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RESEARCH ARTICLE

Detection and differentiation of the coconut lethal yellowing phytoplasma in coconut-growing villages of Grand-Lahou, Côte d'Ivoire

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16SrXXII-B phytoplasma; '*Candidatus* Phytoplasma palmicola', coconut palm (*Cocos nucifera*); lethal yellowing; PCR/RFLP; phylogeny; ribosomal protein gene; *sec*A gene.

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Abstract

Surveys for the Côte d'Ivoire lethal yellowing (CILY) phytoplasma were conducted in eight severely CILY-affected villages of Grand-Lahou in 2015. Leaves, inflorescences and trunk borings were collected from coconut palms showing CILY symptoms and from symptomless trees. Total DNA was extracted from these samples and tested by nested polymerase chain reaction/RFLP and sequence analysis of the *16S rRNA*, ribosomal protein (*rp*) and the translocation protein (*sec*A) genes. The CILY phytoplasma was detected in 82.9% of the symptom-bearing palms collected from all the surveyed villages and from all the plant parts. Trunk borings were recommended as the most suitable plant tissue type for sampling. Results indicate that the CILY phytoplasma may have a westward spread to other coconut-growing areas of Grand-Lahou. CILY phytoplasma strains infecting coconut palms in the western region of Grand-Lahou exhibited unique single nucleotide polymorphisms on the rp sequence compared to the strains from the eastern region. Moreover, single nucleotide polymorphisms on the SecA sequence distinguished the CILY phytoplasma from the Cape St. Paul Wilt Disease phytoplasma in Ghana, and the Lethal Yellowing phytoplasma in Mozambique.

Introduction

Côte d'Ivoire is among the first 24 coconut-producing countries in the world with an annual production of 195 000 tonnes of nuts (Muyengi *et al.,* 2015). It is the top African exporter of copra coconut oil to Europe and West Africa (Allou *et al.,* 2012). Since the 1990s, a lethal yellowing (LY)-type disease of coconut severely affected Grand-Lahou, located in the southern coastal district of the country (Arocha-Rosete *et al.,* 2014). It was, however, not until 2013 that the Côte d'Ivoire Lethal Yellowing (CILY) was associated with a phytoplasma (Konan Konan *et al.,* 2013a). The disease has decimated over 400 ha of coconut plantations (Arocha-*Rosete et al.,* 2014), and it is rapidly spreading into more coconut-growing villages of Grand-Lahou.

LY or Lethal Decline (LD) phytoplasma diseases are the most important threat to coconut production in the world, and are currently affecting about 38 species of palms throughout the Americas and the Caribbean region (Sullivan & Harrison, 2013). LY and LD diseases have been responsible for destroying the livelihoods of many people in Africa, Central America and the Caribbean who depend on the palm trees for nourishment, building materials and income (Strauss, 2009). Phytoplasmas are plant pathogenic bacteria in the class Mollicutes, transmitted by

insect vectors of the Hemiptera order. These pathogens infect hundreds of plant species worldwide, including economically important crops, fruit trees and ornamental plants (Maejima *et al.*, 2014).

Coconut palms affected by CILY die within approximately 6 months after the onset of initial symptoms (Konan Konan *et al.*, 2013b), showing bare trunks known as "telephone poles" for the LY diseases in the Caribbean and Central America (Brown *et al.*, 2007). The situation also resembles that of the Cape St. Paul Wilt Disease (CSPWD) in Ghana that killed approximately 1 million coconut palms during the last 30 years (Eziashi & Omamor, 2010; Danyo, 2011).

The CILY phytoplasma was identified as member of the new subgroup 16SrXXII-B based on *16S rRNA* gene sequence analysis (Arocha-Rosete *et al.*, 2014) and officially named by Harrison *et al.* (2014) as '*Candidatus* Phytoplasma palmicola'-related strains, which embraces both the CSPWD and CILY phytoplasmas, and it is closely related to subgroup 16SrXXII-A '*Ca.* P. palmicola' that includes the LY phytoplasma strain from Mozambique (LYM) and the LD phytoplasma strain from Nigeria (LDN).

Although it is the officially recognised marker for phytoplasma classification (Bertaccini *et al.*, 2014), the *16S rRNA* gene has limitations in differentiating and discriminating closely related phytoplasmas. RFLP typing on non-ribosomal gene sequences like the cell membrane translocation protein (*sec*A) (Hodgetts *et al.*, 2008) and ribosomal proteins (*rp*) genes, *rpIV* and *rpsC* (Martini *et al.*, 2007) have been used for finer phytoplasma differentiation.

The *rp* genes are conserved among bacterial species, and are more variable than the *16S rRNA* gene. They serve as phylogenetic marker useful for bacterial differentiation and classification (Daubin *et al.*, 2002). The *rp* genes have been used to better distinguish within phytoplasma 16Sr groups, including LY phytoplasmas (Martini *et al.*, 2007). Preliminary studies in Ghana report a single nucleotide polymorphism (SNP) in the *rp* gene sequence of the CSPWD phytoplasma strains from the Central and Western Regions, and the Volta Region, indicating their possible geographic differentiation (Pilet *et al.*, 2011).

A high degree of divergence among the phytoplasmas affecting coconut palms was found in the *sec*A gene sequences (Hodgetts *et al.*, 2008). SecA sequence analyses separated them into at least three distinct taxon, reflecting the strain geographic origins: an Americas' group typified by LY, an East African group typified by Tanzanian LD, and a West African group typified by LD from Nigeria and CSPWD from Ghana (Bila *et al.*, 2015). This supported the suitability of the *sec*A gene for the discrimination of the LY-like and LD-like phytoplasmas.

The CSPWD phytoplasma has been predominantly detected in infected coconut palms as the disease progresses (Yankey, 2012). It has been detected more frequently from stem and inflorescences than from leaves (Nipah *et al.*, 2007). However, no information is available on how the CILY phytoplasma is differentially detected from stem, inflorescences or leaves. This could be important in determining the most suitable tissue type for accurate detection of the CILY phytoplasma.

This study was carried out with surveys in eight coconut-growing villages of Grand-Lahou to assess the differential detection of the CILY phytoplasma in the stem, inflorescences and leaves of affected palms. Information on the most likely trend of CILY spread, and the most suitable plant tissue type that supports its detection and its environmentally friendly sampling is provided. Unique SecA sequence SNPs that distinguish the CILY phytoplasma from the CSPWD and the LYM strains, and further unique SNPs on the *rp* gene sequence that distinguish the CILY phytoplasma strains from the eastern and the western areas of Grand-Lahou are also reported.

Materials and methods

Villages of Grand-Lahou surveyed

Grand-Lahou is a coastal town in southern Côte d'Ivoire in the Lagunes District, located on a strip of land in the middle of the Grand-Lahou lagoon, where the Bandama River meets the Gulf of Guinea, 103 km from Abidjan. Surveys for the CILY phytoplasma were conducted in eight villages of Grand-Lahou: Adjadon, Amanikro, Badadon, Braffedon, Doudougbazou, Likpilassié, Palmindustrie V1, and Yaokro (Fig. 1). Using Grand-Lahou downtown as the reference, Braffedon, the easternmost village is 18 km far, while Badadon, the westernmost village is 48 km far away. Palmindustrie V1, Likpilassié, Adjadon, Amanikro, Yaokro and Doudougbazou are located 26, 28, 34, 41, 43 and 45 km from Grand-Lahou, respectively.

Sampling period

Grand-Lahou has an equatorial climate with two dry and two rainy seasons (Whigham *et al.*, 2013). The dry seasons range from December to April, and from August to September. The rainy seasons range from May to July, and from October to November. One-week surveys were performed in March, May, August and September 2015 to collect samples from both the dry and rainy seasons. Two plantations per village were randomly sampled in each survey.

