‘Candidatus Phytoplasma asteris’ strains associated with oil palm lethal wilt in Colombia

Elizabeth Alvarez, Plant Pathology Program, International Center for Tropical Agriculture (CIAT), Cali, Valle del Cauca, Colombia; Juan F. Mejía, CIAT and Department of Agricultural Sciences and Technologies (DipSA), Alma Mater Studiorum, University of Bologna, Italy; Nicoletta Contaldo and Samanta Paltrinieri, DipSA; Bojan Duduk, Institute of Pesticides and Environmental Protection, Belgrade, Serbia; and Assunta Bertaccini, DipSA.

Corresponding author: Elizabeth Alvarez
Email: E.ALVAREZ@CGIAR.ORG

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Abstract

Alvarez, E., Mejía, J. F., Contaldo, N., Paltrinieri, S., Duduk, B., and Bertaccini, A. 

The distribution of lethal wilt, a severe disease of oil palm, is spreading throughout South America. An incidence of about 30% was recorded in four commercial fields in Colombia. In this study, phytoplasmas were detected in symptomatic oil palms by using specific primers, based on 16S rDNA sequences, in nested polymerase chain reaction assays. The phytoplasmas were then identified as ‘Candidatus Phytoplasma asteris’, ribosomal subgroup 16SrI-B, through the use of restriction fragment length polymorphism (RFLP) analysis and sequencing. Cloning and sequencing of 16S rDNA from selected strains, together with phylogenetic analysis, confirmed the classification. Moreover, collective RFLP characterization of the groEL, amp, and rp genes, together with sequence data, distinguished the aster yellows strain detected in Colombian oil-palm samples from other aster yellows phytoplasmas used as reference strains, in particular from an aster yellows strain infecting corn in the same country.

Production of oil palm (Elaeis guineensis Jacq.) is a recent and now a major agricultural activity in Colombia. Because it constitutes a key alternative for generating employment, it is considered strategic for the national economy. However, production has declined by 7.1% since 2002. This drop occurred mainly in northeastern Colombia, where production decreased
by almost 10% (17). The cause of this decrease is a disease known as “lethal wilt of oil palm” (“marchitez letal” in Spanish). Lethal wilt is present in Colombia in the Upía River area, in the oil-palm plantations of Palmar del Oriente (July 1994), Palmas del Casanare (1999), Palmeras Santana (2000), and Palmeras del Upía (2002) (44). By 2010, the disease had severely infected oil palms in these areas, leading to their eradication on about 690 hectares with a total of 97,619 plants (17). Symptoms of lethal wilt usually first appear as vascular discoloration and leaf yellowing when the palm is mature (i.e., flowering and fruiting) at seven years old. These symptoms are followed by leaf drying, wilt, and necrosis of infected tissues, and eventual plant collapse (Fig. 1). Root necrosis often accompanies leaf discoloration. Internal discoloration of trunk tissue may also occur but does not represent a distinctive symptom. Lethal wilt is potentially destructive because it spreads rapidly and causes plant death within 4 to 6 months after symptoms first appear (43).

The pathogen was believed to be a phytoplasma, possibly related to that associated with lethal yellowing disease, which infect other palms such as coconut (2). Phytoplasmas have been associated with diseases in several hundred plant species (6). They are limited to the plants’ phloem tissue, and to insect vectors that feed directly from phloem tissues. Vectors include planthoppers and leafhoppers in the genera *Macrosteles*, *Euscelis*, *Euscelidius*, and *Scaphoideus*, and *Cacopsylla* (52).

Phytoplasmas are associated not only with lethal yellowing in coconut palms in many parts of the world, but also with diverse palm species. Worldwide this disease affects at least 30 species of palm, 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) (39,40). The disease has killed millions of coconut palms (*Cocos nucifera*) throughout the Caribbean, Florida, Mexico, and Central America (23,24,41,42). In addition, a first report of phytoplasmas in symptomatic oil palms was confirmed by electron
microscopy in West New Britain, Papua New Guinea (50). The group 16SrIV lethal yellowing phytoplasma has been shown to be vectored by *Myndus crudus* (American palm cixiid) and possibly also by *Cedusa* species of derbid planthoppers (8). Phytoplasmas closely related to the 16SrIV group have also been reported in date palm and other palm species in USA (22,23). They were also recently detected in weeds such as *Emilia fosbergii*, *Synedrella nodiflora*, and *Vernonia cinerea* (9,10). These weeds are all members of the Asteraceae, and were collected in Jamaica near diseased coconut palms.

