primer mixed in equimolar proportions to the final concentration of 10 µM. PCR thermal conditions for direct and nested-PCR were 94°C for 3 min followed by 35 cycles of 94°C for 15 sec, 54°C for 30 sec and 72°C for 1 min and a final extension step of 72°C for 7 min.

4.3 Appendix C.

Agarose and Polyacrylamide gels

The PCR and restriction digested products were analyzed by electrophoresis in 1% agarose and 6.7% polyacrylamide gels respectively, stained with ethidium bromide and visualised with UV transilluminator.

4.4 Appendix D.

Restriction Fragment Length Polymorphism (RFLP)

After PCR analysis, RFLP was carried out with fast or normal commercials restrictions enzymes (New England, BioLabs Ipswich, MA and Fermentas, Vilnius, Lithuania), following the manufacturer's instructions.

4.5 Appendix E.

Sequences edition and assembling

Sequences were assembled and edited using ChromasPro v1.7.5 (Technelysium Pty Ltd, Tewantin, QLD, Australia) software, aligned using ClustalW as implemented in MEGA v5.1 (Tamura *et al*. 2011) and adjusted manually. They were then compared with selected nucleotide sequences in the NCBI GenBank database (National Center for Biotechnology Information, Bethesda, MD) using BLAST program (version BLASTN 2.2.18).

Phylogenetic tree analysis

Using the MEGA v5.1 software, neighbor-Joining (NJ) (Saito, 1987) trees were constructed using 1,000 replicates for bootstrap analysis (Felsenstein, 1985). Average intra- and inter-group evolutionary divergences were calculated using the Kimura 2-parameter (K2P) distance model (Kimura, 1992). Groups for determining genetic distances were defined based on the 16Sr and '*Ca.* Phytoplasma' classification systems (Lee *et al.*, 1998; IRPCM, 2004; Martini *et al*., 2007; Wei *et al.*, 2007; Makarova *et al*., 2012). The Tamura-Nei model was used to infer the 16S rRNA Maximun Likelihood (ML) trees, and the Tamura 3-parameter model was used for construction of the tuf, rp and groEL ML trees. In both cases a discrete Gamma distribution (+G) was used to model evolutionary rate differences among sites, assuming that a fraction of sites are evolutionarily invariable (+I) (Nei and Kumar, 2000). Regardless of the genes analyzed, the sequences of *A. laidlawii*, were designated as the outgroup to root the different trees.

In silico restriction and SNPs calculation analysis

Publicly available 16S rDNA, ribosomal protein, antigenic membrane protein, elongation factor sequences from different groups/subgroups and '*Ca.* Phytoplasma' species, were retrieved from GenBank and aligned using ClustalW (http://www.clustal.org) and BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) software package. *In silico* restriction analysis and virtual RFLP plotting was performed using pDRAW32 software (http://www.acaclone.com). Each sequence was digested with several restriction enzymes and a virtual 4.0% agarose gel electrophoresis image was plotted for subsequent RFLP pattern comparisons. The virtual RFLP patterns were compared and a similarity coefficient (F) was calculated for each pair of phytoplasma strains according to the formula F52Nxy/(Nx+Ny), described previously (Lee *et al*., 1998; Nei and Li, 1979).

5 Literature

Alhudaib, K., Arocha, Y., Wilson, M., and Jones, P. 2008. First report of a 16SrI, '*Candidatus* Phytoplasma asteris' group phytoplasma associated with a date palm disease in Saudi Arabia. *Plant Pathol*., 57: 366.

Allem, A.C. 2002. The origins and taxonomy of cassava. Cassava: Biology, Production and Utilization, 1-16.

Alma, A., Bosco, D., Danielli, A., Bertaccini, A., Vibio, M., and Arzone, A. 1997. Identification of phytoplasmas in eggs, nymphs and adults of *Scaphoideus titanus* Ball reared on healthy plants. *Insect Mol. Biol*., 6: 115-121.

Alma, A., Davis, R.E., Vibio, M., Danielli, A., Bosco, D., Arzone, A., and Bertaccini, A. 1996. Mixed infection of grapevines in northern Italy by phytoplasmas including 16S rRNA RFLP subgroup 16SrI-B strains previously unreported in this host. *Plant Dis*., 80: 418-421.

Alma, A., Soldi, G., Tedeschi, R., and Marzocchi, C. 2002. Ruolo di *Hyalesthes obsoletus* Signoret (Homoptera Cixiidae) nella trasmissione del Legno nero della vite in Italia. *Petria*, 12: 411-412.

Alvarez, E., and Claroz, J.L. 2003. Characterization and classification of phytoplasma associated with oil palm (*Elaeis guineensis*). Page 284 in: 8th International Congress of Plant Pathology, Christchurch, New Zealand, Australasian Plant Pathology Society ed.

Alvarez E., Llano G.A., and Mejía, J.F. 2012. Cassava diseases. In: Ospina Patiño, Bernardo; Ceballos, Hernán (eds.). Cassava in the third millennium: Modern production, processing, use, and marketing systems. CIAT-CLAYUCA-CTA, Cali, CO. p. 165-199. (Publication CIAT No. 377).

Alvarez, E., Mejía, J.F., Llano, G.A., Loke, J.B., Calari, A., Duduk, B., and Bertaccini, A. 2009. Characterization of a phytoplasma associated with frogskin disease in cassava. *Plant Dis*., 93: 1139-1145.

Álvarez, E., Pardo, J.M., and Mejía, J.F. 2013. Manejo del 'cuero de sapo', enfermedad limitante de la yuca. Cali, CO : Centro Internacional de Agricultura Tropical (CIAT). 12 p. (Publicación CIAT No. 383) ISBN 978-958-694-119-8.

Alvarez, E., Mejia, J.F., Contaldo, N., Paltrinieri, S., Duduk, B., and Bertaccini, A. 2014. '*Candidatus* Phytoplasma asteris' strains associated with oil palm lethal wilt in Colombia. *Plant Dis*., 98(3): 311-318.

Andersen, M.T., Liefting, L.W., Havukkala, I., and Beever, R.E. 2013. Comparison of the complete genome sequence of two closely related isolates of '*Candidatus* Phytoplasma australiense' reveals genome plasticity. *BMC Genomics*, 14: 529.

Anfoka, G.H.A., Khalil, A.B. and Fattash, I. 2003. Detection and molecular charac- terization of a phytoplasma associated with big bud disease of tomatoes in Jordan. *J. Phytopathol*., 151: 223–227.

Angelini, E., Clair, D., Borgo, M., Bertaccini, A., and Boudon-Padieu, E. 2001. Flavescence dorée in France and Italy -occurrence of closely related phytoplasma isolates and their near relationships to Palatinate grapevine yellows and an alder yellows phytoplasma. *Vitis*, 40: 79-86.

Arashida, R., Kakizawa, S., Ishii, Y., Hoshi, A., Jung, H.Y., Kagiwada, S., Yamaji, Y., Oshima, K., and Namba, S. 2008. Cloning and characterization of the antigenic membrane protein (Amp) gene and in situ detection of Amp from malformed flowers infected with Japanese hydrangea phyllody phytoplasma. *Phytopathology*, 98: 769-775.

Arnaud, G., Malembic-Maher, S., Salar, P., Maixner, M., Marcone, C., Boudon-Padieu, E., and Foissac, X. 2007. Multilocus sequence typing confirms the close genetic inter- relatedness between three distinct Flavescence dorée phytoplasma strain clusters and group 16SrV phytoplasmas infecting grapevine and alder in Europe. *Appl. Environ. Microbiol*., 73: 4001-4010.

Arocha, Y., Lopez, M., Pinol, B., Fernandez, M., Picornell, B., Almeida, R., Palenzuela, I., Wilson, M.R., and Jones, P. 2005. '*Candidatus* Phytoplasma graminis' and '*Candidatus* Phytoplasma caricae', two novel phytoplasmas associated with diseases of sugarcane, weeds and papaya in Cuba. *Int. J. Syst. Evol. Microbiol*., 55: 2451-2463.

Arocha, Y., Piñol, B., Almeida, R., Acosta, K., Quiñones, M., Zayas, T., and Lucas, J.A. 2009. First report of phytoplasmas affecting organoponic crops in central and eastern Cuba. *Pl. Pathol*., 58(4): 793-793.

Bai, X., Zhang, J., Ewing, A., Miller, S.A., Radek, A.J., Shevchenko, D.V., Tsukerman, K., Walunas, T., Lapidus, A., Campbell, J.W., and Hogenhout, S.A. 2006. Living with genome instability: the adaptation of phytoplasmas to diverse environments of their insect and plant hosts. *J. Bacteriol*., 188: 3682–3696.

Barbara, D.J., Morton, A., Clark, M.F., and Davies, D.L. 2002. Immunodominant membrane proteins from two phytoplasmas in the aster yellows clade (chlorante aster yellows and clover phyllody) are highly divergent in the major hydrophilic region. *Microbiology*, 148: 157–167.

Bedendo, I.P., Davis, R.E., and Dally, E.L. 1997. Molecular evidence for the presence of maize bushy stunt phytoplasma in corn in Brazil. *Plant Dis*. 81, 957.

Bedendo, I. P., Davis, R. E., and Dally, E. L. 2000. Detection and identification of the maize bushy stunt phytoplasma in corn plants in Brazil using PCR and RFLP. *Int. J. Pest Manag*. 46(1), 73-76.

Berg, M., Davies, D.L., Clark, M.F., Vetten, J., Maier, G., and Seemüller, E. 1999. Isolation of a gene encoding an immunodominant memebrane protein gene in the apple proliferation phytoplasma and expression and characterization of the gene product. *Microbiology*, 145: 1937-1943.

Berges, R., Cousin, M.T., Roux, J., Maurer, R. and Seemüller, E. 1997. Detection of phytoplasma infections in declining *Populus nigra* 'Italica' trees and molecular differentiation of the aster yellows phytoplasmas identified in various *Populus* species. *Europ. J. Forest Path*. 27: 33–43.

