'*Candidatus* Phytoplasma asteris' strains associated with oil palm lethal wilt in Colombia

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Abstract

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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

71 microscopy in West New Britain, Papua New Guinea (50). The group 16SrIV lethal yellowing
72 phytoplasma has been shown to be vectored by *Myndus crudus* (American palm cixiid) and
73 possibly also by *Cedusa* species of derbid planthoppers (8). Phytoplasmas closely related to
74 the 16SrIV group have also been reported in date palm and other palm species in USA (22,23).
75 They were also recently detected in weeds such as *Emilia fosbergii, Synedrella nodiflora*, and
76 *Vernonia cinerea* (9,10). These weeds are all members of the Asteraceae, and were collected
77 in Jamaica near diseased coconut palms.

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

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

93 Phytoplasma identification and classification rely on 16S ribosomal gene analysis to
94 identify '*Candidatus* Phytoplasma' species and distinguish between the 16Sr groups and
95 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, *Tru1*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 *Alu*I and *Tru1*I 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 rpF1/rpR1 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 *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 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 *Tsp509*I 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 *Tru1*I and *Alu*I 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 *groEL*I 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 *Hpy8*I (Table 2). However the further differentiation observed after RFLP analyses with *Alu*I and *Taa*I 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 phytoplasma strains. The fine-scale 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. 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 phytoplasma distribution in woody hosts, as recently described (3,16). 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 AY group, including a MBS strain from Colombia (15). To our knowledge, this is the first study in which the phytoplasma previously associated with lethal wilt of oil palm in Colombia (2) was classified and its molecular identity characterized. The phytoplasma was assigned to the 16SrI-B AY group, which was clearly differentiated from the other reference phytoplasma 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 phytoplasma from group 16SrXI (27), while in Ethiopia a phytoplasma 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.

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474 **Table 1.** Aster yellows-related reference phytoplasma ('*Candidatus* Phytoplasma asteris') strains employed for 16S rDNA, *groEL,*

475 ribosomal protein *l22*, and *s3* characterization

Phytoplasma associated disease (acronym)[a]	**Geographic origin**	**GenBank accession numbers**			**RFLP classification**[b]			
		16S rDNA	***groEL***	***rp***	**16SrI**[c]	***groEL*I**[d]	***rp*I**[e]	**References**
New Jersey aster yellows **(NJ-AY)**	NJ, USA	HM590622	AB599703	-	A	I	A	(31,35,37)
Plantago virescence **(PVM)**	Germany	AY265216	AB599706	AY264867	A	I	A	(5,35,37)
Carrot yellows (ca2006/1)	Serbia	EU215424	AB599708	EU215428	A	I	A	(14,37)
Grey dogwood stunt **(GD1)**	NY, USA	DQ112021	AB599694	AY264864	A	II	M	(32,37)
Periwinkle virescence **(NA)**	Italy	HM590621	AB599702	-	B	III	-	(5,37)
Primula green yellows **(PrG)**	UK	HM590623	AB599696	-	B	III	-	(5,37)
Oilseed rape virescence **(RV)**	France	HM590625	AB599698	-	B	III	-	(5,37)
Carrot yellows (ca2006/9)	Serbia	EU215426	AB599709	EU215430	B	III	B	(14,37)
Primrose virescence **(PRIVA)**	Germany	AY265210	AB599705	-	B(L)	III	B	(35,45)
Aster yellows (AV2192)	Germany	AY180957	AB599687	AY183708	B(L)	III	B	(34,35,37)
Aster yellows **(AVUT)**	Germany	AY265209	AB599686	AY264855	B(M)	III	B	(34,35,37)
Aster yellows **(AY-J)**	France	HM590616	AB599689	-	B	IV	-	(37)

