



## ***Guazuma ulmifolia* (Sterculiaceae), a new natural host of 16SrXV phytoplasma in Costa Rica**

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### **ABSTRACT**

Guacimo trees (*Guazuma ulmifolia*, Sterculiaceae) showing witches' broom symptoms (GWB), small leaves, short internodes, stunting and no flower and fruit production were observed on side roads and fences in different areas of Costa Rica. The occurrence of phytoplasma infection in GWB trees was evaluated by transmission electron microscopy (TEM), and by molecular analyses based on 16S rDNA: nested-PCR/RFLP, sequencing and phylogenetics. Phytoplasmas were observed only in the sieve cells of symptomatic trees by TEM. The infection was confirmed by nested-PCR; amplicons of about 1.2 kb were obtained from all DNA samples from symptomatic trees. The RFLP analysis generated patterns identical among GWB samples and showed a relationship of this phytoplasma to hibiscus witches' broom group (16SrXV). The 16S rDNA sequence (1460 nt), obtained from the P1A/16S-SR semi-nested-PCR products of two phytoplasma strains, shared 98.8% similarity with '*Candidatus* Phytoplasma brasiliense' (GenBank accession: AF147708). The virtual RFLP pattern indicated a similarity coefficient of 0.95 with 16Sr group XV-A (AF147708), suggesting that the GWB phytoplasma may represent a new subgroup within this group. This is the first report of a phytoplasma infecting the neotropical tree species *G. ulmifolia* and the natural occurrence of a phytoplasma strain closely related to '*Ca. Phytoplasma brasiliense*' in Costa Rica.

**Key words:** '*Ca. Phytoplasma brasiliense*', 16S rRNA, guacimo witches'-broom, nested-PCR, RFLP.

### **RESUMEN**

#### ***Guazuma ulmifolia* (Sterculiaceae), un nuevo hospedero natural de fitoplasmas del grupo 16SrXV en Costa Rica**

En varias zonas de Costa Rica se observaron árboles de guácimo (*Guazuma ulmifolia*, Sterculiaceae) con síntomas de escoba de bruja (GWB), hoja pequeña, acortamiento de entrenudos, dando al árbol un aspecto general de enanismo. La infección por fitoplasmas en los árboles de guácimo se evaluó mediante microscopía electrónica de transmisión (TEM), análisis moleculares del 16S rDNA mediante PCR anidado, RFLP's, secuenciación y filogenia. En la TEM, los fitoplasmas se observaron sólo en las células del floema de los árboles sintomáticos. La infección se confirmó por PCR anidada, los productos amplificados de aproximadamente 1,2 kb se obtuvieron para todas las muestras sintomáticas evaluadas. El análisis de RFLP generó patrones idénticos entre las muestras con GWB y mostró relación de este fitoplasma con el de la "escoba de bruja del hibisco" (16SrXV). La secuencia de ADNr 16S (1460 nt) de los productos obtenidos por PCR semi-anidado (P1A/16S-SR) de dos muestras de GWB mostraron 98,8% de similitud con "*Candidatus* Phytoplasma brasiliense" (GenBank, registro AF147708). El patrón RFLP virtual reveló 95% de similitud con el grupo 16Sr XV-A (AF147708), lo que sugiere que el fitoplasma GWB puede representar un nuevo subgrupo dentro del 16Sr XV. Este es el primer informe de un fitoplasma infectando a la especie neotropical *G. ulmifolia* y de la ocurrencia natural de un fitoplasma estrechamente relacionado con "*Ca. Phytoplasma brasiliense*" en Costa Rica.