Bertaccini, A. 2007. Phytoplasmas: diversity, taxonomy, and epidemiology. *Front. Biosci.*, 12: 673-689.

Bertaccini, A., 2008. Phytoplasma diseases and climate change. II Taller Internacional de Fitoplasmas, 23 y 25 de Septiembre, Las Habana, Cuba, CD Rom.

Bertaccini, A., and Duduk, B. 2009. Phytoplasma and phytoplasma diseases: a review of recent research. *Phytopath. Medit*., 48: 355-378.

Bertaccini, A., Davis, R.E., and Lee, I-M. 1992. *In vitro* micropropagation for maintenance of mycoplasmalike organisms in infected plant tissues. *Hort. Science*, 27: 1041-1043.

Bertaccini, A., Vibio, M., and Stefani, E. 1995. Detection and molecular characterization of phytoplasmas infecting grapevine in Liguria (Italy). *Phytopath. Medit*., 34: 137-141.

Bertaccini, A., Mittempergher, L., and Vibio, M. 1996. Identification of phytoplasmas associated with a decline of European hackberry (*Celtis australis*). *Ann. Appl. Biol*., 128(2): 245-253.

Bertaccini, A., Carraro, L., Davies, D., Laimer da Câmara Machado, M., Martini, M., Paltrinieri, S., and Seemüller, E. 2000. Micropropagation of a collection of phytoplasma strains in periwinkle and other host plants. Page 101 in: 13th Congress of IOM, Fukuoka, Japan.

Bertaccini, A., Contaldo, N., Calari, A., Paltrinieri, S., Windsor, H.M., and Windsor, D. 2010. Preliminary results of axenic growth of phytoplasmas from micropropagated infected periwinkle shoots. 18th Congress IOM, Chianciano Terme, Italy, July 11-16, n. 147: 153.

Bertamini, M., and Nedunchezhian, N. 2001. Effect of phytoplasma, stolbur-subgroup (Bois noir-BN)] of photosynthetic pigments, saccarides, ribulose-1,5-bisphosphate carboxylase, nitrate and nitrite reductases and photosynthetic activities in field-grow grapevine (*Vitis vinifera* L. cv Chardonnay) leaves. *Photosynthetica*, 39: 119-122.

Bertamini, M., Nedunchezhian, N., Tomasi, F., and Grando, M.S. 2002. Phytoplasma [Stolbur-subgroup (Bois Noir-BN)] infection inhibitis photosynthetic pigments, ribulose- 1,5-biphosphate carboxylase and photosynthetic activities in field grown grapevine (*Vitis vinifera* L. cv. Chardonnay). *Physiol. and Mol. Plant Pathol*., 61: 357-366.

Bianco, P.A., Bulgari, D., Casati, P., and Quaglino, F. 2011. Conventional and novel strategies for the phytoplasma diseases containment. *Phytopath. Moll*., 1(2): 77-82.

Bisognin, C., Schneider, B., Salm, H., Grando, M.S., Jarausch, W., Moll, E., and Seemüller, E. 2008. Apple proliferation resistance in apomictic rootstocks and its relationship to phytoplasma concentration and simple sequence repeat genotypes. *Phytopathology*, 98: 153-158.

Blomquist, C.L., and Kirkpatrick, B.C. 2002. Identification of phytoplasma taxa and insect vectors of peach yellow leaf roll disease in California. *Plant Dis*., 86: 759-763.

Blomquist, C.L., Barbara, D.J., Davies, D.L., Clark, M.F., and Kirkpatrick, B.C. 2001. An immunodominant membrane protein gene from the western X-disease phytoplasma is distinct from those of other phytoplasmas. *Microbiology*, 147: 571-580.

Botti, S., and Bertaccini, A. 2007. Grapevine yellows in northern Italy: molecular identification of Flavescence dorée phytoplasma strains and of Bois noir phytoplasmas. *J. Appl. Microbiol*., 103: 2325-2330.

Boudon-Padieu, E. 2002. Flavescence dorée of the grapevine: knowledge and new developments in epidemiology, etiology and diagnosis. *ATTI Giornate Fitopatologiche*, 1: 15–34.

Brioso, P.S.T., Montano, H.G., Figueiredo, D.V., Poltronieri, L.S., and Furlan Jr J. 2006. Amarelecimento fatal do dendezeiro: seqüenciamento parcial do fitoplasma associado. *Summa Phytopathol.*, 32: 50.

Brown, S.E., Been, B.O., and McLaughlin, W.A. 2006. Detection and variability of the lethal yellowing group (16SrIV) phytoplasma in the *Cedusa* sp. (Hemiptera: Auchenorrhyncha: Derbidae) in Jamaica. *Ann. Appl. Biol*., 149: 53-62.

Brown, S.E., Been, B.O., and McLaughlin, W.A. 2008a. First report of lethal yellowing group (16SrIV) of phytoplasmas in Vernonia cinerea in Jamaica. *Plant Dis*., 92: 1132.

Brown, S.E., Been, B.O., and McLaughlin, W.A. 2008b. First report of the presence of the lethal yellowing group (16SrIV) of phytoplasmas in the weeds *Emilia fosbergii* and *Synedrella nodiflora* in Jamaica. *Plant Pathol*., 57: 770.

Calvert, L.A., and Thresh, J. 2002. The viruses and virus diseases of cassava. Cassava: biology, production and utilization, 237-260.

Calvert, L.A., Cuervo, M., Lozano, I., Villareal, N., and Arroyave, J. 2008. Identification of three strains of a virus associated with cassava plants affected by frogskin disease. *J. Phytopath*., 156: 647-653.

Calvert, L., and Cuervo, M. 2002. Cuero de Sapo: yuca en el tercer milenio. Sistemas modernos de producción, procesamiento, utilización y comercialización. Eds: B. Ospina y H. Caballos. p. 264-268.

Carvajal-Yepes, M., Olaya, C., Lozano, I., Cuervo, M., Castaño, M., and Cuellar, W.J. 2013. Unraveling complex viral infections in cassava (*Manihot esculenta* Crantz) from Colombia. Virus Res. http://dx.doi.org/10.1016/j.virusres.2013.12.011

Castiblanco C., Etter A., and Aide, M. 2013. Oil palm in Colombia: a model of future expansion. *Environ. Sci. Pol*., 27: 172-83.

Castro, S., and Romero, J. 2002. The association of clover proliferation phytoplasma with stolbur disease of pepper in Spain. *J. Phytopathol*., 150: 25–29.

Ceballos, H. 2012. Cassva in Colombia and the world: new prospects for a millennial crop. In: Ospina Patiño, Bernardo; Ceballos, Hernán (eds.). Cassava in the third millennium: Modern production, processing, use, and marketing systems. CIAT-CLAYUCA-CTA, Cali, CO. p. 165-199. (Publication CIAT No. 377).

Chaparro-Martínez, E.I., and Trujillo-Pinto, G. 2001. First report of frog skin disease in cassava (*Manihot esculenta*) in Venezuela. *Plant Dis*., 85(12): 1285.

Chen, J., Pu, X., Deng, X, Liu S., Li H., and Civerolo, E. 2008. A phytoplasma closely related to the pigeon pea witches'-broom phytoplasma (16SrIX) is associated with citrus huanglongbing symptoms in the state of São Paulo, Brazil. *Phytopathology*, 98: 977-984.

Chen, M.H., and Hiruki, C. 1975. Electron microscopy of mycoplasma-like bodies associated with clover proliferation disease. *Proc. Am. Phytopathol. Soc*., 2: 52.

Choueiri, E., Salar, P., Jreijiri, F., El-Zammar, S., Massaad, R., Abdul-Nour, H., Bové, J.M., Danet, J.L., and Foissac, X. 2007. Occurrence and distribution of 'Candidatus Phytoplasma trifolii' associated with diseases of solanaceous crops in Lebanon. *Eur. J. Pl. Pathol*., 118: 411–416.

Christensen, N.M., Axelsen, K.B., Nicolaisen, M., and Schultz, A. 2005. Phytoplasmas and their interactions with their hosts. *Trend Pl. Sci*., 10: 526-535.

Cieślińska, M. 2011. Less common phytoplasmas infecting stone fruit trees. *J. Pl. Prot. Res*., 51(4): 435-440.

Cimerman, A., Pacifico, D., Salar, P., Marzachì, C., and Foissac, X. 2009. Striking diversity of vmp1, a variable gene encoding a putative membrane protein of the stolbur phytoplasma. *Appl. Environ. Microbiol*., 75: 2951–2957.

Contaldo, N., Bertaccini, A., Paltrinieri, S., and Windosor, H.M. 2012. Axenic culture of plant pathogenic phytoplasmas. *Phytopath. Medit*., 51: 607-617.

Contaldo, N., Bertaccini, A., Paltrinieri, S., Windsor, D., and Windsor H. 2013. Cultivation of several phytoplasmas from a micropropagated plant collection. *Petria*, 23: 13-18.

Cronje, P., Tymon, A.M., Jones, P. and Bailey, R.A. 1998. Association of a phytoplasma with a yellow leaf syndrome of sugarcane in Africa. *Annals App. Biol*. 133, 177–186.

Cronjé, P., Dabek, A.J., Jones, P., and Tymon, A.M. 2000. Slow decline: a new disease of mature date palms in North Africa associated with a phytoplasma. *Pl. Pathol*., 49: 804.

D'Amelio, R., Marzachì, C., and Bosco, D. 2010. Activity of benzothiadiazole on chrysanthemum yellows phytoplasma ('*Candidatus* Phytoplasma asteris') infection in daisy plants. *Crop Protection*, 29(10): 1094-1099.

Daire, X., Clair, D., Reinert, W., and Boudon-Padieu, E. 1997. Detection and differentiation of grapevine yellows phytoplasmas belonging to the elm yellows group and to the stolbur subgroup by PCR amplification of nonribosomal DNA. *Eur. J. Pl. Pathol*., 103: 507-514.