More recently, phytoplasmas from other 16S ribosomal groups have been associated with symptoms in palms in other parts of the world. In Saudi Arabia, a 16SrI group was found associated with the Al-Wijam disease of date palm (1). In North Sudan, a 16SrXIV group, ‘*Ca. P. cynodontis*’, commonly associated with bermudagrass, was found in date palm showing slow decline (11). Recently, a 16SrI phytoplasma was associated with coconut yellow decline and oil palm in Malaysia (39,40). Similarly, the Weligama wilt disease of coconut in Sri Lanka was associated with phytoplasmas belonging to the 16SrXI ‘*Ca. P. oryzae*’ group. Phytoplasmas from two phylogenetic groups, 16SrXI and 16SrXIII, Mexican periwinkle virescence, were associated with Kalimantan wilt in Indonesia (51).

In South America, symptoms similar to those observed in Colombia were also described from oil palms in Brazil suffering from a disease known as “fatal yellowing” (7,38). Although the cause of this disease is still unknown, the symptoms and distribution of the problem in both Colombian and Brazilian plantations suggest that infectious agents are involved (49). A preliminary study also detected phytoplasmas in symptomatic plants in commercial crops of the susceptible oil-palm hybrid (*Elaeis guineensis × Elaeis oleifera*) (2) in Colombia.

Phytoplasma identification and classification rely on 16S ribosomal gene analysis to identify ‘*Candidatus Phytoplasma*’ species and distinguish between the 16Sr groups and subgroups (6). In particular, ‘*Candidatus Phytoplasma asteris*’ (‘*Ca. P. asteris*’) is classified in
the 16SrI group, in which at least 18 subgroups are recognized (30). Finer differentiation can also be obtained by studying polymorphisms on other genes (35,37) in order to monitor the spread of specific phytoplasma strains.

The large survey carried out in this work allowed us to verify phytoplasma presence in diverse tissues from diseased oil palms collected in four areas of Colombia affected by “marchitez letal”. The detected aster yellows strains were then characterized on four phytoplasma genes with a multilocus typing technique that allowed comparison and distinction of the strains infecting diseased oil palm from reference strains (5) and from a strain infecting corn in Colombia.

**Materials and Methods**

**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).

Samples were taken from each symptomatic and asymptomatic plant by collecting entire meristems and about 50 to 100 g from each of three tissue types: chlorotic leaves, spears, and inflorescences. Three 10 × 10 cm segments were also excised from the base of the trunk, together with ten 25-cm-long root segments from the root ball of each palm at 50 cm from the collar. From 44 symptomatic trees 85 samples from different tissues were tested; about half of these samples were collected from palms with severe symptoms (see below). Comparable tissues from seven 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.

**Detecting and identifying phytoplasmas.** DNA was extracted from 0.4 to 1.0 g of tissues from each plant sample as according to previously described protocols (19,44). Tissue samples were frozen and ground in liquid nitrogen using a sterilized mortar and pestle. After the final ethanol precipitation, nucleic acid extracts were resuspended in 30 to 50 µL of Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0; and 1 mM EDTA, pH 8.0) and stored at -20°C.

The phytoplasma universal primer pair P1/P7 (13,46) was used to amplify DNA from the 16Sr region and the beginning of the 23S rDNA genes, including the internal spacer region. Nested-PCR assays were performed on amplicons diluted at 1:29 with sterilized high-performance-liquid-chromatography-grade water, using primers R16F2n/R2 (20). Each PCR 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.

Positive controls employed 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 were extracted from the original host plant, as for maize bushy stunt from Colombia (Table 1). Samples
devoid of DNA template and from asymptomatic oil palms were added as negative controls for the PCR reactions.

