'*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 palm by using specific primers, based on 16S ribosomal DNA (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 se-

quencing. 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 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 palm in these areas, leading to their eradication on about 690 ha 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 7 years old. These symptoms are followed by leaf drying, wilt, 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 infects other palm species 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 *Macrostelus*, *Euscelis*, *Euscelidius*, *Scaphoideus*, and *Cacopsylla* (52).

Phytoplasmas are associated not only with lethal yellowing in coconut palm 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), *P. canariensis* (Canary Island date palm), and *E. guineensis* (African oil palm) (39,40). The disease has killed millions of coconut palm trees (*Cocos nucifera*) throughout the Caribbean, Florida, Mexico, and Central America (23,24,41,42). In addition, a first report of phytoplasmas in symptomatic oil palm trees 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* spp. of derbid planthoppers (8). Phytoplasmas closely related to the 16SrIV group have also been reported in date palm and other palm species in the United States (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 family and were collected in Jamaica near diseased coconut palm.

More recently, phytoplasmas from other 16S ribosomal groups have been associated with symptoms in palm 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, '*Candidatus Phytoplasma 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 palm 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

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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* × *E. oleifera*) (2) in Colombia.

Phytoplasma identification and classification rely on 16S ribosomal gene analysis to identify ‘*Ca. P.*’ species and distinguish between the 16Sr groups and subgroups (6). In particular, ‘*Ca. P. asteris*’ is classified in the 16Srl 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 palm collected in four areas of Colombia affected by “marchitez letal”. The detected aster yellows (AY) 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 7 asymptomatic oil palm trees 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 plantation A: Palmar del Oriente S.A. (located at 4°30′15″ N and 72°56′20″ W), plantation B: Palmas del Casanare S.A. (4°35′58.33″ N and 72°50′58.74″ W), plantation C: Palmeras Santana Ltda. (4°32′24.18″ N and 72°52′51.38″ W), and plantation 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-by-10-cm segments were also excised from the base of the trunk, together with 10 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 palm trees 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). In total, 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, 2 or 3 as having moderate symptoms, and 3 or 4 as having severe symptoms. The ability to detect 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, 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 ribosomal DNA (rDNA) genes, including the internal spacer region. Nested polymerase chain reaction (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, 1× buffer, *Taq* polymerase (Sigma-Aldrich) at 0.05 U/µl, 0.2 mM dNTPs (Invitrogen Life Technologies), and 0.4 µM each primer.

Positive controls employed for the molecular analyses included DNA from phytoplasma reference strains that represented different ribosomal 16S rDNA subgroups. These strains either had been 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 palm trees 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.), using the

Fig. 1. Two diseased oil palm plants in Colombia, **A**, one with mild symptoms (severity score of 1 or 2) and **B**, the other with severe symptoms (severity score of 3 or 4) of lethal wilt.

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 ethidium bromide at 0.75 µg/ml, and analyzed in a Stratagene Eagle Eye II video system.

The 98 amplicons obtained with the R16F2n/R16R2 primers (1.2 kb) were then digested with restriction enzymes *TruI* and *HhaI* (Fermentas), 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). 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 [NCBI]).

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*' phytoplasma 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 analysis, using the Close-Neighbor-Interchange algorithm, with the initial tree created by random addition for 10 replications of neighbor-join-

ing 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 the 16S rDNA gene (one per plant).

The samples were employed for amplification in nested PCR with *groEL* primers AYgroesF/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. Restriction fragment length polymorphism (RFLP) analyses were carried out on amplicons using *AluI* and *TruI* restriction enzymes (Fermentas) 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, because it has finer 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

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

Disease (acronym) ^b	Geographic origin	GenBank accession numbers			RFLP classification ^a			References
		16S rDNA	<i>groEL</i>	<i>rp</i>	16SrI	<i>groELI</i>	<i>rpI</i>	
New Jersey aster yellows (NJ-AY)	New Jersey	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
Gray dogwood stunt (GD1)	New York	DQ112021	AB599694	AY264864	A	II	M	32,37
Periwinkle virescence (NA)	Italy	HM590621	AB599702	–	B	III	–	5,37
Primula green yellows (PrG)	United Kingdom	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,UP ^c
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)	Mississippi	AY265213	–	AY264863	E	–	E	30
Aster yellows apricot (A-AY)	Spain	AY265211	AB599699	AY264866	F	VIII	N	30,37
Strawberry multiplier (STRAWB2)	Florida	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 Different letter represent diverse restriction fragment length polymorphism (RFLP) subgroups in the 16S rDNA and *rpI* genes of aster yellows strains and different Roman number represent diverse RFLP groups in the *groEL* gene; (?) refers to a strain with interoperon heterogeneity that is tentatively classified in this subgroup; – refers to a sequence not available in the GenBank.