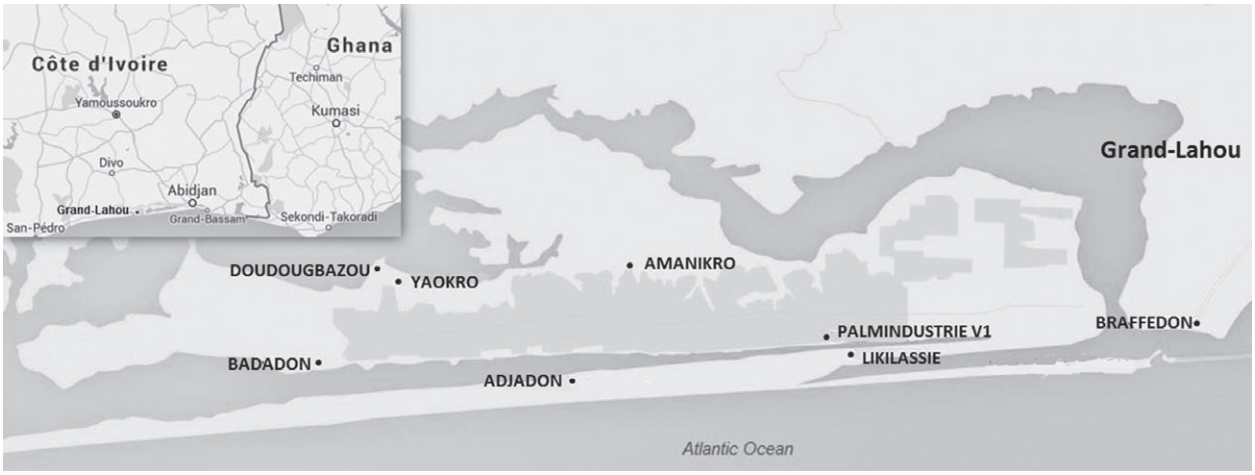

Figure 1 Map showing the eight villages of Grand-Lahou surveyed for the CILY phytoplasma presence during 2015 in Côte d'Ivoire.

Sample collection

All samples were collected from the West African Tall (WAT) or PB121 (Malayan yellow dwarf × WAT) palm ecotypes, the only ones cultivated in the Grand-Lahou region. Twelve symptomless (1 WAT and 11 PB121), and 70 CILY-symptomatic coconut trees (13 WATs and 57 PB121) were sampled for CILY phytoplasma detection. In order to determine the best plant part and disease stage for the effective detection of the CILY phytoplasma, 74 trunk borings, 39 inflorescences and 47 leaves (the youngest inner leaf or spear leaf) were collected (Table 1) from palms representative of each disease stage: 22 of disease stage one (ds1); 30 of disease stage two (ds2); and 18 of disease stage three (ds3). Symptoms of disease stages were identified following the symptom description by Konan Konan *et al.* (2013b). Stage one is referred to as a leaf yellowing starting from the older leaves; necrosis of the mature flowers as they emerge from the spathe; premature nut drop, and the starting of the blackening of inflorescences. Stage two included foliar yellowing progressing towards the younger leaves; browning and desiccation of some of the yellowed leaves, and blackening and necrosis of the inflorescences. Stage three corresponded to brown leaves hanging down to form a skirt around the trunk for several weeks before falling as yellowing continues covering the younger leaves; the inflorescences are fully necrotic and black, and the apical meristem (bud) dies.

Trunk borings were collected by boring into the trunk using an 8 cm long drill bit sterilised in 70% ethanol before collecting each sample (Harrison *et al.,* 2013). Leaves were collected from symptomless and symptomatic palms exhibiting CILY stages ds1, ds2 and ds3, while inflorescences were only sampled from palms exhibiting CILY stages one and two. For palm trees with disease stage three, most of the inflorescences were not collected due to the high level of necrosis and/or blackening. Six trunk boring samples collected from the Western Region of Ghana from symptomless and CSPWD-affected palms (WAT ecotype) from disease stages one, two and three were donated by Dr. N. Yankey of CSIR-OPRI, Ghana.

Total DNA extraction and reference controls

Leaf, inflorescences and trunk boring samples were transferred in sealable plastic bags to the lab, and refrigerated at 4°C until nucleic acid extraction. Total DNA was extracted from the samples using the CTAB extraction procedure as reported by Harrison *et al.* (2013). Total DNA from a coconut palm infected with LYM, 16SrXXII-A strain ('*Ca.* P. palmicola') provided from J. Bila from Mozambique, and the total DNAs extracted from samples from Ghana were used as reference controls.

Polymerase chain reaction

For all polymerase chain reaction (PCR) reactions, 50 ng of DNA template was added to a 50-μL PCR reaction (DreamTaq Green PCR Master Mix, ThermoFisher Scientific, Canada) containing 1 μM of each primer. Universal primers P1 (Deng & Hiruki, 1991) and P7 (Schneider *et al.,* 1995) nested with CSPWD phytoplasma primers G813F/AwkaSR (Tymon *et al.,* 1998) were used to amplify the partial *16S rRNA*, intergenic spacer and *23S* genes of the CILY phytoplasma. One microlitre of the 40-fold diluted P1/P7 PCR products was used in the nested PCR.

The *sec*A gene was initially amplified with the primer pair SecAfor1/SecArev3 (Dickinson & Hodgetts, 2013).

Table 1 Coconut palm parts sampled in the villages of Grand-Lahou from palm trees showing CILY symptoms, and PCR results for the CILY phytoplasma detection (G813/AwkaSR) NC: not collected; Infloresc: inflorescences

Disease stage	Palm number	PCR positive/sampled palm parts: Trunk borings	Leaves	Infloresc	% of palms positive to CILY phytoplasma
ds1	1	0/1	0/1	NC	–
	2	1/1	0/1	1/1	+
	3	1/1	0/1	1/1	+
	4	1/2	0/1	NC	+
	5	1/1	0/1	1/1	+
	6	1/1	0/1	NC	+
	7	1/1	0/1	0/1	+
	8	0/1	NC	1/1	+
	9	0/1	NC	1/1	+
	10	1/1	NC	NC	+
	11	2/2	NC	0/1	+
	12	1/1	0/1	2/2	+
	13	1/1	NC	NC	+
	14	0/1	NC	NC	–
	15	0/1	0/1	0/2	–
	16	1/1	0/1	1/2	+
	17	0/1	0/1	0/1	–
	18	0/1	0/1	0/1	–
	19	0/1	0/1	0/1	–
	20	1/1	1/1	NC	+
	21	1/1	1/1	1/2	+
	22	0/1	0/1	1/1	+
	Total	14/24 (58.3%)	2/16 (12.5%)	10/19 (52.6%)	16/22 (72.7%)
ds2	1	1/1	1/1	1/1	+
	2	1/1	0/1	0/1	+
	3	1/1	0/1	NC	+
	4	1/1	0/1	NC	+
	5	0/1	0/1	NC	–
	6	2/3	0/1	1/1	+
	7	1/1	0/1	NC	+
	8	1/1	0/1	1/1	+
	9	2/2	NC	NC	+
	10	0/1	1/1	NC	+
	11	1/1	1/1	2/2	+
	12	0/1	0/1	NC	–
	13	0/1	1/1	NC	+
	14	1/1	0/1	1/2	+
	15	1/1	NC	NC	+
	16	0/1	NC	1/2	+
	17	1/1	0/1	NC	+
	18	1/1	NC	NC	+
	19	1/1	NC	NC	+
	20	1/1	0/1	1/1	+
	21	1/1	1/1	1/2	+
	22	1/1	1/1	1/1	+
	23	0/1	1/1	1/1	+
	24	1/1	1/1	1/1	+
	25	1/1	NC	NC	+
	26	1/1	NC	1/1	+
	27	1/1	0/1	NC	+
	28	0/1	0/1	NC	–
	29	1/1	NC	NC	+
	30	NC	1/1	NC	+
	Total	24/32 (75.0%)	9/22 (40.9%)	13/17 (76.5%)	27/30 (90.%)
ds3	1	0/1	0/1	NC	–
	2	NC	NC	0/1	–
	3	1/1	NC	NC	+
	4	1/1	1/1	NC	+
	5	2/3	0/1	NC	+
	6	1/1	NC	NC	+
	7	1/1	NC	NC	+
	8	1/1	NC	1/1	+
	9	1/1	NC	NC	+
	10	1/1	1/1	NC	+
	11	1/1	NC	NC	+
	12	1/1	1/1	NC	+
	13	1/1	0/1	NC	+
	14	1/1	NC	NC	+
	15	1/1	NC	NC	+
	16	NC	0/1	NC	–
	17	1/1	0/1	1/1	+
	18	1/1	0/1	NC	+
	Total	16/18 (88.9%)	3/9 (33.3%)	2/3 (66.7%)	15/18 (83.3%)

The direct PCR product was diluted 30-fold and used as a DNA template for nested PCR with primers SecAfor5/SecArev2.