Carrot yellows (ca2006/5rrnA)	Serbia	EU215425/	AB599711	EU215429	B(?)	IV	-	(14,37)
Carrot yellows (ca2006/5rrnB)	Serbia	GQ175789						
Maize bushy stunt **(MBS Col)**	Colombia	HQ530152	AB599712	KF434319	B	V	-	(15,37,Unpublished)
Maize bushy stunt (MBS)	Mexico	AY265208	–	AY264858	B	–	L	(30)
Oil palm lethal wilt **(OP47)**	Colombia	JX681021	JX681023	KF434318	B	V	-	This work
Leontodon yellows **(LEO)**	Italy	HM590620	AB599701	-	C	VI	-	(5,37)
Carrot yellows **(CA)**	Italy	HM448473	AB599690	-	C	VI	-	(5,37)
Clover phyllody **(KVE)**	France	AY265217	–	AY264861	C	–	C	(30,35)
Clover phyllody **(KVF)**	France	HQ530150	AB599695	-	C	VII	-	(34,37)
Potato purple top **(PPT)**	France	HQ530151	AB599704	-	C	VII	-	(34,37)
Paulownia witches' broom (PaWB)	Taiwan	AY265206	AB124810	AY264857	D	–	D	(28,30)
Blueberry stunt (BBS3)	MI, USA	AY265213	–	AY264863	E	–	E	(30)
Aster yellows apricot **(A-AY)**	Spain	AY265211	AB599699	AY264866	F	VIII	N	(30,37)
Strawberry multiplier (STRAWB2)	FL, USA	U96616	–	U96617	K	–	J	(30)
Ipomea obscura witches' broom (IOWB)	Taiwan	AY265205	–	AY264859	N	–	F	(30,34)
Populus decline (PopD)	Serbia	HM590626	AB599710	-	P	IX	-	(37)

[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

492 **Table 2.** Differential SNP positions in ribosomal protein *s3* sequences, of fifteen '*Candidatus* Phytoplasma asteris' strains compared with OP47

493 (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 making 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.

497 **Table 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		Reference
				nt	aa	
Oil palm lethal wilt **(OP47)**	Colombia	JX681022	B	100	100	This work
Maize bushy stunt (MBS Col)	Colombia	KF434320	B	100	100	Unpublished
Paulownia witches' broom (PaWB)	Taiwan	AB124810	D	95.3	89.5	(28)
Onion yellows (OYW)	Japan	AB124806	B	98.6	95.7	(28)
Periwinkle leaf yellowing (PLY)	Taiwan	GQ845122	–	98.3	94.8	Unpublished
Chrysanthemum yellows phytoplasma (CY)	Italy	DQ787852	B	95.3	89.5	(18)
Onion yellows (OY-M)	Japan	AB124807	B	97.6	95.2	(28)
Onion yellows (OY-NIM)	Japan	AB124808	B	96.3	95.2	(28)
Mulberry dwarf (MD)		AB124809	–	91.6	93.8	(28)
Rape virescence (RV)	France	AF244540	B	97.6	84.2	(4)
Bermudagrass white leaf (AYBG)	Thailand	AB124811	B	97.9	95.2	(28)
Iceland poppy yellows (IPY)	Japan	AB242234	B	98.3	95.2	(28)

Eggplant dwarf (ED)	Japan	AB242231	B	98.4	94.8	(28)
Sumac witches' broom (SWB)	Japan	AB242236	–	90.6	95.3	(28)
Porcelain vine witches' broom (PvWB)	Korea	AB242237	–	92.3	80.9	(28)
Lettuce yellows (LeY)	Japan	AB242233	B	98.1	83.7	(28)
Marguerite yellows (MarY)	Japan	AB242235	B	98.7	94.4	(28)
Tomato yellows (TY)	Japan	AB242232	B	98.4	96.1	(28)

[a] – 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 *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. 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*I digested.