Davis, R.E., and Lee, I-M. 1993. Cluster specific polymerase chain reaction amplification of 16S rDNA sequences for detection and identification of mycoplasmalike organisms. *Phytopathology*, 83: 1008-1011.

Davis, R.E., Jomantiene, R., Dally, E.L., and Wolf, T.K. 1998. Phytoplasmas associated with grapevine yellows in Virginia belong to group 16SrI, subgroup A (tomato big bud phytoplasma subgroup), and group 16SrIII, new subgroup I. *Vitis*, 37: 131-137.

Davis, R.E., Jomantiene, R., Kalvelyte, A., and Dally, E.L. 2003a. Differential amplification of sequence heterogenous ribosomal RNA genes and classification of the *Fragaria multicipita* phytoplasma. *Microbiol. Res*., 158: 229-236.

Davis, R.E., Jomantiene, R., Zhao, Y., and Dally, E.L. 2003b. Folate biosynthesis pseudogenes, ψ fol P and ψ fol K, and an O-Sialoglycoprotein endopeptidase gene homolog in the phytoplasma genome. *DNA and Cell Biol*., 22: 697-706.

Davis, R.E., Dally, E., Zhao, Y., Lee, I-M., Jomantiene, R., Detweiler, A.J., and Putnam, M.L. 2010. First report of a new subgroup 16SrIX-E ('*Candidatus* Phytoplasma phoenicium'-related) phytoplasma associated with juniper witches broom disease in Oregon, USA. *Plant Pathol*., 59: 1161.

Davis, R.E., Zhao, Y., Dally, E.L., Jomantiene, R., Lee, I-M., Wei, W., and Kitajima, E.W. 2012. '*Candidatus* Phytoplasma sudamericanum', a novel taxon, and strain PassWB- Br4, a new subgroup 16SrIII-V phytoplasma, from diseased passion fruit (*Passiflora edulis* f. *flavicarpa* Deg.). *Int. J. Syst. Evol. Microbiol*., 62: 984–989.

Davis, R.E., Zhao, Y., Dally, E., Lee, I-M., Jomantiene, R., and Douglas, S.M. 2013. '*Candidatus* Phytoplasma pruni', a novel taxon associated with X-disease of stone fruits, *Prunus* spp.: multilocus characterization based on 16S rRNA, secY, and ribosomal protein genes. *Int. J. Syst. Bacteriol*., 63: 766–776.

Davis, R.I., Arocha, Y., Jones, P., and Malay, A. 2005. First report of the association of phytoplasmas with plant diseases in the territory of Wallis and Futuna. *Austral. Pl. Pathol*., 34(3): 417-418.

Deng, S., and Hiruki, C. 1991. Amplification of 16S rRNA genes from culturable and non- culturable mollicutes. *J. Microbiol. Meth*., 14: 53-61.

Desmidts, M. and Rassel, A. 1974. Presence of mycoplasma-like organisms in plant species affected by yellows symptoms in Upper Volta – relationships with cotton phyllody. *FAO Pl. Prot. Bull.*, 22: 138–141.

Doi, Y., Teranaka, M., Yora, K., and Asuyama, H. 1967. Mycoplasma or PLT grouplike microrganisms found in the phloem elements of plants infected with mulberry dwarf, potato witches' broom, aster yellows or pawlownia witches' broom. *Ann. Phytopath. Soc. Japan*, 33: 259-266.

Duduk, B., and Bertaccini, A. 2006. Corn with symptoms of reddening: new host of stolbur phytoplasma. *Plant Dis*., 90: 1313-1319.

Duduk, B., Mejía, J.F., Paltrinieri, S., Contaldo, N., Alvarez, E., Varón, F., and Bertaccini, A. 2008. Molecular differentiation of phytoplasmas affecting corn in Colombia and Serbia. In CDrom: II Taller Internacional de Fitoplasmas, La Habana, Cuba.

Duduk, B., Mejia, J.F., Calari, A., and Bertaccini, A. 2008. Identification of 16SrIX group phytoplasmas infecting Colombian periwinkles and molecular characterization on several genes. 17th Congress IOM, Tienjin, China, 6-11 July, 112: 83.

Duduk, B., Calari, A., Paltrinieri, S., Duduk, N., and Bertaccini, A. 2009. Multi-gene analysis for differentiation of aster yellows phytoplasmas infecting carrots in Serbia. *Ann. Appl. Biol*., 154: 219-229.

Duduk, B., Tian, J.B, Contaldo, N., Fan, X.P., Paltrinieri, S., Chen, Q.F., Zhao, Q.F., and Bertaccini, A. 2009. Occurrence of phytoplasmas related to stolbur and to '*Candidatus* Phytoplasma japonicum' in woody host plants in China. *J. Phytopath*., 158: 100-104.

Ebbert, M.A., Jeffers, D.P., Harrison, N.A., and Nault, L.R. 2001. Lack of specificity in the interaction between two maize stunting pathogens and field collected *Dalbulus* leafhoppers. *Entomol. Exp. and Aplicata*, 101: 49-57.

Ember, I., Acs, Z., Munyaneza, J. E., Crosslin, J.M., and Kolber, M. 2011. Survey and molecular detection of phytoplasmas associated with potato in Romania and southern Russia. *Eur J. Plant Path*., 130(3): 367-377.

Engelbrecht, M., Joubert, J., and Burger, T.J. 2010. First report of aster yellows phytoplasma in grapevines in South Africa. *Plant Dis*., 94: 373.

EPPO/CABI (European and Mediterranean Plant Protection Organization, and CAB International). 1996. Apple proliferation phytoplasma. Pages 959-962 in: Quarantine Pests for Europe. 2nd edn. Eds I.M. Smith, D.G. McNamara, P.R. Scott and K.M. Harris. Wallingford, UK: CAB International.

Eroglu, S., Ozbek, H., and Sahin, F. 2010. First report of group 16SrXII phytoplasma causing stolbur disease in potato plants in the eastern and southern Anatolia regions of Turkey. *Plant Dis*., 94: 1374.

FAOstat, F. 2014. Agriculture organization of the United Nations. Statistical database.

FEDEPALMA. 2010. Es possible controlar la marchitez letal si se aplican las recommendaciones de Cenipalma. *El Palmicultor*, 458:12-15.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, 39: 783–791.

Fernandez, E.G.B., Calari, A., Hanzer, V., Katinger, H., Bertaccini, A., and Laimer, M. 2007. Phytoplasma infected plants in Austrian forests: role as a reservoir?. *Bull. Insectol*., 60(2): 391.

Firrao, G., Garcia Chapa, M., and Marzachì, C. 2007. Phytoplasmas: genetics, diagnosis and relationships with the palnt and insect host. *Front. Bioscie*., 12: 1352-1375.

Firrao, G., Martini, M., Ermacora, P., Loi, N., Torelli, E., Foissac, X., and Palmano, S. 2013. Genome wide sequence analysis grants unbiased definition of species boundaries in '*Candidatus* Phytoplasma'. *Syst. Appl. Microbiol*., 36(8): 539-548.

Flôres, D., Haas, I.C., Canale, M.C., and Bedendo, I.P. 2013. Molecular identification of a 16SrIII-B phytoplasma associated with cassava witches' broom disease. *Eu. J. Pl. Pathol*., 137(2): 237-242.

Foissac, X., and Wilson, M.R. 2010. Current and possible future distributions of phytoplasma diseases and their vectors. In: Weintraub, P. G., and Jones, P. (Eds.). (2010). Phytoplasmas: genomes, plant hosts and vectors. Cabi. 331 p.

Franco L., Contaldo N., Martinez C., Calari A., Paltrinieri S., Duduk B., Bertaccini A. 2010. Complex phytoplasma infection in declining liquidambar trees in Colombia. 18th Congress of the International Organization for Mycoplasmology, Chianciano Terme, Italy, July 11-16, n. 153: 157.

Frison, E.A., and Feliu, E. Eds. 1991. FAO/IBPGR Technical guidelines for the safe movement of cassava germplasm. Vol. 8. Bioversity International.

Gajardo, A., Fiore, N, Prodan, S., Paltrinieri, S., Botti, S., Pino, A.M., Zamorano, A., Montealegre, J., and Bertaccini, A. 2009. Phytoplasmas associated with grapevine yellows disease in Chile. *Plant Dis*., 93: 789-796.

Galdeano, E., Guzmán, F.A., Fernández, F., and Conci, L.R. 2013. Genetic diversity of 16SrIII group phytoplasmas in Argentina. Predominance of subgroups 16SrIII-J and B and two new subgroups 16SrIII-W and X. *Eu. J. Pl. Pathol*., 137(4): 753-764.

Galetto, L., Fletcher, J., Bosco, D., Turina, M., Wayadande, A., and Marzachì, C. 2008. Characterization of putative membrane protein genes of the '*Candidatus* Phytoplasma asteris', chrysanthemum yellows isolate. *Can. J. Microbiol*., 54: 341-351.

Garcia-Chapa, M., Medina, V., Viruel, M.A., Lavina, A., and Battle, A. 2003. Seasonal detection of pear decline phytoplasma by nested-PCR in different pear cultivars. *Plant Pathol*., 52: 513–520.

Garnier, M. 2000. The stolbur phytoplasma: a ubiquitous agent. *C.R. Ac. Agric. de France*, 86: 27–33.

Gilbert, G.S., and Parker, I.M. 2008. Porroca: an emerging disease of coconut in Central America. *Plant Dis*., 92(5): 826-830.

Gilbertson, L., and Dellaporta, S.L. 1983. Molecular extraction DNA protocols. Pages 395–397 in: Molecular biology of plants. C. S. Harbor, ed. Cold Spring Harbor Laboratory, New York.

Girsova, N., Bottner, K.D., Mozhaeva, K.A., Kastalyeva, T.B.,Owens, R.A., and Lee, I-M. 2008. Molecular detection and identification of group 16SrI and 16SrXII phytoplasmas associated with diseased potatoes in Russia. *Plant Dis*., 92: 654.