CoxF3 and CoxB3 primers that amplify the plant cytochrome oxidase (*cox*) gene were used to confirm the PCR negative samples to assess for PCR inhibitor as recommended by Dickinson & Hodgetts (2013).

Ten samples per village (Braffedon, Palmindustrie V1, Amanikro, Badadon, Adjadon, Doudougbazou and Yaokro), previously confirmed as positive by *sec*A and G813/AwkaSR nested PCR assays, were subjected to amplification and sequencing of their *rp* gene sequences. The *rp* gene was amplified with primers rpLYF1 (forward) and rpLYR1 (reverse) designed by Marinho *et al.* (2006), with a slight modification of the forward primer. Primer sequences are rpLYF1: 5′-TTTAA AGAAGGTATTAACATGA-3; and rpLYR1: 5′-TAATACC TATAACTCCGTG-3′. *Consensus* rp sequences were deposited in GeneBank and one sequence per village was selected for sequence comparison to determine a possible differentiation among the CILY phytoplasma strains from the eastern and western areas.

Thirty-five PCR cycles were performed for both P1/P7 and G813F/AwkaSR PCR assays. PCR cycling conditions for P1/P7 primers were 40 s (3 min for the initial denaturation) at 94°C, 40 s at 56°C and 1 min and 40 s (10 min for the final extension) at 72°C. For primers G813F/AwkaSR, one cycle at 94°C for 2 min was followed by 30 cycles of 94°C for 30 s, 53°C for 1 min, and 72°C for 1 min; and one cycle at 72°C for 10 min. For primers rpLYF1/R1, one cycle at 94°C for 5 min was followed by 35 cycles of 94°C for 30 s, 50°C for 1 min, and 72°C for 2 min; and one cycle at 72°C for 7 min. For primers SecAfor1/SecArev3 and SecAfor5/SecArev2, PCR cycling was the same as for primers G813F/AwkaSR (Dickinson & Hodgetts, 2013).

Five microlitres of each of the PCR products were separated in a 1.5% agarose gel and visualised with SYBR Safe DNA Gel Stain (Invitrogen, USA) in an Alpha Imager (Alpha Innotech, USA).

Determining the best plant tissue type for CILY phytoplasma detection

The CILY phytoplasma detection percentages based on nested PCR amplification with primers G813/AwkaSR were compared with the samples collected in the eight locations for each plant tissue type and disease stage. Pearson Chi-square test was applied to correlate the detection percentage of the CILY phytoplasma with each disease stage, and with each plant part, stem (trunk borings), leaves or inflorescences; and to assess any differential detection of the CILY phytoplasma.

Sequencing, restriction fragment length polymorphism (RFLP), and phylogenetic analyses

Three representative G813/AwkaSR, SecA, and rp amplicons from each disease stage (one, two, three) for each village surveyed were purified on spin columns (E.Z.N.A. Cycle Pure, Omega bio-tek, USA), cloned according to manufacturer's instructions (p-GEMT Easy Vector Systems, Promega, USA) and sequenced bidirectionally using M13F/M13R primers (Centre for the Analysis of Genome Evolution and Function, CAGEF, University of Toronto). The *consensus* 16S rDNA, SecA and rp sequences were deposited in GenBank (Accession numbers are shown in Figs 2–4), and compared by BLAST (Altschul *et al.*, 1990) with sequences available in GenBank including phytoplasmas of 16SrIV and 16SrXXII groups. Sequences obtained were aligned using Clustal W (Thompson *et al.*, 1994) and phylogenetic trees were constructed using the neighbour-joining method with MEGA version 4.0 (Kumar *et al.*, 2004) with default values and 1,000 replicates for bootstrap analysis. *Bacillus cereus*, *Bacillus subtilis* and *Acholeplasma palmae* were used as the out-groups to root the trees.

Ten microliters of the G813/AwkaSR and SecA PCR amplicons were digested with *Tsp509*I, *Rsa*I, *Hae*III, *Alu*I, *Taq*I, *Hin*fI, and *Mbo*II restriction endonucleases (New England Biolabs, Canada), following manufacturer's recommendations. RFLP profiles were visualised in a 3% agarose gel stained with SYBR^R Safe DNA Gel Stain (Invitrogen, USA) in a gel documenter (Alpha Innotech, USA).

G813/AwkaSR and SecA sequences were assessed with *in silico* restriction endonucleases using the virtual gel plotting program pDRAW32 (http://www.acaclone.com) to identify the enzymes yielding polymorphisms that could distinguish among the phytoplasma strains.

Results

CILY phytoplasma detection in coconut palms

A total of 160 samples of leaves, trunk borings, and inflorescences were collected from CILY symptomatic palms. Coconut palms were considered positive for CILY based on the detection of the CILY phytoplasma in a sole plant part sampled. Ninety-three samples from the symptomatic palms gave positive results after nested PCR with primers P1/P7 and G813/AwkaSR (Table 2). These corresponded to samples from 54 trunk borings, 25 inflorescences, and 14 leaves. SecA amplicons were obtained from 89 samples positive to G813/AwkaSR PCR. The *cox* gene was amplified from the four PCR negative samples, ruling out the possibility of PCR inhibition and confirmed these as true negative samples. Rp amplicons were obtained for all the eight CILY phytoplasma-G813/AwkaSR positive trunk boring samples selected from each village. Inflorescence samples from four symptomless trees from the village Badadon yielded G813/AwkaSR and SecA amplicons. No amplicons were obtained for any of the primer combinations for the rest of the trunk boring or leaf samples from the symptomless coconut palms tested.

The CILY phytoplasma was detected in 82.9% of the symptomatic palm trees surveyed and the percentages of CILY phytoplasma detection with primers G813/AwkaSR varied across the villages of Grand-Lahou. Samples collected from Badadon showed the highest detection percentage (92.9%) followed by Braffedon (91.7%), then Palmindustrie V1 (90.0%), Adjadon (88.9%), Doudougbazou (83.3%), Yaokro (66.7%), Likpilassié (66.7%) and Amanikro (57.1%) (Table 2).