Fig. 4. Phylogenetic trees constructed by maximum parsimony analysis of (**A**) 16S rDNA gene sequences and (**B**) *groEL* gene sequence from selected phytoplasma strains. Strains employed were paulownia witches' broom (PaWB); *Ipomea obscura* witches' broom (IOWB); oil-palm lethal wilt (OP47); maize bushy stunt (MBS); primula green yellows (PrG); maize bushy stunt Colombia (MBS Col); periwinkle virescence (NA); aster yellows apricot (A-AY); blueberry stunt (BBS3); strawberry multiplier (STRAWB2); carrot yellows (ca2006/5rrnA; ca2006/5rrnB; ca2006/5); carrot yellows (ca2006/9); '*Ca.* P. asteris' strain OY-M (NC_005303); aster yellows (AY2192); aster yellows (AVUT); oilseed rape virescence (RV); primrose virescence (PRIVA); aster yellows (AY-J); *Populus* decline (PopD); grey dogwood stunt (GD1); plantago virescence (PVM); New Jersey aster yellows (NJ-AY); carrot yellows (ca2006/1); carrot yellows (CA); clover phyllody England (KVE); clover phyllody France (KVF); leontodon yellows (LEO); potato purple top (PPT);. '*Ca.* P. australiense' (NC 010544); '*Ca.* P. mali' strain AT (NC 011047) and *Acholeplasma laidlawii* PG-8A (CP000896). Numbers on the branches are bootstrap values obtained for 1,000 replicates (only values above 60% are shown).

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 *Tru*I (**A**); *Taa*I (**B**); *Hpy8*I (**C**), and *Alu*I (**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

549 rape virescence (RV); potato purple top (PPT). Markers: phiX174, phiX174 *Hae*III digested;

550 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.
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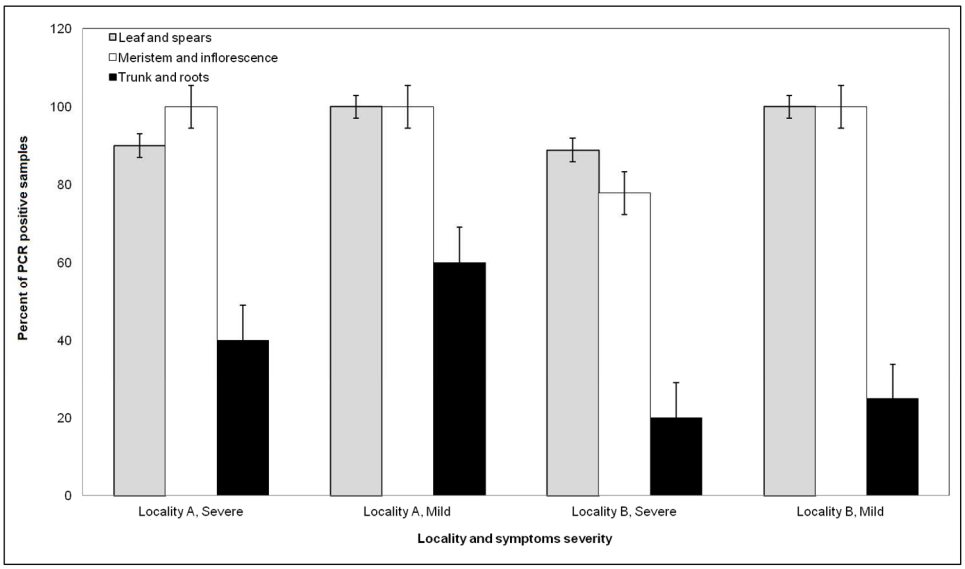