**Palabras-clave:** '*Ca. Phytoplasma brasiliense*', 16S rRNA, escoba de bruja del guacimo, PCR anidada, RFLP.

*Guazuma ulmifolia* Lam. (Sterculiaceae) is a middle-sized tree, which can reach 20 m in height. It is pantropical, semideciduous, heliophytic, a characteristic pioneer of second-growth broad-leaf forests. This species occurs naturally throughout Latin American, being found from Mexico to the Northern Region of Argentina (Francis, 1991; Tapia-Pastrana, 2007). It is commonly known as "guacimo", "guacima", "mutamba" and other names. The fruits and foliage are eaten by domestic animals and wildlife, and timber is an important source of firewood in rural areas. Additionally, this tree is widely used in neotropical American folk-medicine for the treatment of a variety of

diseases, including gastrointestinal disorders and stomach aches; diabetes; malaria and syphilis (Magos et al., 2008). The phytochemical studies carried out with *G. ulmifolia* have led to the isolation of different substances, some of them related to antihypertensive activities (Hoer et al., 1996; Magos et al., 2008), others to *in vitro* antimicrobial (Camporinese et al., 2003) or antiviral properties (Felipe et al., 2006).

Recently, several trees of *G. ulmifolia* exhibited symptoms of witches'-broom (WB): reduced leaf size, short internodes and proliferation of axillary shoots (Figure 1-A), as well as reduced overall size (Figure 1-B), and no

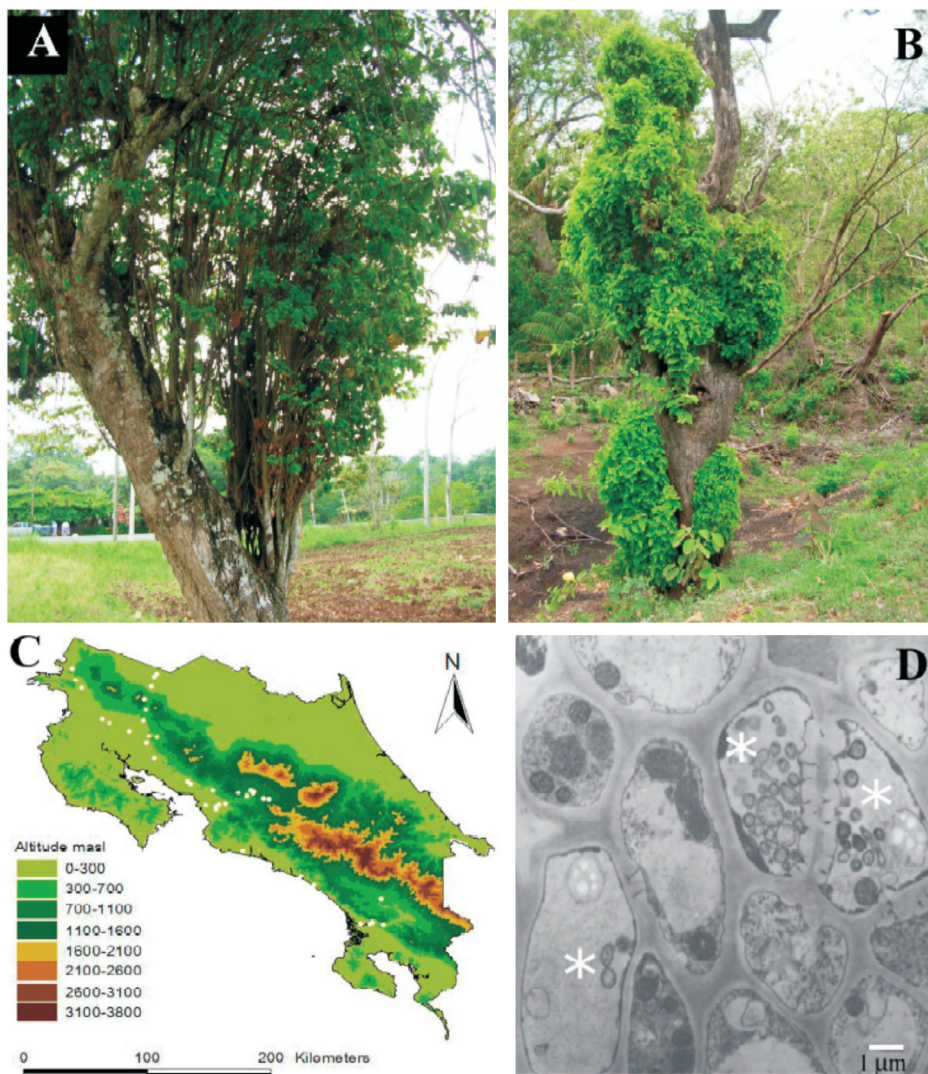
production of flowers and fruit. Symptomatic guacimo trees were found beside fences and on side roads in Costa Rica along the Pacific coast from the border with Nicaragua to nearly the border with Panama [provinces of Guanacaste (La Cruz, Liberia, Cañas) and Puntarenas (Jaco, Caldera, Parrita, Palmar Sur)], in the Northern and South-Western areas of Alajuela Province (Upala, Atenas, Tacaes) and the Western area of San Jose Province (Santa Ana) (Figure 1-C). The symptoms were reminiscent of diseases caused by phytoplasmas (Bertaccini, 2007); therefore, to determine the association of phytoplasmas with guacimo witches'-broom (GWB) disease, analyses of samples based on transmission electron microscopy, nested-PCR, RFLP, sequencing and phylogenetics were carried out.