Direct and nested-PCR assays were carried out in a PTC-100 thermal cycler with a heated lid (MJ Research, Inc., Waltham, MA), using the following thermal profile: 30 s (90 s for the first cycle) of denaturation at 94°C, annealing for 50 s at 55°C, and extension of the primer for 80 s (10 min in the final cycle) at 72°C. For primer pair R16F2n/R16R2, amplifying about 1,200 bp within the 16S rDNA region in nested-PCR assays, the annealing temperature was 50°C. The PCR products were visualized in a 1.5% agarose gel, stained with 0.75 µg/ml ethidium bromide, and analyzed in a Stratagene Eagle Eye® II video system (La Jolla, CA).

The 98 amplicons obtained with the R16F2n/R16R2 primers (1.2 kb) were then digested with restriction enzymes, *Tru*I and *Hha*I (Fermentas, Vilnius, Lithuania), following the manufacturer’s instructions. Separation of bands generated from restriction digestion was performed in 6.7% polyacrylamide gels. The DNA was then stained and visualized as described above.

Direct sequencing in both directions [using primers P1/F1 (12) 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. They were then 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 were performed using ClustalX and BioEdit (21,48). 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 *Candidatus* phytoplasmas strains using *Acholeplasma laidlawii* as the outgroup. GenBank accession numbers and other sources of 16S rRNA gene sequences used in
phylogenetic analyses are given in Table 1. Phylogenetic trees were constructed with maximum parsimony (MP) analysis, using the Close-Neighbor-Interchange algorithm, with the initial tree created by random addition for 10 replications of neighbor-joining (NJ) method, using MEGA version 5 (47). For all methods, all default values (gaps excluded) were performed with 1,000 replications for bootstrap analysis to estimate stability and support for the clades.

Strain characterization on groEL, rp, and amp genes. These gene regions were chosen because they were useful in distinguishing among phytoplasma strains in several studies (28,35,37). Amplification was carried out on 44 positive oil-palm samples obtained from previous phytoplasma identification on 16S rDNA gene.

The samples were employed for amplification in nested-PCR with groEL primers AYgroELF/AYampR, followed by AYgroELF/AYgroELR amplicons, diluted at 1:30 as described in published protocols (36,37). The negative and positive controls were as described above. RFLP analyses were carried out on amplicons using AluI and Tru1I restriction enzymes (Fermentas, Vilnius, Lithuania) according to the manufacturer’s instructions. Restriction fragments were separated as described above. 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 1), as described above.

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, as it has fine resolving power for differentiating distinct phytoplasma strains in 16S rDNA subgroups (35). The 44 oil-palm samples were employed for direct amplification with the rpFI/rpRI primer pair (33), 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 *Tru*I, *Hpy*8I, *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 SNPs presence in comparison with reference strains was also carried out using Mega version 5 (47).

The *amp* gene codes for a surface membrane protein that was recently reported as being involved in insect to phytoplasma transmission. It is therefore also suitable for phytoplasma strain differentiation (4,28). Direct PCR assays with Amp-N1/C1 primers, which amplify 702 bp of the *amp* gene, were carried out according to reported procedures (29). The 44 oil-palm samples tested and the negative and positive controls were all as described above. RFLP profiles generated with *Tru*I and *Tsp*509I were compared with those of the reference strains (Table 1). Direct sequencing and sequence assembly were performed on the amplicon from sample OP47. A phylogenetic tree was produced using available reference strains (Table 1) as described above. 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 2).

**Results**

**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. 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 template without nucleic acid, were negative according to nested PCR.

Symptoms were evaluated and compared with phytoplasma detection percentages in the diverse oil-palm tissues showing symptoms at different stages in two localities (A and B) (Fig. 2). Leaves or spears showed 86% to 100% incidence of phytoplasma detection in 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 (Fig. 3A). Phytoplasma strain OP47, obtained from a palm hybrid growing in a Palmar del Oriente field, was then selected for further molecular characterization. The 1,491-bp 16S rDNA sequence was deposited in GenBank under accession number JX681021 (Table 1), and showed 99% with a number of strains in group 16SrI, ‘Ca. P. asteris’. The sequence of strain OP47 was then employed for phylogenetic analysis and 20 equally parsimonious trees were constructed, using 27 additional strains of aster yellows (AY) phytoplasmas from different crops (Table 1). Results confirmed its placement in the 16SrI group (Fig. 4A).