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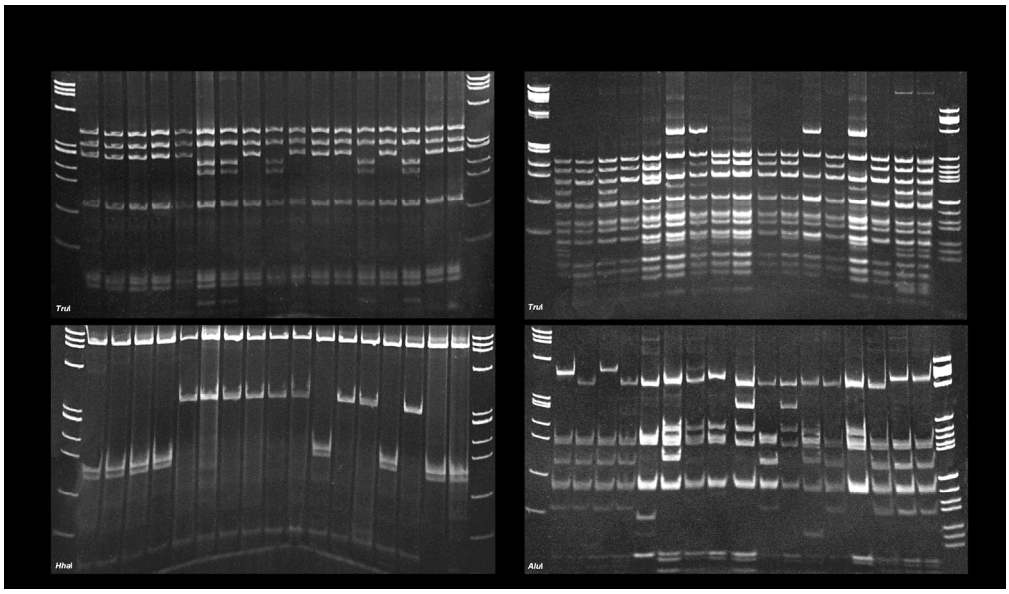

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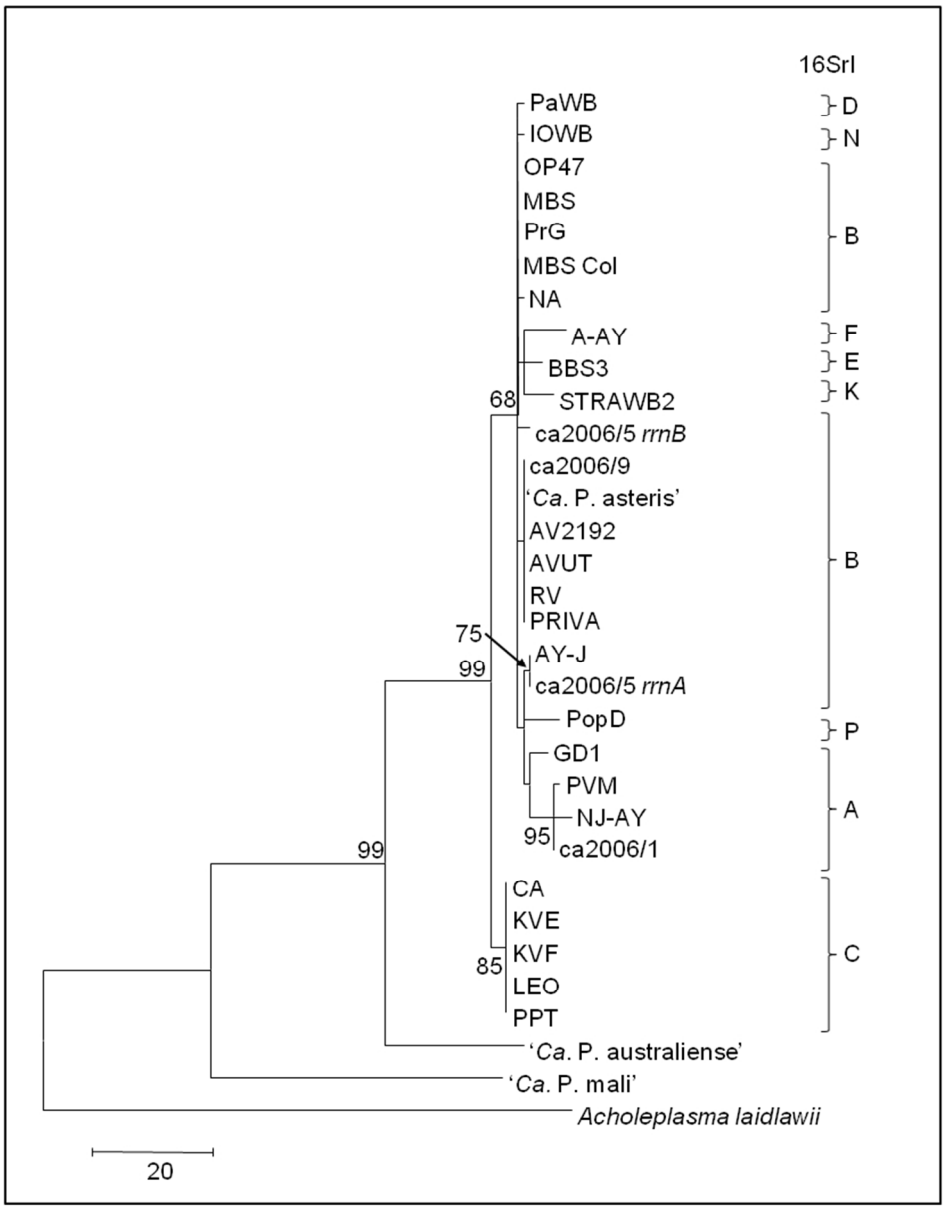