Samples were collected from symptomatic guacimo trees in October 2009 in Costa Rica at different points below 1000 masl (Latitude/ Longitude data of some sampling points: 10,9720/ -85,6210; 10,0810/ -84,7840; 10,0180/ -84,6000; 10,9080/ -85,0220; 9,9070/ -84,5110;

9,9910/ -84,4160; 10,5648/ -85,1038; 10,6497 /-85,0895; 8,9507/ -83,3603). Petioles and leaf midribs were used for analysis in the present study. DNA extracted from *Secchium edule* infected with aster yellows phytoplasma (16SrI-B) (Villalobos et al., 2002) was used as a positive control.

Pieces of midrib and petiole (1-2 mm long) from symptomatic and healthy guacimo leaves were fixed with Karnovsky solution (Karnovsky, 1965) in 0.05 M cacodylate buffer and post-fixed with osmium tetroxide (1%) for transmission electron microscopy (TEM). The samples were dehydrated using an ethanol/propilen oxide series and finally embedded and polymerized using epoxy resin (Spurr's medium). A double-staining of sections was performed with uranyl acetate and lead citrate to be observed with a Hitachi H-7100 electron microscope (Tokyo, Japan) at 100kV.

DNA was extracted from leaf midribs, following in the initial steps a protocol modified from Lee et al. (1993). Approximately 1 g of leaf midribs, frozen in liquid nitrogen,



**FIGURE 1** - A-B Witch's broom symptoms observed in guacimo trees (*Guazuma ulmifolia* Sterculiaceae). A. proliferation of axillary shoots; B. short internodes and small leaves; C. Distribution map of the disease (white dots) in Costa Rica; D. Phloem elements (\*) of a symptomatic guacimo tree showing pleomorphic phytoplasma bodies inside.

was pulverized in a pre-chilled mortar. Then 7 mL of grinding buffer and 6 mL of ethylene glycol monomethyl ether were added. The samples were clarified by centrifugation using a Sorvall rotor SS-34 at 2500 g for 5 minutes (min). The supernatant was collected and centrifuged at high speed 24000 g for 30 min. The pellet was re-suspended in 2 mL of CTAB 2% and two aliquots (about 600 µL) were collected and incubated at 60°C for 30 min, with sporadic agitation. The next steps of DNA extraction were carried out according to Martini et al. (2009). The extracted DNA was quantified by Nanodrop ND1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and diluted in sterile water to obtain a concentration of about 20 ng/µL.

A nested PCR amplification was carried out to confirm the presence of phytoplasmas, with universal primer pairs, P1/16S-SR (Lee et al., 2004), followed by R16F2n/R2 (Gundersen & Lee, 1996) after a 1:30 dilution of the direct PCR products. Amplifications were performed with an automated thermal cycler PTC-100 (MJ Research, Cambridge, MA, USA) in 25 µL reactions containing 200 µM of each of the four dNTPs, 0.4 µM of each primer, 1.5 mM MgCl<sub>2</sub>, 0.625 units of GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA) and 1 µL of diluted DNA. The PCR program consisted of 38 cycles: denaturation at 94°C for 1 min (2 min for the first cycle), annealing at 55°C for 1 min, and extension at 72°C for 2 min (10 min for the last cycle). Five µL of the amplified products were electrophoresed through a 1% agarose gel, stained in ethidium bromide, and visualized on a UV transilluminator.