**Strain characterization on groEL, rp, and amp genes.** The expected length (about 1.4 kb) of the amplicons of the partial groEL gene was amplified from 21 of the 44 oil-palm samples tested. They showed identical RFLP profiles after TruI and AluI digestion. This profile was identical to the one observed in maize bushy stunt (MBS) strain from Colombia, thus differentiating aster yellows phytoplasmas in oil palm from other AY strains and assigning them to the groELI RFLP subgroup V (Fig. 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 (Fig. 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 restriction profiles that were identical to each other and allowed clear differentiation.
of the two oil-palm phytoplasma strains from all the other AY strains, including maize from
Colombia (Fig. 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 from Colombia in the restriction site Hpy8I (Table 2).
However the further differentiation observed after RFLP analyses with AluI and Taal that
allow distinguishing oil palm aster yellows phytoplasma from maize as well as from all other
reference strains employed was not retrieved in SNPs comparison, presumably due to the
position of the sequenced fragment outside of the rp gene.

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 the one from
maize (data not shown). Sequencing and alignment for oil-palm strain 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 of aster yellows phytoplasmas available in GenBank
(Table 3). The phylogenetic tree confirmed the differentiation of phytoplasmas from oil palm
and maize in Colombia from other strains tested (data not shown).

Discussion

The results of this study confirmed the association of a phytoplasma strain related to ‘Ca.
P. asteris’ with oil palms severely affected by a 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
were 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 aster yellows phytoplasmas is clear.
The 16S rDNA is a valuable classification tool, but it is not always able to discriminate phytoplasm strains. The fine-scale molecular characterization of the phytoplasm from oil palm indicates that it can be differentiated from all other phytoplasmas in the same ribosomal subgroup enclosing those infecting corn in Colombia. Multilocus sequence analysis on amp, groEL and rp genes indicated that they could be useful molecular markers to follow 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 phytoplasm distribution in woody hosts, as recently described (3,16). The amplification of other genes allowed finer characterization of the phytoplasm strain infecting oil palms in Colombia, and indicated that it can be differentiated from all the other phytoplasm strains in the AY group, including a MBS strain from Colombia (15). To our knowledge, this is the first study in which the phytoplasm previously associated with lethal wilt of oil palm in Colombia (2) was classified and its molecular identity characterized. The phytoplasm was assigned to the 16SrI-B AY group, which was clearly differentiated from the other reference phytoplasm strains.

The association of more than one group of phytoplasmas with a specific set of disease symptoms at different locations is not unusual. Napier grass stunt disease in Kenya was shown to be associated with a phytoplasm from group 16SrXI (27), while in Ethiopia a phytoplasm from group 16SrIII was found associated with the same symptoms in Napier grass (26). These findings suggest that such phytoplasmas are being transmitted among plant species at these locations, although vectors have yet to be identified.

The epidemiological and etiological significance of the ability of phytoplasmas to move among plant species and into coconut and oil palm is unclear. 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 (52). 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 (25). 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 determining how these organisms are evolving and adapting to old and new plant and insect hosts.

To our knowledge, this is the first time where a multigenic characterization of conserved genes other than 16S rDNA distinguished an AY strain in a specific host plant. The close association of this pathogen with oil palm lethal wilt disease was also confirmed since all symptomatic plants tested positive for the phytoplasma. In spite of 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 provide important information and tools that can be employed to further study the disease. The epidemiology and insect vector identity can be defined for planning disease management strategies and contains further epidemics.

**Acknowledgements**

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Literature Cited


Table 1. Aster yellows-related reference phytoplasma (*Candidatus Phytoplasma asteris*) strains employed for 16S rDNA, groEL, ribosomal protein l22, and s3 characterization

<table>
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<th>RFLP classification&lt;sup&gt;b&lt;/sup&gt;</th>
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<sup>a</sup> Acronym used in phytoplasma literature

<sup>b</sup> RFLP classification: A, I, A; B, III, B; B(L), III, B; B(M), III, B

<sup>c</sup> Accession numbers for 16S rDNA, groEL, and rpl genes

References: (31,35,37)
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Strains in bold were used as references for PCR-RFLP analysis.