Gomes, E. A., Jardim, S. N., Guimarães, C. T., Souza, I. R. P. D., and Oliveira, E. D. 2004. Genetic variability of Brazilian phytoplasma and spiroplasma isolated from maize plants. *Pesq. Agrop. Bras*., 39(1), 61-65.

González, G., San Pedro, J., Arencibia, N., Ruiz, M., Fernández, J., Valdés, S., and Quiala, I. 2002. Virus y fitoplamas en el cultivo del maíz (*Zea mays*, L.) en Cuba. Distibución y diagnóstico. *Fitosanidad*, 6(4), 3-6.

Granett, A.L. and Gilmer, R.M. 1971. Mycoplasmas associated with X-diseases in various *Prunus* species. *Phytopathology*, 61: 1036–1037.

Gundersen-Rindal, D.E. and Lee, I-M. 1996. Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. *Phytopath. Medit*., 35: 144-151.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Ac. Symp. Series*, 41: 95-98.

Hanboonsong, Y., Choosai, C., Panyim, S. and Damak, S. 2002. Transovarial transmission of sugarcane white leaf phytoplasma in the insect vector *Matsumuratettix hiroglyphicus* (Matsumura). *Insect. Mol. Biol*., 11: 97-103.

Harrison, N.A., Bourne, C.M., Cox, R.L., Tsai, J.H., and Richardson, P.A. 1992. DNA probes for detection of mycoplasmalike organisms associated with lethal yellowing disease of palms in Florida. *Phytopathology*, 82: 216-224.

Harrison, N.A., Richardson, P.A., Tsai, J.H., Ebbert, M.A., and Kramer, J.B. 1996. PCR assay for detection of the phytoplasma associated with maize bushy stunt disease. *Pl. Dis*., 80: 263-269.

Harrison, N.A., Myrie, W., Jones, P., Carpio, M.L., Castillo, M., Doyle, M.M., and Oropeza, C. 2002a. 16S rRNA interoperon sequence heterogeneity distinguishes strain populations of palm lethal yellowing phytoplasma in the Caribbean region. *Ann. Appl. Biol*., 141:183-193.

Harrison, N.A., Womack, M., and Carpio, M.L. 2002b. Detection and characterization of a lethal yellowing (16SrIV) group phytoplasma in Canary Island date palms affected by lethal decline in Texas. *Plant. Dis*., 86: 676-681.

Harrison, N.A., Helmick, E.E., and Elliot, M.L. 2008. Lethal yellowing-type diseases of palms associated with phytoplasmas newly identified in Florida, USA. *Ann. Appl. Biol*., 153: 85-89.

Harrison, N.A., Helmick, E.E., and Elliott, M.L. 2009. First report of a phytoplasma-associated lethal decline disease of Sabal palmetto in Florida, USA. *Plant Pathol*., 58: 789.

Harrison, N., Davis, R. E., Oropeza, C., Helmick, E., Narvaez, M., Eden-Green, S., Dollet, M., and Dickinson, M. 2014. '*Candidatus* Phytoplasma palmicola', a novel taxon associated with a lethal yellowing-type disease (LYD) of coconut (*Cocos nucifera* L.) in Mozambique. *Int. J. Syst. Evol. Microbiol*., doi: 10.1099/ijs.0.060053-0.

Hiruki C and Wang K. 2004. Clover proliferation phytoplasma: '*Candidatus* Phytoplasma trifolii'. *Int. J. Syst. Evol. Microbiol*.,, 54(4): 1349- 1353.

Himeno, M., Neriya, Y., Minato, N., Miura, C., Sugawara, K., Ishii, Y., Yamaji, Y., Kakizawa, S., Oshima, K., Namba, S. 2011. Unique morphological changes in plant pathogenic phytoplasma-infected petunia flowers are related to transcriptional regulation of floral homeotic genes in an organ-specific manner. *Plant J*., 67: 971–979.

Hogenhout, S. A., Oshima, K., Ammar, E-D., Kakizawa, S., Kingdom, H.N., and Namba, S. 2008. Phytoplasmas: bacteria that manipulate plants and insects. *Mol. Plant Pathol*., 9: 403–423.

Hoshi, A., Oshima, K., Kakizawa, S., Ishii, Y., Ozeki J., Hashimoto, M., Komatsu, K., Kagiwada, S., Yamaji, Y., and Namba, S. 2009. A unique virulence factor for proliferation and dwarfism in plants identified from a phytopathogenic bacterium. *PNAS USA*, 106: 6416-6421.

Hosseini, P., Bahar, M., Madani, G., and Zirak, L. 2011. Molecular characterization of phytoplasmas associated with potato purple top disease in Iran. *J. Phytopathol*., 159: 241– 246.

Howeler, R.. 2012. Recent trends in production and utilization of cassava in Asia. In: Howeler, Reinhardt H. (ed.). The cassava handbook: A reference manual based on the asian regional cassava training course, held in Thailand. CIAT, Bangkok, TH. p. 258-304.

IRPCM. 2004. '*Candidatus* Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonise plant phloem and insects. *Int. J. Syst. Evol. Microbiol*., 54: 1243-1255.

Ishii, Y., Kakizawa, S., Hoshi, A., Maejima, K., Kagiwada, S., Yamaji, Y., Oshima, K., and Namba, S. 2009. In the non-insect-transmissible line of onion yellows phytoplasma (OY- NIM), the plasmid-encoded transmembrane protein ORF3 lacks the major promoter region. *Microbiology*, 155: 2058-2067.

Ishiie, T., Doi, Y., Yora, K., and Asuyama, H. 1967. Suppressive effects of antibiotics of tetracycline group on symptom developement of mulberry dwarf disease. *Ann. Phytopath. Soc. Japan*, 33: 267-275.

Jacobs, K.A., Lee, I-M., Griffiths, H.M., Miller, F.D.Jr., and Bottner, K.D. 2003. A new member of the clover proliferation phytoplasma group (16SrVI) associated with elm yellows in Illinois. *Plant Dis*., 87: 241-246.

Jagoueix-Eveillard, S., Tarendau, F., Guolter, K., Danet, J.L., Bové, J.M., and Garnier, M. 2001. Catharanthus roseus genes regulated differentially by mollicute infections. *Mol. Plant-Microbe Interact*., 14: 225-233.

Jayasinghe, U., Pineda, B., and Lozano, J.C. 1984. Antholysis in cassava (*Manihot esculenta* Crantz) possibly caused by mycoplasma-like organisms. *J. Phytopath*., 109(4): 295-300.

Jomantiene, R., and Davis, R.E. 2006. Clusters of diverse genes existing as multiple, sequence-variable mosaics in a phytoplasma genome. *FEMS Microbiol. Lett*., 255: 59-65.

Jomantiene, R., Zhao, Y., and Davis, R.E. 2007. Sequence-variable mosaics: composites of recurrent transposition characterizing the genomes of phylogenetically diverse phytoplasmas. *DNA Cell Biol.*, 26: 557-564. Erratum 26: 695.

Jones, P., Arocha, Y., Sheriff, T., Proud, J., Abebe, G., and Hanson, J. 2007. A stunting syndrome of Napier grass in Ethiopia is associated with a 16SrIII group phytoplasma. *Plant Pathol*., 56: 345.

Jones, P., Cockbain, A.J., and Freigoun, S.O. 1984. Association of a mycoplasma-like organism with broad bean phyllody in the Sudan. *Plant Pathol*., 33: 599–602.

Jones, P., Devonshire, B.J., Holman, T.J., and Ajanga, S. 2004. Napier grass stunt: a new disease associated with a 16SrXI group phytoplasma in Kenya. *Plant Pathol*., 53: 519.

Jović, J., Cvrković, T., Mitrović, M., Petrović, A., Krstić, O., Krnjajić, S., and Toševski, I. 2011. Multigene sequence data and genetic diversity among '*Candidatus* Phytoplasma ulmi' strains infecting *Ulmus* spp. in Serbia. *Plant Pathol*., 60(2): 356-368.

Jung, H-Y., Sawayanagi, T., Kakizawa, S., Nishigawa, H., Wei, W., Oshima, K., Miyata, S., Ugaki, M., Hibi, T., and Namba, S. 2003a. '*Candidatus* Phytoplasma ziziphi', a novel phytoplasma taxon associated with jujube witches' broom disease. *Int. J. Syst. Evol. Microbiol*., 53: 1037-1041.

Jung, H-Y., Sawayanagi, T., Wongkaew, P., Kakizawa, S., Nishigawa, H., Wei, W., Oshima, K., Miyata, S., Ugaki, M., Hibi, T, and Namba, S. 2003b. '*Candidatus* Phytoplasma oryzae', a novel phytoplasma taxon associated with rice yellow dwarf disease. *Int. J. Syst. Evol. Microbiol*., 53: 1925-1929.

Jung, H-Y., Miyata, S., Oshima, K., Kakizawa, S., Nishigawa, H., Wei, W., Suzuki, S., Ugaki, M., Hibi, T., and Namba, S. 2003c. First complete nucleotide sequence and heterologous gene organization of the two rRNA operons in the phytoplasma genome. *DNA and Cell Biol*., 22: 209-215.

Junquera, A., Bedendo, I., and Pascholati, S. 2004. Biochemical changes in corn plants infected by the maize bushy stunt phytoplasmas. *Physiol. Mol. Pl. Pathol*., 65: 181-185.

Kakizawa, S., Oshima, K., Kuboyama, T., Nishigawa, H., Jung, H-Y., Sawayanagi, T., Tsuchizaki, T., Miyata, S., Ugaki, M., and Namba, S. 2001. Cloning and expression analysis of phytoplasma protein translocation genes. *Mol. Plant-Microbe Interact*., 14: 1043–1050.