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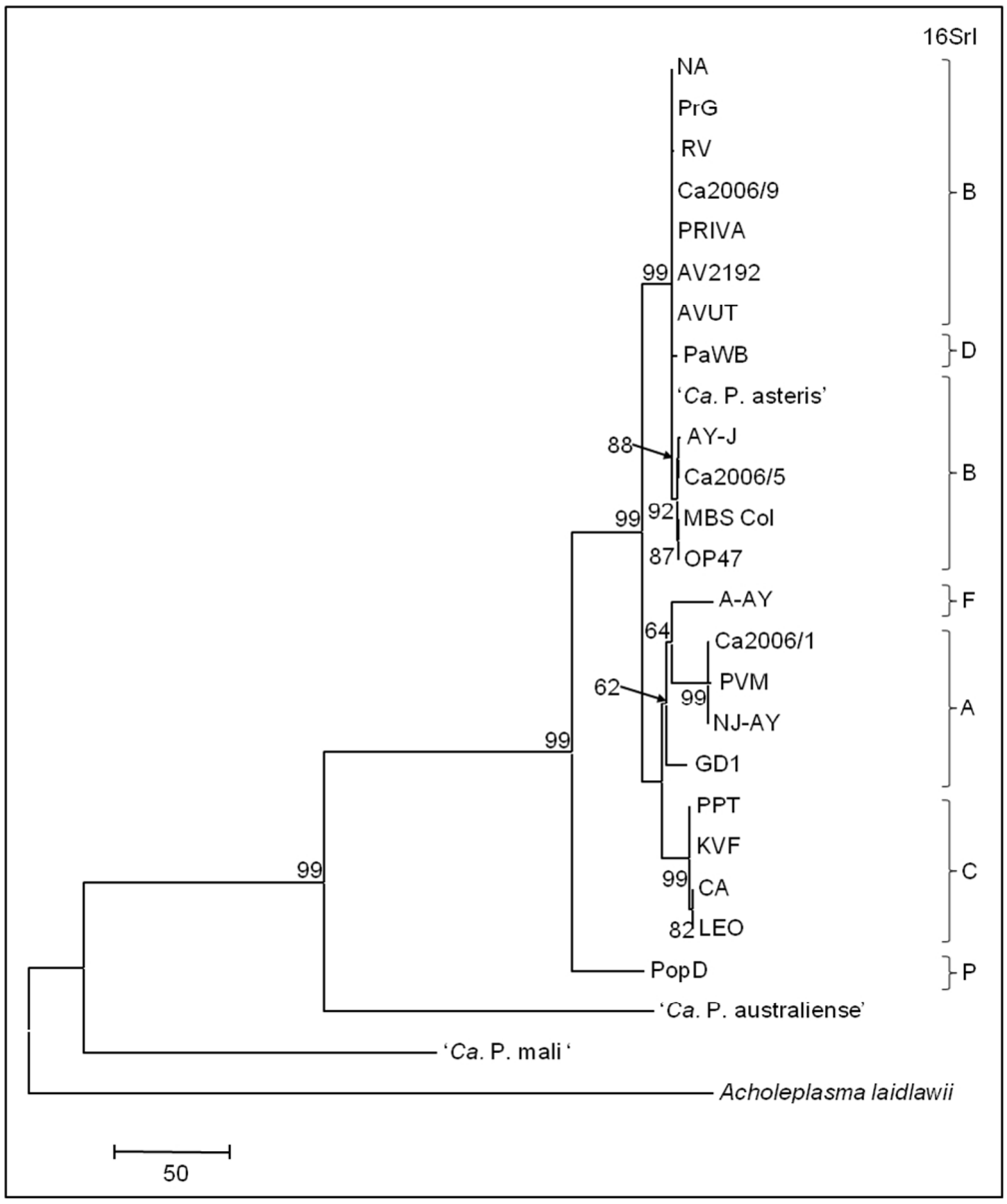