Restriction fragment length polymorphism (RFLPs) of nested PCR products obtained from three GWB phytoplasma infected samples were analysed by single restriction endonuclease digestion with *AluI*, *HaeIII*, *HinfI*, *HpaII*, *TruI*, *TaqI*, *Tsp509I* (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. The restriction products were then separated by electrophoresis through 5% polyacrylamide gel stained and visualized as described above. Two semi-nested PCR products obtained with P1A/16S-SR primer pair (Lee et al., 2004) were purified using a Wizard® SV Gel and the PCR Clean-Up System Kit (Promega, Madison, WI, USA). Sequencing was performed with an automated DNA sequencer (ABI Prism Model 3730) at the Genelab (ENEA Casaccia, Rome, Italy) using the primers P1A, 16S-SR and the internal primer 16S(RT) F1 (Martini et al., unpublished). The obtained nucleotide sequences (about 1460 nt) were compared with those present in GenBank using the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The nucleotide sequences were deposited in GenBank.

Both 16S rRNA gene sequences of GWB phytoplasma strains along with 26 previously described and seven incidentally cited '*Candidatus* Phytoplasma' species, and *Acholeplasma palmae* (ATCC 49389T) were aligned using CLUSTAL V (Higgins & Sharp, 1989) from the Lasergene software MegAlign program. Cladistic analyses

were performed using PAUP version 4.0 (Swofford, 2002). *A. palmae* was used as the outgroup. Bootstrap analyses (1000 replicates) were performed to estimate the stability and support for the inferred clades. Virtual RFLP analysis of 16S rDNA F2nR2 fragments was conducted using the *iPhyClassifier* program (Zhao et al., 2009) available on the web site of USDA in Beltsville, MD, USA (<http://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi>).