Disease stages one, two and three were present throughout all the coconut plantations of the surveyed villages, and the CILY phytoplasma was detected from samples representative of all disease stages. The detection percentages do not differ significantly ($P = 0.000327$; $\chi^2 = 20.93$): they increased as the disease stages progressed scoring 72.7% for samples from coconut trees exhibiting stage one symptoms; 90.0% for those from

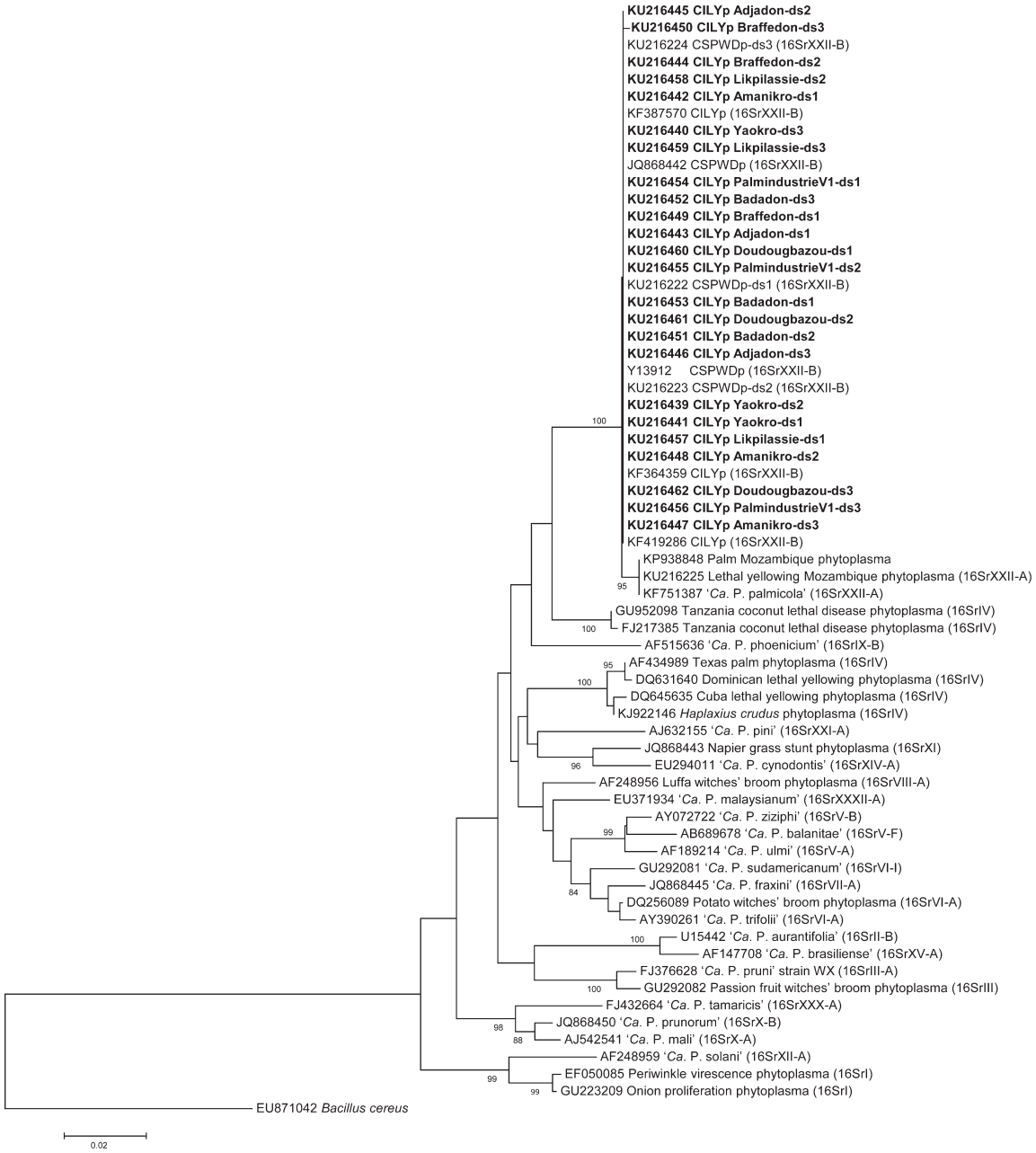

Figure 2 Phylogenetic tree constructed using the neighbour-joining algorithm based on the G813/AwkaSR sequences of the CILY phytoplasma and reference 16Sr phytoplasma groups. '*Ca.* P. sp': '*Candidatus* Phytoplasma sp.' *B. cereus:* outgroup to root the tree; ds: disease stage. CILYp: CILY phytoplasma; CSPWDp: Cape St. Paul Wilt Disease phytoplasma. In bold sequences of phytoplasma strains from Côte d'Ivoire palms; GenBank numbers on the branches and in parenthesis ribosomal group/subgroup when available.

stage two; and 83.3% for samples collected from coconut palms showing stage three symptoms (Table 1). In addition, the CILY phytoplasma was detected in 45 out of 57 PB121 trees surveyed (79.0%), while all the 13 WAT ecotypes sampled from the eight villages resulted infected by the CILY phytoplasma (Table 2).

Detection of the CILY phytoplasma also varied across the different plant parts sampled with the highest rates from trunk borings (54/74 positive to G813/AwkaSR nested PCR, 72.9%) followed closely by the inflorescences (25/39 positive to G813/AwkaSR PCR, 64.1%), then leaves (14/47 positive to G813/AwkaSR PCR, 29.8%)

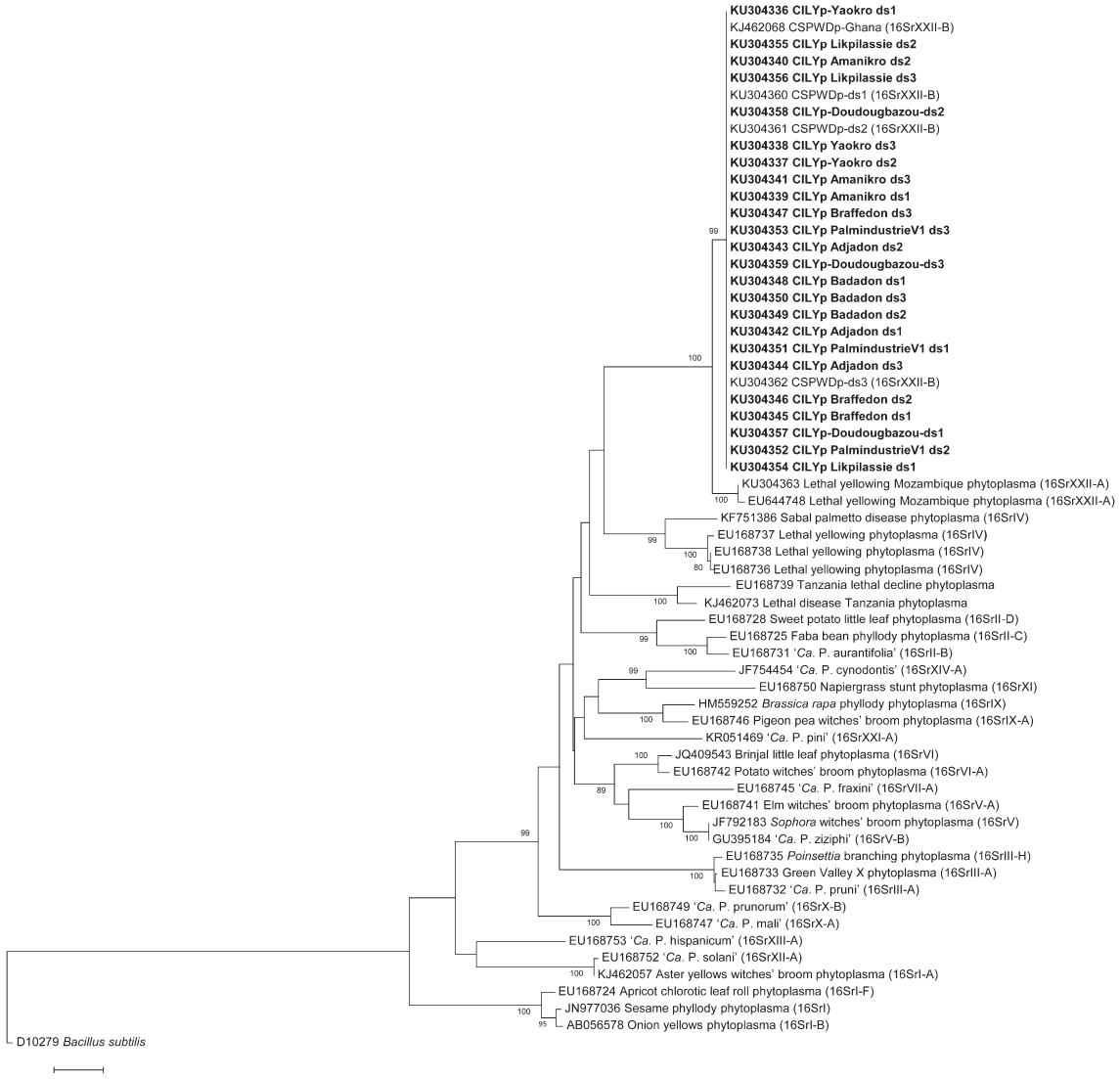

Figure 3 Phylogenetic tree constructed using the neighbour-joining algorithm based on the SecA sequences of the CILY phytoplasma and reference 16Sr phytoplasma groups. '*Ca*. P. sp': '*Candidatus* Phytoplasma sp.'. *B. subtilis,* outgroup to root the tree; ds: disease stage; CILYp, CILY phytoplasma; V1: Palmindustrie V1; CSPWDp, Cape St. Paul Wilt Disease phytoplasma. In bold sequences of phytoplasma strains from Côte d'Ivoire palms; GenBank numbers on the branches and in parenthesis ribosomal group/subgroup when available.