(?) refers to a strain with interoperator heterogeneity that is tentatively classified in this subgroup; – refers to a sequence not available in the GenBank.

Different letter represent diverse RFLP subgroups in the 16S rDNA gene of aster yellows strains

Different Roman number represent diverse RFLP groups in the groEL gene

Different letter represent diverse RFLP groups in the rp gene
Table 2. Differential SNP positions in ribosomal protein s3 sequences, of fifteen 'Candidatus Phytoplasma asteris' strains compared with OP47 (Oil palm lethal wilt).

<table>
<thead>
<tr>
<th>Strain</th>
<th>18</th>
<th>124*</th>
<th>139</th>
<th>252</th>
<th>277*</th>
<th>287*</th>
<th>285*</th>
<th>444*</th>
<th>486</th>
<th>571</th>
<th>667*</th>
<th>673</th>
</tr>
</thead>
<tbody>
<tr>
<td>OP47</td>
<td>C</td>
<td>C</td>
<td>A</td>
<td>T</td>
<td>A</td>
<td>A</td>
<td>C</td>
<td>T</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>MBS Col</td>
<td>.</td>
<td>A</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
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<tr>
<td>MBS</td>
<td>T</td>
<td>.</td>
<td>.</td>
<td>G</td>
<td>.</td>
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<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>PVM</td>
<td>T</td>
<td>.</td>
<td>G</td>
<td>C</td>
<td>G</td>
<td>C</td>
<td>T</td>
<td>A</td>
<td>T</td>
<td>G</td>
<td>T</td>
<td>G</td>
</tr>
<tr>
<td>ca2006/1</td>
<td>T</td>
<td>.</td>
<td>G</td>
<td>C</td>
<td>G</td>
<td>C</td>
<td>T</td>
<td>A</td>
<td>T</td>
<td>G</td>
<td>T</td>
<td>G</td>
</tr>
<tr>
<td>GD1</td>
<td>T</td>
<td>.</td>
<td>G</td>
<td>C</td>
<td>G</td>
<td>C</td>
<td>T</td>
<td>A</td>
<td>.</td>
<td>G</td>
<td>T</td>
<td>G</td>
</tr>
<tr>
<td>ca2006/5</td>
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<td>.</td>
<td>.</td>
<td>G</td>
<td>.</td>
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<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>G</td>
</tr>
<tr>
<td>KVE</td>
<td>T</td>
<td>.</td>
<td>G</td>
<td>C</td>
<td>G</td>
<td>C</td>
<td>A</td>
<td>A</td>
<td>T</td>
<td>G</td>
<td>.</td>
<td>G</td>
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</tbody>
</table>
SNPs making differential restriction sites for RFLP differentiation: 124 and 278 (Hpy8I), 277 and 285 (TruI), 444 (AluI), 667 (Taal). Dots represent nucleotides identical to the OP47 consensus sequence.
Table 3. Aster yellows-related reference phytoplasma strains employed for amp characterization, and their homology percentages.