Kakizawa, S., Oshima, K., Nishigawa, H., Jung, H-Y., Wei, W., Suzuki, S., Tanaka, M., Miyata, S., Ugaki, M., and Namba, S. 2004. Secretion oimmunodominant membrane protein from onion yellows phytoplasma through the Sec protein-translocation system in *Escherichia coli*. *Microbiology*, 150: 135–142.

Kakizawa, S., Oshima, K., Jung, H-Y., Suzuki, S., Nishigawa, H., Arashida, R., Miyata, S., Ugaki, M., Kishino, H., and Namba, S. 2006. Positive selection acting on a surface membrane protein of the plant-pathogenic phytoplasmas. *J. Bacteriol*., 188: 3424-3428.

Kawakita, H., Saiki, T., Wei, W., Mitsuhashi, W., Watanabe, K., and Sato, M. 2000. Identification of mulberry dwarf phytoplasmas in the genital organs and eggs of leafhopper *Hishimonoides sellatiformis*. *Phytopathology*, 90: 909-914.

Kelly, P.L., Reeder, R., Kokoa, P., Arocha, Y., Nixon, T., and Fox, A. 2011. First report of a phytoplasma identified in coconut palms (*Cocos nucifera*) with lethal yellowing-like symptoms in Papua New Guinea. *New Dis. Rep*., 23: 9.

Kirkpatrick, B.C., Stenger, B.C., Morris, T.J., and Purcell, A.H. 1987. Cloning and detection of DNA from a nonculturable plant pathogenic mycoplasma-like organism. *Science*, 238: 197-200.

Kirkpatrick, B.C., Uyemoto, J.K., and Purcell, A.H. 1995. X-disease. In: Compendium of stone fruit diseases. American Phytopathological Society, St. Paul, USA.

Kison, H., and Seemüller, E. 2001. Differences in strain virulence of the European stone fruit yellows phytoplasma and susceptibility of stone fruit trees on various rootstocks to this pathogen. *J. Phytopathol*., 149: 533-541.

Koji S., Fujinuma S., Midega C., Mohamed H., Ishikawa T., Wilson M., Asche M., Degelo S., Adati T., and Pickett, J.A. 2012. Seasonal abundance of *Maiestas banda* (Hemiptera: Cicadellidae), a vector of phytoplasma, and other leafhoppers and planthoppers (Hemiptera: Delphacidae) associated with Napier grass (*Pennisetum purpureum*) in Kenya. *J. Pest Sc*., 85(1): 37-46.

Kube, M., Schneider, B., Kuhl, H., Dandekar, T., Heitmann, K., Migdo, A.M., Reinhardt, R., and Seemüller, E. 2008. The linear chromosome of the plant-pathogenic mycoplasma '*Candidatus* Phytoplasma mali'. *BMC Genomics*, 9: 306.

Kube, M., Mitrovic, J., Duduk, B., Rabus, R., and Seemüller, E. 2012. Current view on phytoplasma genomes and encoded metabolism. *The Sci. World J*., 25 pp..

Kuboyama, T., Huang, C., Lu, X., Sawayanagi, T., Kanazawa, T., Kagami, T., Matsuda, I., Tsuchizaki, T., and Namba, S. 1998. A plasmid isolated from phytopathogenic onion yellows phytoplasma and its heterogeneity in the pathogenic phytoplasma mutant. *Mol. Plant Microbe Interact*., 11: 1031-1037.

Landi F., Prandini A., Paltrinieri S., Mori N., Bertaccini A. 2007. Detection of different types of phytoplasmas in stone fruit orchards in northern Italy. *Bull. Insectol*., 60(2): 163–164.

Langer, M., and Maixner, M. 2004. Molecular characterisation of grapevine yellows associated phytoplasmas of the stolburgroup based on RFLP-analysis of non-ribosomal DNA. *Vitis*, 43: 191-199.

Lee, I-M., Davis, R.E. and Hiruki, C. 1991. Genetic interrelatedness among clover proliferation mycoplasmalike organisms (MLOs) and other MLOs investigated by nucleic acid hybridization and restriction fragment length polymorphism analyses. *Appl. Environ. Microbiol*., 57: 3565–3569.

Lee, I-M., Davis, R.E., Chen, T-A., Chiykowski, L.N., Fletcher, J., Hiruki, C., and Schaff, D.A. 1992. A genotype-base system for identification and classification of mycoplasmalike organisms (MLOs) in the aster yellows MLO strain cluster. *Phytopathology*, 82: 977-986.

Lee, I-M., Gundersen, D.E., Hammond, R.W., and Davis, R.E. 1994. Use of mycoplasmalike organism (MLO) group-specific oligonucleotide primers for nested-PCR assays to detect mixed-MLO infections in a single host plant. *Phytopathology*, 84: 559-566.

Lee, I-M., Bertaccini, A., Vibio, M., and Gundersen, D. E. 1995. Detection of multiple phytoplasmas in perennial fruit trees with decline symptoms in Italy. *Phytopathology*, 85: 728-735.

Lee, I-M., Gundersen-Rindal, D.E., Davis, R.E., and Bartoszyk, I.M. 1998a. Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. *Int. J. Syst. Bacteriol*., 48: 1153-1169.

Lee, I-M., Gundersen-Rindal, D.E., and Bertaccini, A. 1998b. Phytoplasma: ecology and genomic diversity. *Phytopathology*, 88: 1359-1366.

Lee, I-M., Davis, R.E. and Gundersen-Rindal, D.E. 2000. Phytoplasma: phytopathogenic mollicutes. Ann. Rev. Microbiol., 54: 221–255.

Lee, I-M., Martini, M., Bottner, K.D., Dane, R.A., Black, M.C., and Troxclair, N. 2003. Ecological implications from a molecular analysis of phytoplasmas involved in an aster yellows epidemic in various crops in Texas. *Phytopathology*, 93: 1368-1377.

Lee, I-M., Gundersen-Rindal, D.E., Davis, R.E., Bottner, K.D., Marcone, C., and Seemüller, E. 2004. '*Candidatus* Phytoplasma asteris', a novel phytoplasma taxon associated with aster yellows and related diseases. *Int. J. Syst. Evol. Microbiol*., 54: 1037-1048.

Lee, I. M., Bottner, K. D., Munyaneza, J. E., Secor, G. A., and Gudmestad, N. C. 2004a. Clover proliferation group (16SrVI) subgroup A (16SrVI-A) phytoplasma is a probable causal agent of potato purple top disease in Washington and Oregon. *Plant Dis*. 88(4), 429-429.

Lee, I-M., Martini, M., Marcone, C., and Zhu, S.F. 2004b. Classification of phytoplasma strains in the elm yellows group (16SrV) and proposal of '*Candidatus* Phytoplasma ulmi' for the phytoplasma associated with elm yellows. *Int. J. Syst. Evol. Microbiol*., 54: 337- 347.

Lee, I-M., Zhao, Y., and Bottner, K.D. 2005. Novel insertion sequence-like elements in phytoplasma strains of the aster yellows group are putative new members of the IS3 family. *FEMS Microbiol. Lett*., 242: 353-360.

Lee, I-M., Zhao, Y., and Bottner, K.D. 2006. SecY gene sequence analysis for finer differentiation of diverse strains in the aster yellows phytoplasma group. *Mol. Cell. Probes*, 20: 87-91.

Lee, I-M., Zhao, Y., Davis, R.E., Wei, W., and Martini, M. 2007. Prospects of DNA-based systems for differentiation and classification of phytoplasmas. *Bull. of Insect*., 60(2): 239.

Lee, I. M., Zhao, Y., Davis, R.E., Weintraub, P.G., and Jones, P. 2010. Prospects of multiple gene-based systems for differentiation and classification of phytoplasmas. Phytoplasmas: genomes, plant hosts and vectors, 51-63.

Lee, I-M., Bottner-Parker, K.D., Zhao, Y., Villalobos, W., and Moreira, L. 2011. '*Candidatus* Phytoplasma costaricanum' a new phytoplasma associated with a newly emerging disease in soybean in Costa Rica. *Int. J. Syst. Evol. Microbiol*., 61: 2822-2826.

Lee, I-M., Bottner-Parker, K. D., Zhao, Y., Bertaccini, A., Davis, R. E. 2012. Differentiation and classification of phytoplasmas in the pigeon pea witches' broom group (16SrIX): an update based on multiple gene sequence analysis. *Int. J. Syst. Evol. Microbiol*., 62: 2279-2285.

Lepka, P., Atitt, M., Moll, E., and Seemüller, E. 1999. Effect of phytoplasmal infection on concentration and translocation of carbohydrates and amino acids in periwinkle and tobacco. *Physiol. Mol. Plant Pathol*., 55: 59-68.

Liefting, L.W., Andersen, M.T., Beever, R. E., Gardner, R.C., and Forster, R.L. 1996. Sequence heterogeneity in the two 16S rRNA genes of *Phormium* yellow leaf phytoplasma. *Appl. Environ. Microbiol*., 62: 3133-3139.

Liefting, L.W., Veerakone, S., Ward, L.I., and Clover, G.R.G. 2009. First report of '*Candidatus* Phytoplasma australiense' in potato. *Plant Dis*., 93: 969.

Lim, P.O., and Sears, B.B. 1992. Evolutionary relationships of a plant pathogenic mycoplasma-like organism and *Acholeplasma laidlawii* deduced from two ribosomal protein gene sequences. *J. Bacteriol.*, 174: 2606–2611.

Lim, P.O., and Sears, B.B. 1992. Evolutionary relationships of plant-pathogenic mycoplasmalike organisms and *Acholeplasma laidlawii* deduced from two ribosomal protein gene sequences. *J. Bacteriol*., 174: 2606-2611.

Lozano, J.C. 1981. Field problems in cassava (No. 16). CIAT.

MacLean, A.M., Sugio, A., Makarova, O.V., Findlay, K.C., Grieve, V.M., Toth, R., Nicolaisen, M, and Hogenhout, S. 2011. Phytoplasma effector SAP54 induces indeterminate leaf-like flower development in Arabidopsis plants. *Plant Physiol*., 157: 831– 841.