($P = 1.59e^{-5}$; $\chi^2 = 22.10$). Although the CILY phytoplasma was detected from all the coconut plant tissues tested, results indicate that sink tissues, such as stems and inflorescences, are the optimal plant parts for phytoplasma detection (Table 1).

Phylogenetic analysis

Phylogenetic trees constructed on the G813/AwkaSR (Fig. 2), SecA (Fig. 3) and rp (Fig. 4) sequences were in agreement and confirmed the assignment of the CILY and CSPWD phytoplasmas to the 16SrXXII–B subgroup ('*Ca*. P. palmicola'-related strains). Phylogeny also supports the three phytoplasma taxon distinguished by their geographical origins: North America (LY), East Africa (Tanzania LD, LDT), and West Africa (CSPWD and CILY).

16S ribosomal and 16/23S intergenic spacer, *rp*, and *sec*A gene sequences

G813/AwkaSR, rp, and SecA consensus sequences were deposited in GenBank under accession numbers specified in Figs. 2–4 (G813/AwkaSR, SecA and rp, respectively). CILY phytoplasma G813/AwkaSR sequences showed a

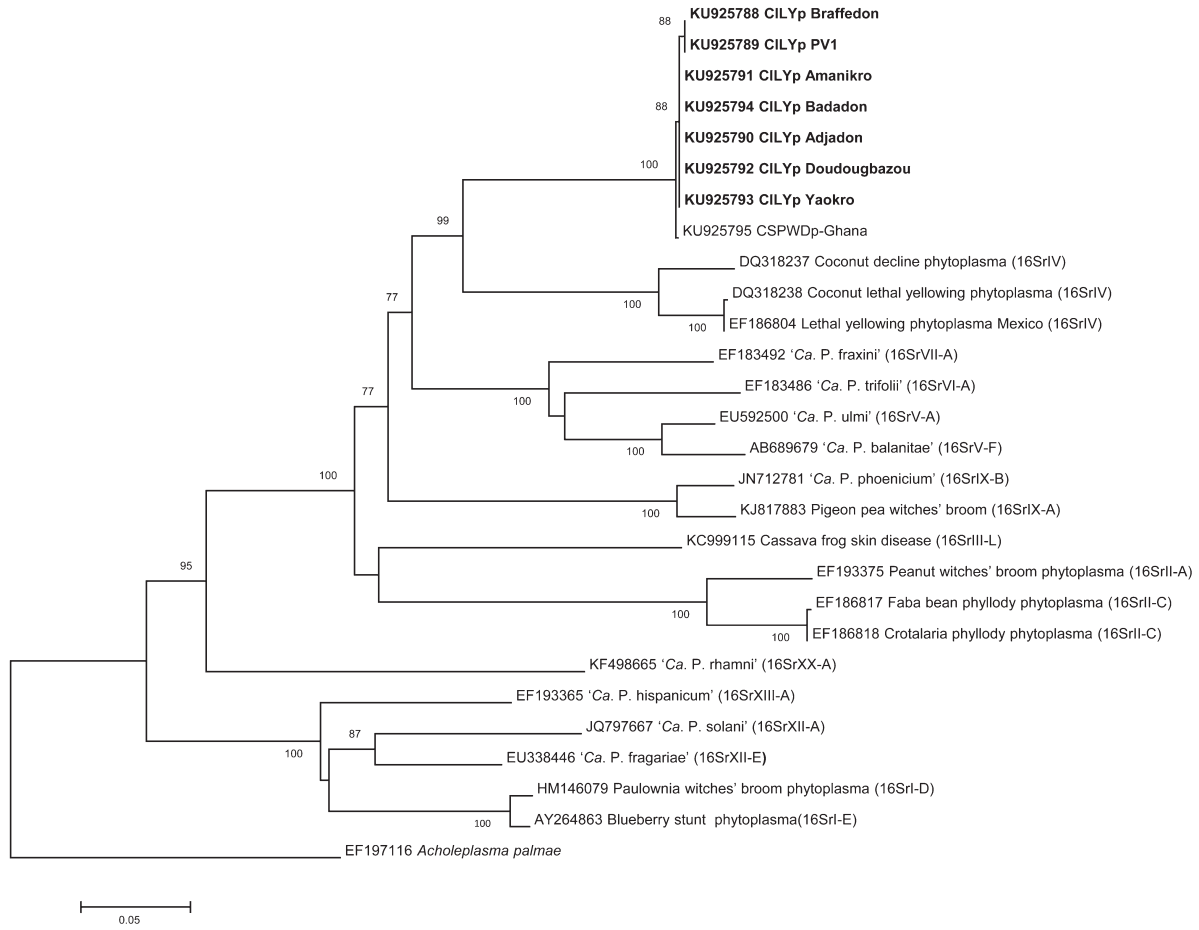

Figure 4 Phylogenetic tree constructed using the neighbour-joining algorithm based on the rp sequences of the CILY phytoplasma and reference 16Sr phytoplasma groups. '*Ca*. P. sp': '*Candidatus* Phytoplasma sp.'; CILYp, CILY phytoplasma; CSPWDp, Cape St. Paul Wilt Disease phytoplasma.; *A. palmae*, outgroup to root the tree. In bold sequences of phytoplasma strains from Côte d'Ivoire palms; GenBank numbers on the branches and in parenthesis ribosomal group/subgroup when available.

99.7% and 99.28% identity, respectively, when aligned to sequences from the CSPWD and LYM phytoplasmas. CILY phytoplasma SecA sequences showed a 99.3% and 96% identity, respectively, when aligned to sequences from the CSPWD and LYM phytoplasmas. Comparison of the full SecAfor5/rev2 sequences among the 16SrXXII phytoplasma strains, CILY [GenBank accession numbers, (AC): KU3043336-59, CSPWD, AC: KU304360-62, KJ462068], and LYM (AC: KU304363) indicated that a unique SNP in base position C/A^{36} is present among these CILY phytoplasma strains (Fig. 5).

SecA sequences of the LYM phytoplasma showed four signature substitutions G/A^{50}, T/C^{77}, G/T^{83}, A/G^{89}, compared to the CSPWD and CILY phytoplasma strains. All CILY phytoplasma rp sequences showed a range of 99.4%–99.7% identities when aligned to the rp sequence from the CSPWD phytoplasma.

Ribosomal protein gene sequences *rplV-rpsC* from Braffedon and Palmindustrie V1 (easternmost villages) were 100% identical, and differed of 99.65% from those of the western villages (Amanikro, Adjadon, Doudougbazou, Badadon, and Yaokro). Two SNPs on the rp sequences differentiated the CILY (AC: KU925788-94) and CSPWD (AC: KU925795) phytoplasma strains, i.e. T/G^{387} and G/T^{423} (Fig. 6). Moreover, CILY strains from the easternmost areas Braffedon and Palmindustrie V1 showed unique SNPs, T/G^{29} and C/A^{56}, respectively; and an addition of T at position 59 (Fig. 6). These SNPs were not found in the rp sequences of the CILY phytoplasma strains from the other five western villages. All the CILY strains shared the rp nucleotide sequence 'ACGTCAAATAAT' (positions 291–302) that characterises the Ghanaian CSPWD phytoplasma strains (Pilet *et al.*, 2011) from the Central and Western Regions, where the disease is still active since the 1964 and 1983 outbreaks.