<table>
<thead>
<tr>
<th>Phytoplasma associated disease (acronym)</th>
<th>Geographic origin</th>
<th>GenBank accession number</th>
<th>16SrI RFLP classification</th>
<th>% nucleotide (nt) and amino acid (aa) identity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil palm lethal wilt (OP47)</td>
<td>Colombia</td>
<td>JX681022</td>
<td>B</td>
<td>100 100</td>
<td>This work</td>
</tr>
<tr>
<td>Maize bushy stunt (MBS Col)</td>
<td>Colombia</td>
<td>KF434320</td>
<td>B</td>
<td>100 100</td>
<td>Unpublished</td>
</tr>
<tr>
<td>Paulownia witches’ broom (PaWB)</td>
<td>Taiwan</td>
<td>AB124810</td>
<td>D</td>
<td>95.3 89.5</td>
<td>(28)</td>
</tr>
<tr>
<td>Onion yellows (OYW)</td>
<td>Japan</td>
<td>AB124806</td>
<td>B</td>
<td>98.6 95.7</td>
<td>(28)</td>
</tr>
<tr>
<td>Periwinkle leaf yellowing (PLY)</td>
<td>Taiwan</td>
<td>GQ845122</td>
<td>–</td>
<td>98.3 94.8</td>
<td>Unpublished</td>
</tr>
<tr>
<td>Chrysanthemum yellows phytoplasma (CY)</td>
<td>Italy</td>
<td>DQ787852</td>
<td>B</td>
<td>95.3 89.5</td>
<td>(18)</td>
</tr>
<tr>
<td>Onion yellows (OY-M)</td>
<td>Japan</td>
<td>AB124807</td>
<td>B</td>
<td>97.6 95.2</td>
<td>(28)</td>
</tr>
<tr>
<td>Onion yellows (OY-NIM)</td>
<td>Japan</td>
<td>AB124808</td>
<td>B</td>
<td>96.3 95.2</td>
<td>(28)</td>
</tr>
<tr>
<td>Mulberry dwarf (MD)</td>
<td></td>
<td>AB124809</td>
<td>–</td>
<td>91.6 93.8</td>
<td>(28)</td>
</tr>
<tr>
<td>Rape virescence (RV)</td>
<td>France</td>
<td>AF244540</td>
<td>B</td>
<td>97.6 84.2</td>
<td>(4)</td>
</tr>
<tr>
<td>Bermudagrass white leaf (AYBG)</td>
<td>Thailand</td>
<td>AB124811</td>
<td>B</td>
<td>97.9 95.2</td>
<td>(28)</td>
</tr>
<tr>
<td>Iceland poppy yellows (IPY)</td>
<td>Japan</td>
<td>AB242234</td>
<td>B</td>
<td>98.3 95.2</td>
<td>(28)</td>
</tr>
<tr>
<td>Disease</td>
<td>Country</td>
<td>Accession</td>
<td>Ribosomal Group</td>
<td>Similarity 1</td>
<td>Similarity 2</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>---------</td>
<td>-----------</td>
<td>-----------------</td>
<td>--------------</td>
<td>--------------</td>
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<tr>
<td>Eggplant dwarf (ED)</td>
<td>Japan</td>
<td>AB242231</td>
<td>B</td>
<td>98.4</td>
<td>94.8</td>
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<tr>
<td>Sumac witches' broom (SWB)</td>
<td>Japan</td>
<td>AB242236</td>
<td>–</td>
<td>90.6</td>
<td>95.3</td>
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<tr>
<td>Porcelain vine witches’ broom (PvWB)</td>
<td>Korea</td>
<td>AB242237</td>
<td>–</td>
<td>92.3</td>
<td>80.9</td>
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<td>Lettuce yellows (LeY)</td>
<td>Japan</td>
<td>AB242233</td>
<td>B</td>
<td>98.1</td>
<td>83.7</td>
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<tr>
<td>Marguerite yellows (MarY)</td>
<td>Japan</td>
<td>AB242235</td>
<td>B</td>
<td>98.7</td>
<td>94.4</td>
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<td>Tomato yellows (TY)</td>
<td>Japan</td>
<td>AB242232</td>
<td>B</td>
<td>98.4</td>
<td>96.1</td>
</tr>
</tbody>
</table>

*– means “not described as a ribosomal group”.

In bold oil palm phytoplasma strain used for similarity comparison.
Figure legends

Fig. 1. Two diseased oil palms in Colombia, one with mild symptoms (severity score of 2) (A) and the other with severe symptoms (severity score of 4) (B) of lethal wilt.

Fig. 2. Oil palm wilt symptoms observed in relation to percentage of phytoplasma detection 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).