Maixner, M. 1994. Transmission of German grapevine yellows (Vergilbungskrankheit) by the planthopper *Hyalesthes obsoletus* (Auchenorrhyncha: Cixiidae). *Vitis*, 33: 103–104.

Makarova, O.V., Contaldo, N., Paltrinieri, S., Kawube, G., Bertaccini, A., and Nicolaisen, M. 2012. DNA barcoding for universal identification of '*Candidatus* Phytoplasmas' using a fragment of the elongation factor Tu gene. *PLOS ONE*, 7(12): e52092.

Marcone, C., Lee, I-M., Davis, R.E., Ragozzino, A., and Seemüller, E. 2000. Classification of aster yellows-group phytoplasmas based on combined analyses of rRNA and tuf gene sequences. *Int. J. Syst. Evol. Microbiol*., 50: 1703-1713.

Marcone, C., Ragozzino, A., Camele, I., Rana, G. L., and Seemüller, E. 2001. Updating and extending genetic characterization and classification of phytoplasmas from wild and cultivated plants in southern Italy. *J. Plant Pathol*. 83(2), 133-138.

Mardi, M., Khayam Nekouei, S.M., Karimi Farsad, L., Ehya, F., Shabani, M., Shafiee, M., Tabatabaei, M., Safarnejad, M.R., Jouzani, G.S., and Salekdeh, G.H. 2011. Witches' broom disease of Mexican lime trees: disaster to be addressed before it will be too late. *Bull. Insectol*., 64(Suppl.): S205-S206.

Martini, M., Murari, E., Mori, N., and Bertaccini, A. 1999. Identification and epidemic distribution of two Flavescence dorée-related phytoplasmas in Veneto (Italy). *Plant Dis*., 83: 925-930.

Martini, M., Botti, S., Marcone, C., Marzachì, C., Casati, P., Bianco, P.A., Benedetti, R., and Bertaccini, A. 2002. Genetic variability among Flavescence dorée phytoplasmas from different origins in Italy and France. *Mol. Cell. Probes*, 16: 197-208.

Martini, M., Lee, I-M., Bottner, K.D., Zhao, Y., Botti, S., Bertaccini, A., Harrison, N.A., Carraro, L., Marcone, C., Khan, J., and Osler, R. 2007. Ribosomal protein gene-based filogeny for finer differentiation and classification of phytoplasmas. *Int. J. Syst. Evol. Microbiol*., 57: 2037-2051.

Maust, B.E., Espadas, F., Talavera, C., Aguilar, M., Santamaría, J.M., and Oropeza, C. 2003. Changes in carbohydrate metabolism in coconut palms infected with the lethal yellowing phytoplasma. *Phytopathology*, 93: 976-981.

Mejia J.F., Paltrinieri S., Rincon E., Galeano N.F., Gaitan A.L., Pardo J.M., Alvarez E., Duduk B., and Bertaccini, A. 2010. Characterization of phytoplasma strains associated with coffee crispiness and nogal cafetero witches broom in Colombia. 18th Congress IOM, Chianciano Terme, Italy, July 11-16, n. 147: 153.

Mejia, J.F., Contaldo, N., Paltrinieri, S., Pardo, J.M., Rios, C. A., Alvarez, A., and Bertaccini, A. 2011. Molecular detection and identification of group 16SrV and 16SrXII phytoplasmas associated with potatoes in Colombia. *Bull. Insectol*., 64(suppl.): S97-S98.

Mejia, J.F., Zamora, L., Duduk, B., Quiñones, M. L., Bertaccini, A., and Contaldo, N. 2013. Caratterizzazione molecolare di ceppi di '*Candidatus* Phytoplasma asteris' da mais. *Petria*, 23: 135-138.

Mello, A.P.D.O.A., Eckstein, B., Flores, D., Kreyci, P.F., Bedendo, I. P. 2011. Identification by computer-simulated RFLP of phytoplasmas associated with eggplant giant calyx representative of two subgroups, a lineage of 16SrIII-J and the new subgroup 16SrIII-U. *Int. J. Syst. Evol. Microbiol*., 61: 1454-1461.

Mitra, D.K. 1988. Little leaf disease of eggplant. In: Maramorosch, K. and Raychaudhuri, S.P. (eds) Mycoplasma Disease of Crops. Springer-Verlag, New York, pp. 343–348.

Mitrović, J., Contaldo, N., Paltrinieri, S., Mejía, J. F., Mori, N., Bertaccini, A., and Duduk, B. 2011a. The use of groEL gene in characterisation of aster yellows phytoplasmas in field collected samples. *Bull. Insectol*., 64(Suppl.): S17-S18.

Mitrović, J., Kakizawa, S., Duduk, B., Oshima, K., Namba, S., and Bertaccini, A. 2011b. The cpn60 gene as an additional marker for finer differentiation of '*Candidatus* Phytoplasma asteris' related strains. *Ann. Appl. Biol*., 159: 41-48.

Miura, C., Sugawara, K., Neriya, Y., Minato, N., Keima, T., Himeno, M., Maejima, K., Komatsu, K., Yamaji, Y., Oshima, K., and Namba, S. 2012. Functional characterization and gene expression profiling of superoxide dismutase from plant pathogenic phytoplasma. *Gene*, 510: 107–112.

Miyata, S., Oshima, K., Kakizawa, S., Nishigawa, H., Jung, H-Y., Kuboyama, T., Ugaki, U., and Namba, S. 2003. Two different thymidylate kinase gene homologues, including one that has catalytic activity, are encoded in the onion yellows phytoplasma genome. *Microbiology*, 149: 2243-2250.

Moghal, S. M., Zidgali, A.D., and Moustafa, S.S. 1998. Natural host range and reactions of citrus species to witches' broom disease of lime (WBDL) in Oman. Proceedings of the IPM Conference, Sultan Qaboos Univ., Muscat: 143-152.

Molino Lova, M., Quaglino, F., Abou-Jawdah, Y., Choueiri, E., Sobh, H., Casati, P., Tedeschi, R., Alma, A., and Bianco, P.A. 2011. Identification of new 16SrIX subgroups, -F and -G, among '*Candidatus* Phytoplasma phoenicium' strains infecting almond, peach and nectarine in Lebanon. *Phytopath. Medit*., 50: 273−282.

Montano, H.G., Davis, R.E., Dally, E.L., Hogenhout, S., Pimentel, P.P., and Brioso, P.S.T. 2001. '*Candidatus* Phytoplasma brasiliense', a new phytoplasma taxon associated with hibiscus witches' broom disease. *Int. J. Syst. Evol. Microbiol*., 51: 1109-1118.

Montano, H.G., Brioso, P.S.T., and Pimentel, J.P. 2007. List of phytoplasma hosts in Brazil. *Bull. Insectol*., 60(2): 129-130.

Mori, N., Bressan, A., Martini, M., Guadagnini, M., Girolami, V., and Bertaccini, A. 2002. Experimental transmission by *Scaphoideus titanus* Ball of two Flavescence dorée-type phytoplasmas. *Vitis*, 41: 99-102.

Mori, N., Pavan, F., Bondavalli, R., Reggiani, N., Paltrinieri, S., and Bertaccini, A. 2008. Factors affecting the spread of "bois noir" disease in north Italy vineyards. *Vitis*, 47: 65-72.

Morton, A., Davies, D.L., Blomquist, C.L., and Barbara, D.J. 2003. Characterization of homologues of the apple proliferation immunodominant membrane protein gene from three related phytoplasmas. *Mol. Pl. Pathol*., 4: 109–114.

Musetti, R., Favali, A., and Pressacco, L. 2000. Histopathology and polyphenol content in plants infected by phytoplasmas. *Cytobios*, 102: 133-147.

Musetti, R., Sanità Di Toppi, L., Martini, M., Ferrini, F., Loschi, A., Favali, M.A., and Osler, R. 2005. Hydrogen peroxide localization and antioxidant status in the recovery of apricot plants from European stone fruit yellows. *Eur. J. Pl. Pathol*., 112: 53-61.

Nei M. and Kumar S. 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.

Nejat, N., Sijam, K., Abdullah, S., Vadamalai, G., and Dickinson, M. 2009. Phytoplasmas associated with disease of coconut in Malaysia: phylogenetic groups and host plant species. *Plant Pathol*., 58: 1152-1160.

Nejat, N., Vadamalai, G., Davis, R.E., Harrison, N.A., Sijam, K., Dickinson, M., Abdullah, S.N., and Zhao, Y. 2012. '*Candidatus* Phytoplasma malaysianum', a novel taxon associated with virescence and phyllody of Madagascar periwinkle (*Catharanthus roseus). Int. J. Syst. Evol. Microbiol*., 63: 540-548.

Nolt, B.L., Pineda, L.B., and Velasco, A.C. 1992. Surveys of cassava plantations in Colombia for virus and virus-like diseases. *Pl. Pathol*., 41(3): 348-354.

Ntushelo, K., Harrison, N.A., and Elliott, M.L. 2012. Comparison of the ribosomal RNA operon from Texas Phoenix decline and lethal yellowing phytoplasmas. *Eur. J. Plant Pathol*., 133: 779-782.

Obura E., Midega, C.A., Masiga, D., Pickett, J.A., Hassan, M., Koji, S., and Khan, Z.R. 2009. *Recilia banda* Kramer (Hemiptera: Cicadellidae), a vector of Napier stunt phytoplasma in Kenya. *Naturwissenschaften*, 96(10): 1169-1176.

Oliveira, S.A.S., Abreu, E.F.M., Araújo, T.S., Oliveira, E.J., Andrade, E.C., Garcia, J.M.P., and Álvarez, E. 2014. First report of a 16SrIII-L phytoplasma associated with frogskin disease in cassava (*Manihot esculenta* Crantz) in Brazil. *Pl. Dis*., 98(1): 153-153.

Olsen, K.M. 2002. Population history of *Manihot esculenta* (Euphorbiaceae) inferred from nuclear DNA sequences. *Mol. Ecol*., 11(5): 901-911.