Table 2 Coconut palms sampled in the villages of Grand-Lahou showing CILY symptoms, and PCR results for the CILY phytoplasma detection (G813/AwkaSR)

Village	Ecotype	PCR positive palms	Number of palms sampled	% palms positive to CILY phytoplasma
Adjadon	PB121	3	4*	
		4	4	
		1	1	
	Total	**8**	**9**	**88.9%**
Amanikro		1	1	
	PB121	2	3	
		0	2	
	WAT	1	1	
	Total	**4**	**7**	**57.1%**
Badadon		5	5	
	PB121	3	4	
		2	2	
		1	1	
	WAT	1	1	
		1	1	
	Total	**13**	**14**	**92.9%**
	PB 121	2	3	
		2	2	
		2	2	
Braffedon		4	4	
	WAT	1	1	
	Total	**11**	**12**	**91.7%**
Doudougbazou	PB 121	1	2	
		2	2	
		2	2	
	Total	**5**	**6**	**83.3%**
Likpilassié	PB 121	0	2	
		2	2	
		2	2	
	Total	**4**	**6**	**66.7%**
Palmindustrie V1		1	2	
	PB 121	2	2	
		2	2	
	WAT	1	1	
		2	2	
		1	1	
	Total	**9**	**10**	**90.0%**
Yaokro	PB 121	1	1	
		2	3	
		1	2	
	Total	**4**	**6**	**66.7%**
		58	**70**	**82.9%**

*Disease stages represented by grey shadows: disease stage 1 (light); disease stage 2 (medium light); disease stage 3 (dark). Bolded numbers correspond to the numbers of palm trees positive to CILY phytoplasma from the total trees tested; and the percentages of detection of the CILY phytoplasma.

RFLP analyses

The *Tsp509*I, *Rsa*I, *Hae*III, *Alu*I, *Taq*I, and *Hin*fI RFLP profiles of both the G813/AwkaSR and SecA sequences of the CILY phytoplasma from trunk borings, leaves and inflorescences, regardless of the disease stage or ecotype were identical to those of the CSPWD phytoplasma from Ghana (data not shown). Only RFLP patterns with *Mbo*II restriction endonuclease on the SecA sequence differentiated the CILY phytoplasma from both the CSPWD and LYM phytoplasmas (Fig. 7).

Discussion

Palmindustrie V1, with 30 000 ha devoted to coconut plantations was the first area in Grand-Lahou where LY-like symptoms were observed in the 1990s (Konan Konan *et al.,* 2013b); however, no pathogen (including phytoplasma) was found associated with the disease by that time. In 2012, a severe CILY outbreak was reported and associated with a phytoplasma (Konan Konan *et al.,* 2013a), and at the beginning of 2013, the disease was spotted in the village of Likpilassié, located at 2 km from Palmindustrie V1.

CILY was subsequently detected in Palmindustrie V1, Braffedon, Adjadon, Amanikro, Yaokro, Doudougbazou, and then Badadon (J. Konan Konan, personal communication). While diseased trees of all stages were observed in Palmindustrie V1, Braffedon exhibited mostly disease stages two and three during 2013. However, 2 years later, in 2015, the progress of the disease towards the terminal stage "telephone pole" was very noticeable in both villages (J. Konan Konan, personal communication).

The fast extent of CILY from the Palmindustrie area to the western coastal villages raised concerns about a possible westward spread of the disease from the initial disease focus. The CILY phytoplasma was consistently detected in all the villages surveyed with the highest detection percentages for Braffedon, Palmindustrie V1 and Badadon. The latter exhibited the highest score for the CILY phytoplasma from symptomatic palms, which supports the successful establishment of the disease towards the western area. Although the current available data are not conclusive, further surveys and additional epidemiological data may confirm the hypothesis on the westward spread of the CILY phytoplasma.

Aerial surveys in Ghana that assessed the limit of spread of CSPWD (Nkansah-Poku *et al.,* 2009) showed that the diseased areas spanned from Ampain (Western Region) in the west to Keta (Volta Region) in the east along the coast. The 1995 CSPWD focus in Ampain, just 60 km away from the border with Côte d'Ivoire, destroyed 70% of the original 25 ha of coconut palm planting (Nkansah-Poku *et al.,* 2009), and became a threat for the Ivoirian coconut groves (Yankey, 2012). However, it is not clear how the disease reached and spread throughout the

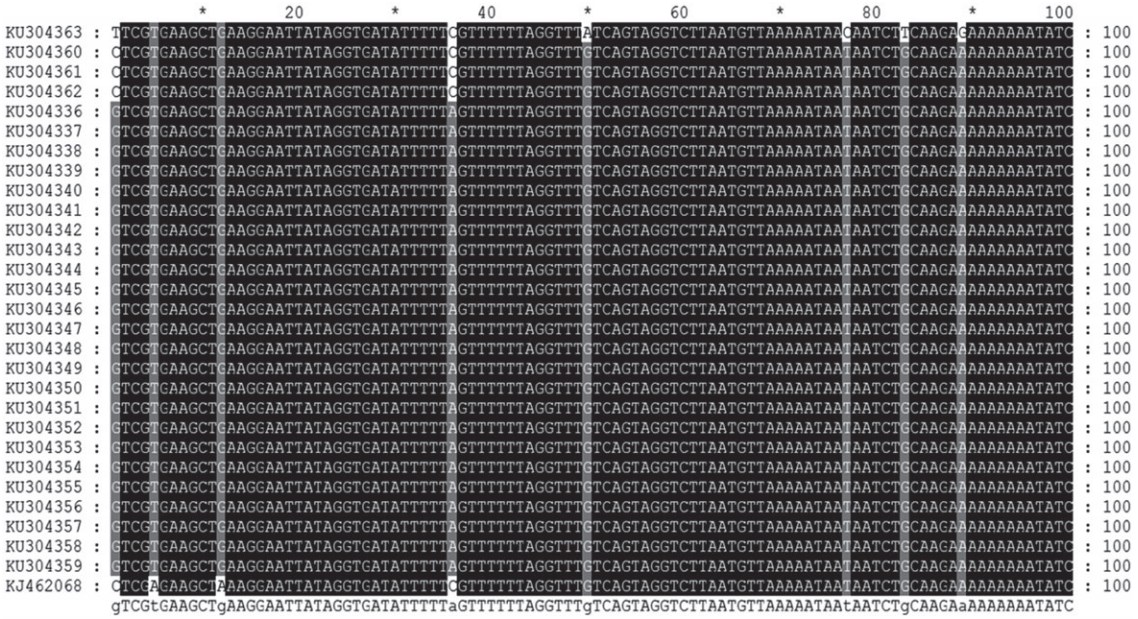

Figure 5 Comparison of the SecAfor5/rev2 sequences among the 16SrXXII phytoplasma strains: 16SrXXII-A, LYM (AC: KU304363), 16SrXXII-B, CILY (AC: KU304336-59), 16SrXXII-B, CSPWD (AC: KU304360, KU304361, KU304362, KJ462068).