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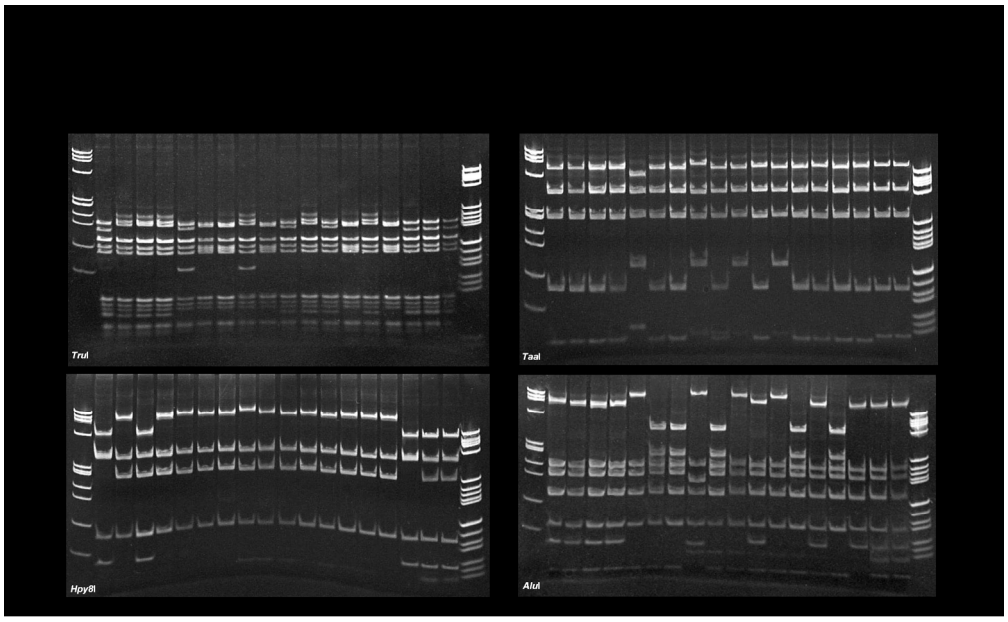

Fig. 5. Restriction fragment length polymorphism (RFLP) patterns of oil-palm phytoplasma strains OP45 and OP47 compared with several reference strains from periwinkle that were amplified with primers rpF1/rpR1 and digested with restriction enzymes TruI (A); TaaI (B); Hpy8I (C), and AluI (D). Strains 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)