Oropeza, C., Cordova, I., Chumba, A., Narváez, M., Sáenz, L., Ashburner, R., and Harrison, N. 2011. Phytoplasma distribution in coconut palms affected by lethal yellowing disease. *Ann. Appl. Biol*., 159: 109-117.

Oshima, K., Kakizawa, S., Nishigawa, H., Kuboyama, T., Miyata, S., Ugaki, M., and Namba, A. 2001. A plasmid of phytoplasma encodes a unique replication protein having both plasmidand virus-like domains: clue to viral ancestry or result of virus/plasmid recombination. *Virology*, 285: 270-277.

Oshima, K., Kakizawa, S., Nishigawa, H., Jung, H-Y., Wei, W., Suzuki, S., Arashida, R., Nakata, D., Miyata, S., Ugaki, M., and Namba, S. 2004. Reductive evolution suggested from the complete genome sequence of a plant-pathogenic phytoplasma. *Nature Genetics*, 36: 27-29.

Oshima, K., Kakizawa, S., Arashida, R., Ishii, Y., Hoshi, A., Hayashi, Y., Kawagida, S., and Namba, S. 2007. Presence of two glycolityc gene clusters in a severe pathogenic line of '*Candidatus* Phytoplasma asteris'. *Mol. Pl. Pathol*., 8: 481-489.

Oshima, K., Ishii, Y., Kakizawa, S., Sugawara, K., Neriya, Y., Himeno, M., Minato, N., Miura, C., Shiraishi, T., Yamaji, Y., and Namba, S. 2011. Dramatic transcriptional changes in an intracellular parasite enable host switching between plant and insect. *PLoS One*, 6: e23242.

Oshima K., Maejima, K., and Namba, S. 2013. Genomic and evolutionary aspects of phytoplasmas. *Front Microbiol*., 4: 230.

Parani, M., Singh, K.N., Rangasamy, S.N.S., and Ramalingam, R.S. 1996. A study on mechanism of phyllody disease resistance in *Sesamum alatum* Thonn. *Current Sci*., 70: 86- 89.

Perera L., Meegahakumbura, M.K., Wijesekara, H.R.T., Fernando, W.B.S., Dickinson, M.J. 2012. A phytoplasma is associated with the Weligama coconut leaf wilt disease in Sri Lanka. *J. Phytopathol*., 94 (1): 204-209.

Pérez, A.P., and Cayón, G. 2010. Metabolism of carbohydrates in oil palms (*Elaeis guineensis* Jacq.) affected by lethal wilt. *Agronomia Colombiana*, 28: 181-187.

Perilla-Henao, L.M., Dickinson, M., and Franco-Lara, L. 2012. First report of '*Candidatus* Phytoplasma asteris' affecting woody hosts (*Fraxinus uhdei*, *Populus nigra*, *Pittosporum undulatum*, and *Croton* spp.) in Colombia. *Pl. Dis*., 96(9): 1372.

Prince, J.P., Davis, R.E., Wolf, T.K., Lee, I-M., Mogen, B., Dally, E., Bertaccini, A., Credi, R., and Barba, M. 1993. Molecular detection of diverse mycoplasma-like organisms (MLOs) associated with grapevine yellows and their classification with aster yellows, X- disease, and elm yellows MLOs. *Phytopathology*, 83: 1130-1137.

Prota, V.A., Garau, R., Paltrinieri, S., Botti, S., Nahdi, S., Calari, A., and Bertaccini, A. 2007. Molecular identification of phytoplasmas infecting myrtle plantations in Sardinia (Italy). *Bull. Insectol*., 60(2): 383-384.

Quaglino F., Zhao, Y., Casati, P., Bulgari, D., Bianco, P.A., Wei, W., and Davis, R.E. 2013. '*Candidatus* Phytoplasma solani', a novel taxon associated with stolbur- and bois noir-related diseases of plants. *Int. J. Syst. Evol. Microbiol*., 63: 2879–2894.

Rodríguez Vargas, G. 1997. Análisis del estado actual del" cuero del sapo"(complejo viral) y la tecnología empleada por los productores de yuca en la Región Huetar Norte de Costa Rica. Instituto Tecnológico de Costa Rica, Santa Carlos. Universidad de Costa Rica, (UCR). Undergraduate thesis.

Saccardo, F., Martini, M., Palmano, S., Ermacora, P., Scortichini, M., Loi, N., and Firrao, G. 2012. Genome drafts of four phytoplasma strains of the ribosomal group 16SrIII. *Microbiology*, 158(11): 2805-2814.

Saitou, N., and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*., 4: 406–425.

Salehi, M. and Izadpanah, K. 1995. Almond brooming. In: 12th Iranian Plant Protection Congress, Karaj, Iran, Entomological Society of Iran, Iran, p. 238.

Salehi, M., Izadpanah, K., and Heydarnejad, J. 2006. Characterization of a new almond witches' broom phytoplasma in Iran. *J. Phytopath*., 154: 386–391.

Santos-Cervantes, M.E., Chávez-Medina, J.A., Acosta-Pardini, J., Flores-Zamora, G.L., Méndez-Lozano, J., and Leyva-López, N.E. 2010. Genetic diversity and geographical distribution of phytoplasmas associated with potato purple top disease in Mexico. *Plant Dis*., 94(4): 388-395.

Schneider, B., and Seemüller, E. 1994. Presence of two set of ribosomal genes in phytopatogenic mollicutes. *Appl. Environ. Microbiol*., 60: 3409-3412.

Schneider, B., Cousins, M.T., Klinkong, S. and Seemüller, E. 1995. Taxonomic related- ness and phylogenetic positions of phytoplasmas associated with diseases of faba bean, sunnhemp, sesame, soybean, and eggplant. *J. Pl. Dis. Prot*., 102: 225–232.

Schneider, B., Gibb, K.S., and Seemüller, E. 1997. Sequence and RFLP analysis of the elongation factor Tu gene used in differentiation and classification of phytoplasmas. *Microbiol.* 143: 3381-3389.

Schneider, B., Gibb, K.S., Padovan, A., Davis, R.I. and De La Rue, S. 1999. Comparison and characterization of tomato big bud and sweet potato little leaf group phytoplasmas. *J. Phytopathol*., 147: 31–40.

Schneider, B., Seemüller, E., Smart, C.D., and Kirkpatrick, B.C. 1995. Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas. Pages 369- 380 in: Molecular and Diagnostic Procedures in Mycoplasmology. S. Razin and J. G. Tully, eds. Academic Press, New York, NY.

Seemüller, E., and Schneider, B., 2004. Taxonomic description of '*Candidatus* Phytoplasma mali' sp. nov., '*Candidatus* Phytoplasma pyri' sp. nov. and '*Candidatus* Phytoplasma prunorum' sp. nov., the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively. *Int. J. Syst. Evol. Microbiol*., 54: 1217-1226.

Seemüller, E., Kiss, E., Sule, S., and Schneider, B. 2010. Multiple infection of apple trees by distinct strains of '*Candidatus* Phytoplasma mali' and its pathological relevance. *Phytopathology*, 100: 863–870.

Šeruga, M., Škorić, D., Botti, S., Paltrinieri, S., Juretić, N., and Bertaccini, A. 2003. Molecular characterization of a phytoplasma from the Aster Yellows (16SrI) group naturally infecting *Populus nigra* L. 'Italica' trees in Croatia. *Forest Pathol*., 33: 113-125.

Sforza, R., Clair, D., Daire, X., Larrue, J., and Boudon-Padieu, E. 1998. The role of *Hyalesthes obsoletus* (Hemiptera: Cixiidae) in the occurrence of bois noir of grapevines in France. *J. Phytopathol*., 146: 549-556.

Sharma, O.P. 1990. Witches' broom disease of sunn hemp (*Crotalaria juncea* L.) by MLO. *Ind. J. Mycol. and Pl. Pathol*., 20: 264–265.

Shaw, M.E., Kirkpatrick, B.C., and Golino, D.A. 1993. The beet leafhopper-transmitted virescence agent causes tomato big bud disease in California. *Pl. Dis*., 77: 290–295.

Siddique, A., Agrawal, G.K., Alam, N., and Krishnareddy, M. 2001. Electron microscopy and molecular characterization of phytoplasmas associated with little leaf disease of brinjal (*Solanum melongena* L.) and periwinkle (*Catharanthus roseus*) in Bangladesh. *J. Phytopathol*., 149: 237–244.

Sinclair, W.A., Griffiths, H.M., and Whitlow, T.H. 1997a. Comparisons of tolerance of ash yellows phytoplasmas in *Fraxinus* species and rootstock-scion combinations. *Pl. Dis*., 81: 395-398.

Sinclair, W.A., Whitlow ,T.H., and Griffiths, H.M. 1997b. Heritable tolerance of ash yellows phytoplasmas in green ash. *Can. J. Forest Res*., 27: 1928-1935.

Souza, A.N., Silva, F.N., Bedendo, I.P., and Carvalho, CM. 2014. A phytoplasma belonging to a 16SrIII-A subgroup and dsRNA virus associated with cassava frogskin disease in Brazil. *Pl. Dis*., http://dx.doi.org/10.1094/PDIS-04-13-0440-RE.

Sugawara, K., Honma, Y., Komatsu, K., Himeno, M., Oshima, K., and Namba, S. 2013. The alteration of plant morphology by small peptides released from the proteolytic processing of the bacterial peptide TENGU. *Pl. Physiol*., 162(4): 2005-2014.

Sugio, A., and Hogenhout, S.A. 2012. The genome biology of phytoplasma: modulators of plants and insects. *Curr. Opin. Microbiol*., 15: 247–254.

Sugio, A., MacLean, A.M., Kingdom, H.N., Grieve, V.M., Manimekalai, R., and Hogenhout, S.A. 2011a. Diverse targets of phytoplasma effectors: from plant development to defense against insects. *Ann. Rev. Phytopathol*., 49: 175–195.