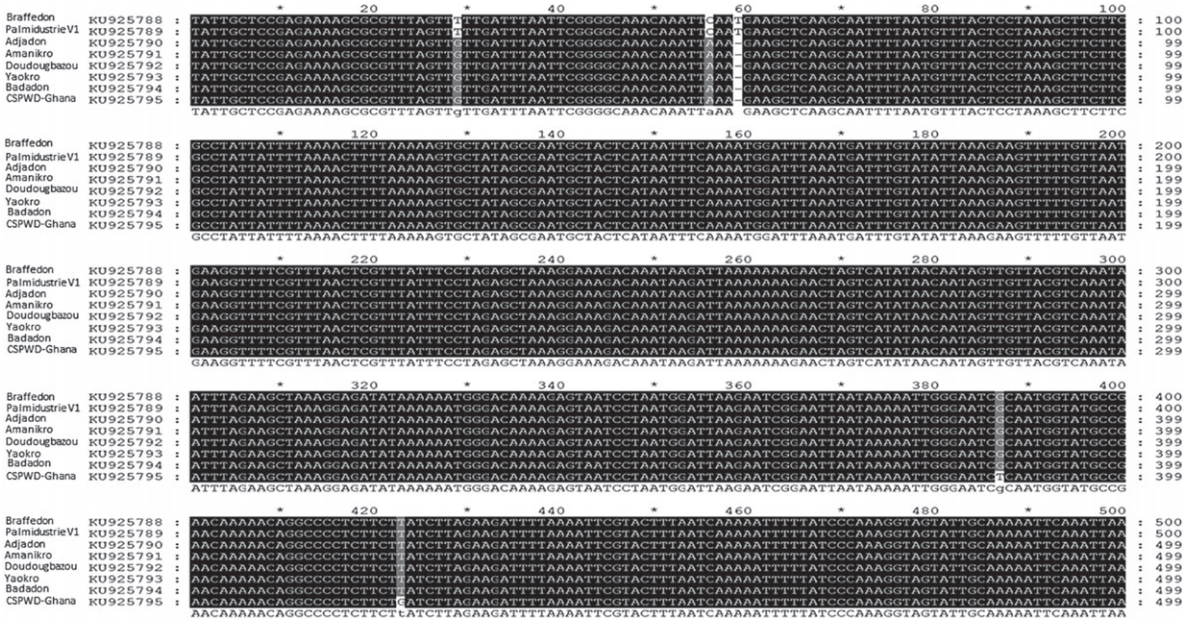

Figure 6 Comparison of the rpLYF1/R1 sequences among the CSPWD phytoplasma (AC: KU925795) and the CILY phytoplasma strains from eastern Braffedon (AC: KU925788) and Palmindustrie V1 (AC: KU925789), and western (AC: KU925790-94) villages of Grand-Lahou.

coconut-growing villages of Grand-Lahou. Further studies may help to elucidate the possible epidemiological constraints that may govern the spread of these two closely related 16SrXXII-B strains, and particularly of CILY within the Grand-Lahou area.

CILY symptom manifestations slightly differ to LY symptoms in north America and the Caribbean. While first stage LY symptoms are recognised by the sole premature nut fall (Brown *et al.,* 2007; Harrison & Elliot, 2005), CILY symptoms of disease stage 1 also include the starting

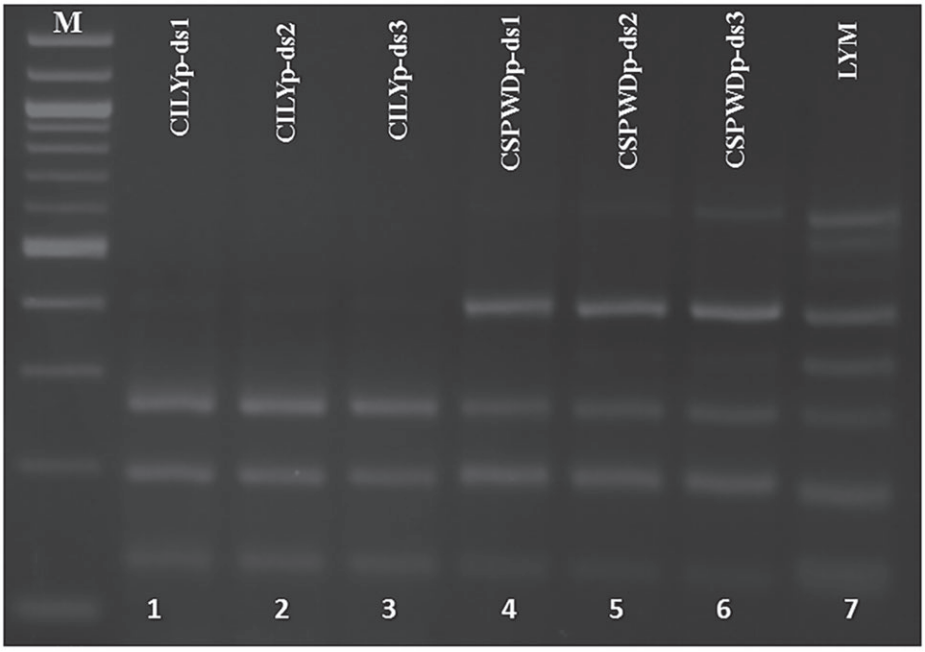

Figure 7 RFLP profiles with *Mbo*II from the 16S rDNA amplicons in a 3% agarose gel showing distinction among the three West African phytoplasma strains. Lane 1, 2, 3: CILY phytoplasmas (CILYp) from coconut palms with CILY symptoms from disease stages ds1, ds2 and ds3, respectively. Lanes 4, 5, 6: CSPWD phytoplasmas (CSPWDp) from coconut palms with CSPWD symptoms from disease stages ds1, ds2 and ds3, respectively. Lane 7: LYM phytoplasma.

of yellowing from the older leaves as well as the starting of the blackening of the inflorescences (Konan Konan *et al.,* 2013b). Similarly in Ghana, CSPWD-affected palms of disease stage 1 show premature nut drop and blackening of the inflorescence with or without leaf yellowing (Dery & Philippe, 1997). For LY, the second stage of the disease implies only necrosis of the inflorescences (Brown *et al.,* 1997; Harrison & Elliot, 2005), while CILY is also characterised by the yellowing of the older leaves progressing to the younger leaves. Yellowing of the frond is not observed until the third stage for LY. For CSPWD in Ghana, the yellowing on leaves is described as present in less than half of the canopy for disease stage 2, more than half of the canopy for disease stage 3 and present in all leaves in the canopy for disease stage 4 (Dery & Philippe, 1997).

For both LY and CILY, the yellowed leaves at the disease stage 3 eventually turn brown, desiccate, and hang down forming a skirt around the trunk before falling (Brown *et al.,* 1997; Konan Konan *et al.,* 2013b). There are a number of epidemiologic considerations that may explain the differences among the LY- and LD-like disease symptom manifestations (Sullivan & Harrison, 2013). Those include geographic distribution patterns, rates of spread, and varietal and host species susceptibility, which have indicated dissimilarities among phytoplasmas and vector species involved with these diseases. The study of epidemiologic factors associated with CILY would definitively contribute to a better understanding of the disease development and the design of more effective control strategies in Grand-Lahou.

Despite the high average percentage of detection of the CILY phytoplasma, symptomless palms were observed in heavily CILY-affected diseased farms, including Badadon, Braffedon and Palmindustrie V1, a phenomenon also reported for CSPWD in Ghana (Nkansah-Poku *et al.,* 2009). Interestingly, the CILY phytoplasma was detected from four symptomless coconut palms from the westernmost village, Badadon, which showed 92.9% of phytoplasma detection. Symptomless infection in palms infected with LY (Maust *et al.,* 2003) and CSPWD (Nipah *et al.,* 2007) phytoplasmas have also been reported. Similar reports have been made in other phytoplasma-infected monocot species like sugarcane (Soufi & Komor, 2014). Those infected asymptomatic trees in Badadon suggest that natural asymptomatic infection from the CILY phytoplasma can occur in Grand-Lahou. Further monitoring is required to assess the symptom development on those CILY phytoplasma-infected asymptomatic palms since commonly all infected palms eventually die from the disease as previously reported (Nkansah-Poku *et al.,* 2009; Eziashi & Omamor, 2010).

The CILY phytoplasma was more consistently detected in sink tissues such as trunk borings and inflorescences

than in source tissues like leaves, regardless of the disease stage, which is in agreement with previous reports (Nipah *et al.,* 2007; Nejat & Vadamalai, 2010; Tomlinson *et al.,* 2010; Oropeza *et al.,* 2011; Yankey *et al.,* 2014; Bila *et al.,* 2015).