Fig. 3. Restriction fragment length polymorphism patterns of oil-palm phytoplasma strains OP45 and OP47 compared with those of several reference strains from periwinkle. (A) Phytoplasma 16S rDNA amplified in nested-PCR with R16F2n/R16R2 primers and digested with $TruI$ and $HhaI$ restriction enzymes. (B) Phytoplasma $groEL$ gene amplified in nested-PCR with AYgroelF/R primers and digested with $TruI$ and $AluI$ restriction enzymes. Strains employed were maize bushy stunt Colombia (MBS Col); periwinkle virescence (NA); aster yellows (AY-J); primula green yellows (PrG); grey dogwood stunt (GD1); carrot yellows (CA); clover phyllody-France (KVF); aster yellows from apricot (A-AY); clover phyllody-England (KVE); New Jersey aster yellows (NJ-AY); primrose virescence (PRIVA); plantago virescence (PVM); leontodon yellows (LEO); oilseed rape virescence (RV); potato purple top (PPT); aster yellows Germany (AVUT) Markers: phiX174, phiX174 $Hae$III digested; and pBR322, pBR322 $Hae$III digested.
Fig. 4. Phylogenetic trees constructed by maximum parsimony analysis of (A) 16S rDNA 525
gene sequences and (B) groEL gene sequence from selected phytoplasma strains. Strains 526
employed were paulownia witches’ broom (PaWB); Ipomea obscura witches’ broom (IOWB); 527
oil-palm lethal wilt (OP47); maize bushy stunt (MBS); primula green yellows (PrG); maize 528
bushy stunt Colombia (MBS Col); periwinkle virescence (NA); aster yellows apricot (A-AY); 529
blueberry stunt (BBS3); strawberry multiplier (STRAWB2); carrot yellows (ca2006/5rnnA; 530
ca2006/5rrnB; ca2006/5); carrot yellows (ca2006/9); ‘Ca. P. asteris’ strain OY-M 531
(Ch_005303); aster yellows (AY2192); aster yellows (AVUT); oilseed rape virescence (RV); 532
primrose virescence (PRIVA); aster yellows (AY-J); Populus decline (PopD); grey dogwood 533
stunt (GD1); plantago virescence (PVM); New Jersey aster yellows (NJ-AY); carrot yellows 534
(ca2006/1); carrot yellows (CA); clover phyllody England (KVE); clover phyllody France 535
(KVF); leontodon yellows (LEO); potato purple top (PPT); ‘Ca. P. australiense’ (NC 536
010544); ‘Ca. P. mali’ strain AT (NC 011047) and Acholeplasma laidlawii PG-8A 537
(CP000896). Numbers on the branches are bootstrap values obtained for 1,000 replicates (only 538
values above 60% are shown).

Fig. 5. Restriction fragment length polymorphism (RFLP) patterns of oil-palm phytoplasma 541
strains OP45 and OP47 compared with several reference strains from periwinkle that were 542
amplified with primers rpF1/rpR1 and digested with restriction enzymes Trul (A); Taal (B); 543
Hpy8I (C), and Alul (D). Strains employed were maize bushy stunt Colombia (MBS Col); 544
periwinkle virescence (NA); aster yellows (AY-J); primula green yellows (PrG); grey 545
dogwood stunt (GD1); carrot yellows (CA); clover phyllody-France (KVF); aster yellows 546
from apricot (A-AY); clover phyllody-England (KVE); New Jersey aster yellows (NJ-AY); 547
primrose virescence (PRIVA); plantago virescence (PVM); leontodon yellows (LEO); oilseed
rape virescence (RV); potato purple top (PPT). Markers: phiX174, phiX174 *Hae*III digested; and pBR322, pBR322 *Hae*I digested.
Fig. 1. Two diseased oil palms in Colombia, one with mild symptoms (severity score of 2) (A) and the other with severe symptoms (severity score of 4) (B) of lethal wilt.

253x159mm (150 x 150 DPI)
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Fig. 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 TruI (A); TaaI (B); Hpy8I (C), and AluI (D). Strains employed were maize bushy stunt Colombia (MBS Col); periwinkle virescence (NA); aster yellows (AY-J); primula green yellows (PrG); grey dogwood stunt (GD1); carrot yellows (CA); clover phyllody-France (KVF); aster yellows from apricot (A-AY); clover phyllody-England (KVE); New Jersey aster yellows (NJ-AY); primrose virescence (PRIVA); plantago virescence (PVM); leontodon yellows (LEO); oilseed rape virescence (RV); potato purple top (PPT). Markers: phiX174, phiX174 HaeIII digested; and pBR322, pBR322 HaeI digested.

380x232mm (150 x 150 DPI)