^b Phytoplasma-associated disease. Strains in bold were used as references for polymerase chain reaction RFLP analysis.

^c UP = unpublished.

TruI, *Hpy8I*, *TaaI*, and *AluI* were then performed. The *rpF1/rpR1* fragment of OP47 samples was also sequenced as described above and a search for the presence of single-nucleotide polymorphisms (SNPs) in comparison with reference strains was also carried out using MEGA version 5 (47) (Table 2).

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 (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 *TruI* and *Tsp509I* 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; NCBI) (Table 3).

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 palm plants. RFLP patterns from the positive samples were indis-

Table 2. Differential single-nucleotide polymorphism (SNP) positions in ribosomal protein *s3* sequences of 15 *'Candidatus Phytoplasma asteris'* strains compared with OP47 (Oil palm lethal wilt)

Strain	SNP positions in ribosomal protein <i>s3</i> ^a											
	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

^a Asterisk (*) indicates SNPs making differential restriction sites for restriction fragment length polymorphism differentiation: 124 and 278 (*Hpy8I*), 277 and 285 (*TruI*), 444 (*AluI*), and 667 (*TaaI*). 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

Phytoplasma-associated disease (acronym) ^a	Origin ^b	GenBank ^c	16SrI RFLP ^d	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 Oil palm phytoplasma strain used for similarity comparison shown in bold.

^b Geographic origin.

^c GenBank accession number.

^d 16SrI restriction fragment length polymorphism (RFLP) classification; – indicates “not described as a ribosomal group”.

tinguishable 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 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.

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 ribosomal DNA amplified in nested polymerase chain reaction (PCR) with R16F2n/R16R2 primers and digested with *TruI* and *HhaI* restriction enzymes. **B.** Phytoplasma *groEL* gene amplified in nested PCR with AYgroEL/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); gray 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 *HaeIII* digested; and pBR322, pBR322 *HaeI* digested.

Fig. 4. Phylogenetic trees constructed by maximum parsimony analysis of **A**, 16S ribosomal DNA 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 (ca2006/5*rmA*; ca2006/5*rmB*; ca2006/5); carrot yellows (ca2006/9); '*Candidatus* Phytoplasma 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); gray 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 *Achleoplasma laidlawii* PG-8A (CP000896). Numbers on the branches are bootstrap values obtained for 1,000 replicates (only values above 60% are shown).

They showed identical RFLP profiles after *Tru*II and *Alu*I digestion. This profile was identical to the one observed in maize bushy stunt (MBS) strain from Colombia, thus differentiating AY 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 SNP comparison confirmed the differentiation of phytoplasmas from oil palm and maize from Colombia in the restriction site *Hpy*8I (Table 2). However the further differentiation observed after RFLP analyses with *Alu*I and *Taa*I that allow distinguishing oil palm AY phytoplasma from maize as well as from all other reference strains employed was not retrieved in SNP 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 palm 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 AY 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 palm severely affected by a lethal wilt in Colombia. The work carried out expanded knowledge of this oil palm disease because 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 AY 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 palm 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 palm in Colombia, and indicated that it can be differentiated from all the other phytoplasma strains in the AY group, including an MBS strain from Colombia (15). To our knowledge, this is the first study in which the phytoplasma previously associated with lethal wilt of

Fig. 5. Restriction fragment length polymorphism 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 **A**, *TruI*; **B**, *TaqI*; **C**, *Hpy8I*, and **D**, *AluI*. Strains employed were maize bushy stunt Colombia (MBS Col); periwinkle virescence (NA); aster yellows (AY-J); primula green yellows (PrG); gray 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); and potato purple top (PPT). Markers: phiX174, phiX174 *HaeIII* digested; and pBR322, pBR322 *HaeI* digested.

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 toward 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 because 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 containing further epidemics.

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