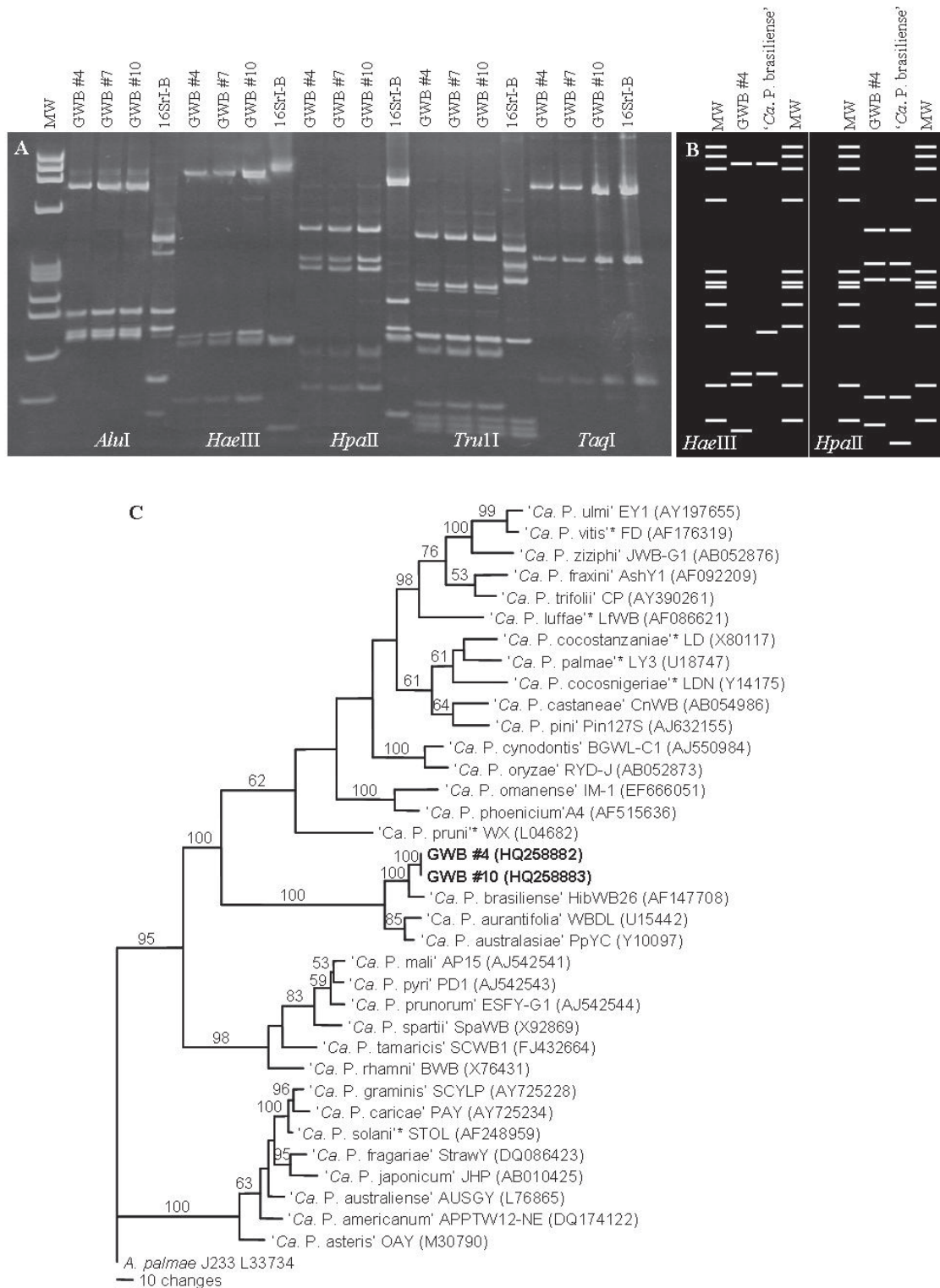
Numerous wall-less structures, round to pleomorphic with diameter between 200 and 500 nm, resembling phytoplasma (Bertaccini, 2007) were only observed in the phloem tissue of leaf midrib and petioles from symptomatic guacimo trees (Figure 1-D). The phytoplasma infection was confirmed by nested-PCR with primer pairs P1/16S-SR followed by R16F2n/R2 showing positive results with the amplification of 1.2 kb DNA fragments from all the tested symptomatic samples. No amplification was obtained from asymptomatic samples tested.

The DNA extraction protocol using ethylene glycol monomethyl ether in the initial steps permitted to obtain good quality DNAs for successful PCR amplifications, which were not achieved using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) and Martini et al. (2009) protocol without modified initial steps. This plant species produces a gummy product during the DNA extraction process and that may be the cause of unsuccessful amplification.

The actual RFLP analysis with the endonucleases *AluI*, *HaeIII*, *HpaII*, *TruI*, *TaqI* (Figure 2-A), *HinfI* and *Tsp509I* (data not shown) of R16F2n/R16R2 PCR products generated patterns identical among the phytoplasmas under study but different from the RFLP pattern of phytoplasma infecting *Sechium edule* (subgroup 16SrI-B, Villalobos et al., 2002). The RFLP results indicated that the phytoplasma associated with GWB belonged to hibiscus witches' broom group (16SrXV), represented by '*Candidatus* Phytoplasma (*Ca. P.*) *brasiliense*' (Montano et al., 2001b; Silva et al., 2009). The 16S rDNA sequence (about 1460 nt), obtained from the P1A/16S-SR semi-nested-PCR products of two phytoplasma strains (GenBank accession numbers: HQ258882 and HQ258883), shared 98.8% similarity with that of the '*Ca. P. brasiliense*' reference strain (GenBank accession: AF147708).