Sugio, A., Kingdom, H.N., MacLean, A.M., Grieve, V.M., and Hogenhout, S.A. 2011b. Phytoplasma protein effector SAP11 enhances insect vector reproduction by manipulating plant development and defense hormone biosynthesis. *PNAS USA*, 108: E1254–E1263.

Suzuki, S., Oshima, K., Kakizawa, S., Arashida, R., Jung, H-Y., Yamaji, Y., Nishigawa, H., Ugaki, M., and Namba, S. 2006. Interaction between the membrane protein of a pathogen and insect microfilament complex determines insect-vector specificity. *PNAS USA*, 103: 4252–4257.

Takinami, Y., Maejima, K., Takahashi, A., Keima, T., Shiraishi, T., Okano, Y., Komatsu, K., Oshima, K and Namba, S. 2013. First report of '*Candidatus* Phytoplasma asteris' infecting hydrangea showing phyllody in Japan. *J. Gen. Pl. Pathol*., 79(3): 209-213.

Tamura, K. 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases. *Mol. Biol. Evol*., 9: 678–687.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol*., 28: 2731-2739.

Taylor, N., Chavarriaga, P., Raemakers, K., Siritunga, D., and Zhang, P. 2004. Development and application of transgenic technologies in cassava. *Pl. Mol. Biol*., 56(4): 671-688.

Tedeschi, R., Ferrato, V., Rossi, J., and Alma, A. 2006. Possible phytoplasma transovarial transmission in the psyllids Cacopsylla melanoneura and Cacopsylla pruni. *Pl. Pathol*., 55(1): 18-24.

Teixeira, D.C., Wulff, N.A., Martins, E.C., Kitajima, E.W., Bassanezi, R., Ayres, A.J., Eveillard, S., Saillard, C., and Bové, J.M. 2009. A phytoplasma related to '*Candidatus* Phytoplasma asteris' detected in citrus showing huanglongbing (yellow shoot disease) symptoms in Guangdong, P.R. China. *Phytopathology*, 99: 236-242.

Thompson, J. D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl. Ac. Res*., 25: 4876-4882.

Torres, E., Botti, S., Rahola, J., Martin, M.P., and Bertaccini, A. 2005. Grapevine yellows diseases in Spain: eight years survey of disease spread and molecular characterization of phytoplasmas involved. *Anales del Jardín botánico de Madrid*, 62: 127-133.

Toruno, T.Y., Seruga Musić, M., Simi, S., Nicolaisen, M., and Hogenhout, S.A. 2010. Phytoplasma PMU1 exists as linear chromosomal and circular extrachromosomal elements and has enhanced expression in insect vectors compared with plant hosts. *Mol. Microbiol*., 77: 1406–1415.

Tovar, J.P., and Torres, E. 2004. Estudio epidemiológico de la enfermedad marchitez letal de la palma de aceite en plantaciones de Villanueva, Casanare–Cenipalma. *Palmas*, 25: 210- 219.

Tran-Nguyen, L.T., Kube, M., Schneider, B., Reinhardt, R., and Gibb, K.S. 2008. Comparative genome analysis of '*Candidatus* Phytoplasma australiense' (subgroup tuf- Australia I; rp-A) and '*Ca*. Phytoplasma asteris' strains OY-M and AY-WB. *J. Bacteriol*., 190: 3979–3991.

Turner, P.D. 1981. Oil palm diseases and disorders. Oxford University Press, Oxford, UK.

Valiunas, D., Jomantiene, R., Ivanauskas, A., Abraitis, R., Staniene, G., Zhao, Y, and Davis, R.E. 2009. First report of a new phytoplasma subgroup, 16SrIII-T, associated with decline disease affecting sweet and sour cherry trees in Lithuania. *Pl. Dis*., 93: 550.

Verdin, E., Salar, P., Danet, J-L., Choueiri, E., Jreijiri, F., El Zammar, S., Gèlie, B., Bové, J, and Garnier, M. 2003. '*Candidatus* Phytoplasma phoeniceum', a new phytoplasma associated with an emerging lethal disease of almond trees in Lebanon and Iran. *Int. J. Syst. Evol. Microbiol*., 53: 833-838.

Villalobos, W., Martini, M., Garita, L., Muñoz, M., Osler, R., and Moreira, L. 2011. *Guazuma ulmifolia* (*Sterculiaceae*), a new natural host of 16SrXV phytoplasma in Costa Rica. *Trop. Plant Pathol*., 36: 2.

Warokka, J. 2005. Studies on the etiology and epidemiology of kalimantan wilt disease of coconut in Indonesia. Dissertation. University of Nottingham, Nottingham, UK.

Wei, W., Kakizawa, S., Jung, H-Y., Suzuki, S., Tanaka, M., Nishigawa, H., Miyata, S., Oshima, K., Ugaki, M., Hibi, T., and Namba, S. 2004a. An antibody against the SecA membrane protein of one phytoplasma reacts with those of phylogenetically different phytoplasmas. *Phytopathology*, 94: 683-686.

Wei, W., Kakizawa, S., Suzuki, S., Jung, H-Y., Nishigawa, H., Miyata, S., Oshima, K., Ugaki, M., Hibi, T., and Namba, S. 2004b. In planta dynamic analysis of onion yellows phytoplasma using localized inoculation by insect transmission. *Phytopathology*, 94: 244- 250.

Wei, W., Davis, R.E., Lee, I-M., and Zhao, Y. 2007. Computer-simulated RFLP analysis of 16S rRNA genes: identification of ten new phytoplasma groups. *Int. J. Syst. Evol. Microbiol*., 57: 1855-1867.

Wei, W., Lee, I., Davis, R. E., Suo, X., and Zhao, Y. 2007a. Virtual RFLP analysis of 16S rDNA sequences identifies new subgroups in the clover proliferation phytoplasma group. *Bull. Insect.*, 60(2): 349.

Wei, W., Lee, I-M., Davis, R.E., Suo, X., and Zhao, Y. 2008a. Automated RFLP pattern comparison and similarity coefficient calculation for rapid delineation of new and distinct phytoplasma 16Sr subgroup lineages. *Int. J. Syst. Evol. Microbiol*., 58: 2368-2377.

Wei, W., Davis, R.E., Jomantiene, R., and Zhao, Y. 2008b. Ancient, recurrent phage attacks and recombination shaped dynamic sequence-variable mosaics at the root of phytoplasma genome evolution. *PNAS USA*, 105: 11827-11832.

Weintraub, P.G., and Beanland, L.A. 2006. Insect vectors of phytoplasmas. *Ann. Rev. Entomol*., 51: 91-111.

Whitcomb, R. F., Chen, T. A., Williamson, D. L., Liao, C., Tully, J. G., Bové, J. M., Rose, D. L., Coan, M. E and Clark, T. B. 1986. *Spiroplasma kunkelii* sp. nov.: characterization of the etiological agent of corn stunt disease. *Int. J. Syst. Bacteriol.* 36(2), 170-178.

Yang, L.L. 1985. Host responses of peanut witches' broom disease. *J. Agricult. Res. China*, 34: 464–468.

Yang, Z.H., Chen, Q.B., Yang, L.F., Li, H.C. and Bai, C.J. 2008. Occurrence of a 16SrII group phytoplasma associated with crotalaria witches' broom in Hainan, China. *Pl. Pathol*., 57: 364.

Yu, Y., Yeh, K., and Lin, C. 1998. An antigenic protein gene of a phytoplasma associated with sweet potato witches' broom. *Microbiology*, 144: 1257-1262.

Zhao. Y., Wei, W., Lee, I-M., Shao, J., Suo, X., and Davis, R.E. 2009. Construction of an interactive online phytoplasma classification tool, iPhyClassifier, and its application in analysis of the peach X-disease phytoplasma group (16SrIII). *Int. J. Syst. Evol. Microbiol*., 59: 2582-2593.

Zhu, S.F., Bertaccini, A., Lee, I-M., Paltrinieri, S., Hadidi, A. 2011. Cherry lethal yellows and decline phytoplasmas. In: Virus and Virus-like Diseases of Pome and Stone Fruits. Ed. A. Hadidi, M. Barba, T. Candresse, and W. Jelkmann. APS Press, St. Paul, Minnesota, USA, 47: 255-257.

Zhu, S.F., Hadidi, A., Gundersen, D.E., Lee, I-M., and Zhang, C.L. 1998. Characterization of the phytoplasmas associated with cherry lethal yellows and jujube witches' broom diseases in China. *Acta Hortic*., 472: 701–714.

Zirak, L., Bahar, M., and Ahoonmanesh, A. 2009. Molecular characterization of phytoplasmas related to peanut witches broom and stolbur groups infecting plum in Iran. *J. Pl. Pathol*., 91: 713–716.

Zirak, L., Bahar, M., and Ahoonmanesh, A. 2010. Molecular characterization of phytoplasmas associated with peach diseases in Iran. *J. Phytopathol*., 158: 105–110.

ACKNOWLEDGMENTS

I am very grateful to all the Phytoplasmology laboratory group at DipSA, for the continuous support and friendship: **Dr. Samanta Paltrinieri**, **Dr. Nicoletta Contaldo** and **Dr. Stefano Ardizzi**

I gratefully thank **Dr. Elizabeth Alvarez** and **Juan Manuel Pardo** from the International Center for Tropical Agriculture (CIAT) - Colombia; **Dr. Tin Maung Aye**, CIAT-Thailand and **Dr. Trinh Xuan Hoat** from the Plant Protection Research Institute (PPRI) - Vietnam for cassava field sampling support

I thank the company Palmar del Oriente S.A., particularly **Fernando Rodríguez** and **Rodrigo Bedoya**, for financial and sampling support; and Palmas del Casanare, Palmeras Santana, Palmeras del Upía, CIAT and Palmas del Sicarare for sampling and technical support.

I am very grateful to **prof. Assunta Bertaccini** for the supervision and confidence in my work.