The virtual RFLP pattern derived from the query 16S rDNA F2nR2 fragment (data not shown) 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 16SrXV group, 16SrXV-B. The enzymes *HaeIII* and *HpaII* distinguished GWB phytoplasma from the closely related '*Ca. P. brasiliense*' (Figure 2-B). The actual laboratory restriction digestion with the key enzyme *HpaII* confirmed the new subgroup pattern (Figure 2-A). The phytoplasma nature of the GWB disease-associated agent was further confirmed by a phylogenetic analysis of its 16S





**FIGURE 2** - RFLP and phylogenetic analyses based on 16S rRNA gene of GWB phytoplasma strains, 16SrXV-B. **A.** Actual restriction patterns derived from digestions using enzymes: *AluI*, *HaeIII*, *HpaII*, *TruI* and *TaqI*. Lanes GWB #4, #7, #10: three different guazuma witches' broom (GWB) phytoplasma strains; 16SrI-B: phytoplasma infecting *Sechium edule*; MW:  $\Phi$ 174 *HaeIII* digested; **B.** Virtual RFLP patterns derived from *in silico* digestions using *HaeIII* and *HpaII* restriction enzymes of GWB #4 phytoplasma strain (16SrXV-B) and 'Ca. P. brasiliense' (AF147708, 16SrXV-A) sequences. MW:  $\Phi$ 174 *HaeIII* digested; **C.** Phylogenetic tree constructed by parsimony analysis of nearly full-length 16S rRNA gene sequences from GWB phytoplasma strains and previously described and incidentally cited 'Candidatus Phytoplasma (Ca. P.)' species. In bold GWB phytoplasma 16S rDNA sequences obtained in this work; other sequences used in this study were retrieved from GenBank.

rRNA gene sequence. The topology of the phylogenetic tree (Figure 2C) clearly demonstrated that the GWB disease-associated agent belonged to the phytoplasma clade and shared a common ancestor with '*Ca. P. brasiliense*'. All the above-mentioned results demonstrated that the phytoplasma under study is a '*Ca. P. brasiliense*'-related strain belonging to a new subgroup (16SrXV-B). This is the first report of the natural occurrence of a phytoplasma strain closely related to '*Ca. P. brasiliense*' (16SrXV-A) in Costa Rica, outside Brazil where this phytoplasma was first reported (Montano et al., 2001a,b; Silva et al., 2009).

This finding contributes knowledge about the diversity of phytoplasma diseases in Costa Rica and Central America, where the phytoplasmas detected are mainly related to groups 16SrI (Aster yellows group) (Lee et al., 2000; Villalobos et al., 2002; Saborío-R. et al. 2007; Moreira et al. 2010), 16SrIV (Coconut lethal yellows group) (Harrison et al., 2002; Roca et al., 2006) and 16SrIX (Pigeon pea witches'-broom group) (Kenyon et al., 1998, 1999). New diseases related to other phytoplasma groups have been also reported in the last years, such as 16SrIII in El Salvador (Parada et al., 2006) and 16SrXXXI in Costa Rica (Villalobos et al., 2009; Lee et al., 2011). Additionally, this is the first report of occurrence of a phytoplasma belonging to "*Ca. Phytoplasma brasiliense*" in a Sterculiaceae tree species. Previous reports of phytoplasmas in a Sterculiaceae weed species, *Waltheria indica*, were done in Brazil (Kitajima & Costa, 1971) and Australia (Schneider et al., 1999; Wilson et al., 2001); the last of these belonged to group 16SrII (Gen Bank accession Y15870).

Further molecular analyses will be necessary for better characterization of several phytoplasma strains from different geographical origins in Costa Rica on the basis of 16S rRNA gene sequences, and more variable genes such as ribosomal protein and *secY* genes. Additionally, studies will be carried out to investigate epidemiological aspects of the disease, host range and potential insect vectors. The information that is currently available does not allow us to determine if this native species is a phytoplasma reservoir for other economically important crops in Costa Rica or throughout Central America.

## ACKNOWLEDGMENTS

The authors thank the Universidad de Costa Rica and Università di Udine for supporting this research and the scientific visit of Dr. M. Martini (October 2009) to Costa Rica.

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TPP 214 - Received 12 December 2010 - Accepted 29 April 2011  
Section Editor: F. Murilo Zerbini