It is well documented that trunk borings are highly recommended for sampling LY-like affected coconut palms since they provide higher DNA yield (Harrison *et al.,* 2013). However, no information is available on the suitability of different plant parts of the CILY-affected coconut palms for the detection of the CILY phytoplasma. Considering the relevance of determining the most effective tissue(s) to sample for diagnostic purposes (Oropeza *et al.,* 2011), our study focused on sampling trunk borings, leaves and inflorescences to determine the most suitable tissue type for the detection of the CILY phytoplasma as an approach to support CILY diagnosis in Grand-Lahou.

The CILY phytoplasma was detected from trunk boring (stem) samples of all disease stages: 58.3% for ds1; 75.0% for ds2 and 88.9% for ds3, as for inflorescences (52.6% for ds1; 76.5% for ds2 and 66.7% for ds3) and leaves (12.5% for ds1; 40.9% for ds2 and 33.3% for ds3). Trunk borings actually exhibited the best suitability for the CILY phytoplasma detection. Previous studies refer to trunk borings as the most reliable source for phytoplasma detection compared to spear leaves and inflorescences (Nejat *et al.,* 2009; Oropeza *et al.,* 2011). This may be related to the abundance of phytoplasmas in the larger stem phloem vascular bundles at the periphery of the monocot species, including coconut palms as previously indicated by Oropeza *et al.* (2011).

The detection of the CILY phytoplasma showed an increasing trend for trunk borings from ds1 to ds3 compared to leaves and inflorescences, which only showed an increase of the CILY phytoplasma titre at disease stages 1 and 2, and dropped at disease stage 3. It is worth to mention that results related to the detection of the CILY phytoplasma from inflorescences sampled from coconut palms at disease stage 3 are not conclusive since most of the inflorescences were not tested due to their bad conditions at sampling. Most of the inflorescences at this stage were rotten and exhibited a high level of necrosis and blackening, so they were not collected for testing since plant material sampled under those conditions are very likely to generate PCR inhibitors that would prevent the detection of the CILY phytoplasma (Nejat *et al.,* 2009).

The lower level of detection of the CILY phytoplasma from leaves at disease stage 3 may be related to the fact that those leaves were mostly intermediate or mature, rather than young, and most of them displayed rotten conditions when sampling. In fact, previous studies on the distribution of the LY phytoplasma in coconut palms confirm that the phytoplasma titres are very low or minimal, or non-detectable in source tissues like mature and intermediate leaves compared to sink tissues such as the stem (Thomas & Norris, 1980; Oropeza *et al.,* 2011).

Oropeza *et al.* (2011) reported an interesting pattern of detection level for the LY phytoplasma from stem, leaves and inflorescences, which was lowest in LY phytoplasma-infected symptomless palms, and increased from disease stages 1 to 3, before decreasing towards the final stages of the disease development. Similarly to the CILY phytoplasma, an increasing trend from disease stage 1 to stage 3 in the titre of the CSPWD phytoplasma in Ghana, measured by quantitative real-time from trunk borings of CSPWD-affected palms was reported (Yankey, 2012). In this case, the amount of the phytoplasma in the stage 2 palms was about twice as much as that in the stage 1 palm, while the amount in the stage 3 palms was as much as three-fold greater than the amounts in the stage 2 palms. Such increasing trend of the phytoplasma pathogen may be related to the fact that a significant build-up of phytoplasma titres in palms at disease stages 2 and 3 of infection may speed up the disease development process. In fact, Oropeza *et al.* (2011) suggested, based on the detection of the LY phytoplasma in stem tissues throughout all the subsequent stages, that stem should be the part of the palm where phytoplasmas first move from leaves after foliar feeding of inoculative insect vectors, and then multiply and become distributed to other parts of the coconut palm.

Although all the plant parts screened in our study (stems, inflorescences and leaves) were suitable for the CILY phytoplasma detection, trunk borings were the parts that provided the best yields for the CILY phytoplasma from CILY-affected coconut palms, and proved to be very suitable for the early detection of the phytoplasma from symptomless palms. Moreover, collecting sawdust from trunk borings is a simpler and non-destructive sampling approach (Oropeza *et al.,* 2011; Harrison *et al.,* 2013). Since climbers are not commonly available in Grand-Lahou, sampling of leaves and inflorescences mostly involve the felling of coconut palms, which becomes a problem for farmers; besides it is labour intensive and time consuming. Therefore, our results strongly recommend the trunk borings as the most practical and effective choice for sampling coconut palms for the detection of the CILY phytoplasma in Grand-Lahou, and to support further CILY epidemiology studies.

Pilet *et al.* (2011) suggested an independent evolution of the two CSPWD *foci* that occurred in the Western and Central regions from the one present in the Volta region based on a SNP at position 346 in their *rp* gene sequences that distinguished them. For the CILY phytoplasma strains, the unique SNPs found in the CILY phytoplasma rp sequences from Braffedon and Palmidustrie

V1 distinguished them from the CILY phytoplasma strains from the other villages. This is a preliminary study based on the selection of 10 samples per village for the first exploratory screening using the *rp* gene in order to determine any differentiation between western and eastern CILY phytoplasma strains. Nevertheless, a larger sampling is ongoing to support a similar hypothesis to that of CSPWD.

Interestingly, the nucleotide sequence "ACGTCAAATAAT" reported by Pilet *et al.* (2011) as characteristic for the CSPWD phytoplasma strains of the Central and West Regions was found in all the CILY phytoplasma strains. This may support the hypothesis that the CILY phytoplasma affecting Grand-Lahou may be originated and spread from either Central or West Regions in Ghana.

Analysis based on the *sec*A and *rp* genes suggest that there is a diversity among the CILY phytoplasma strains infecting coconut palms in Grand-Lahou. Assays based on the *sec*A gene have been successfully used to detect phytoplasmas associated with diseases in Poaceae (Bekele *et al.,* 2011) and recently in pine trees in Lithuania (Valiunas *et al.,* 2015). It has been also crucial in distinguishing phytoplasmas of group 16SrIV (LY) in palms (Ntushelo *et al.,* 2013), and also for the detection and identification of the CSPWD phytoplasma in Ghana (Yankey *et al.,* 2014), and '*Ca*. P. palmicola' in Mozambique (Bila *et al.,* 2015).

In our study, the CILY phytoplasma was distinguished from the CSPWD and LYM phytoplasma strains based on *Mbo*II profiles on the *sec*A gene sequences. This supports previous differentiation among the CILY, CSPWD and LYW phytoplasma strains based on the *16S rRNA* gene sequences (Arocha-Rosete *et al.,* 2014) amplified with the universal primers R16F2n/R2 and digested with endonuclease restriction enzymes *Alu*I, *Bfa*I, *Hae*III, *Hpa*I, *Hpa*II and *Mse*I. However, the amplification of a larger genomic region as that flanked by primers R16F2n/R2 (~1.2 kb) may be problematic, particularly for a large number of samples since it is too long for routine diagnostics (Makarova *et al.,* 2012) compared to the *sec*A shorter fragment (~480 bp). This is despite the fact that the *sec*A is a single-copy gene in the phytoplasma genome, whilst the rRNA operon is present in two copies (Hodgetts *et al.,* 2008). Our findings suggest that the *sec*A nested PCR/*Mbo*II system performs as well than the R16F2n/R2 fragment, and thus provides an easier procedure for the routine detection and identification of the CILY phytoplasma. Therefore, the use of the nested *sec*A PCR in combination with *Mbo*II RFLP is recommended as a useful alternative approach to distinguish between '*Ca*. P. palmicola' strains and to support the CILY epidemiologic screening.

This is the first study on determining the detection percentages of the CILY phytoplasma in those coconut-growing villages of Grand-Lahou, and recommending trunk borings as the most suitable plant tissue type for sampling purposes. It is also the first preliminary characterisation of the CILY phytoplasma strains based on the *rp* gene. Other less conserved non-ribosomal genes should be used to assess the genetic diversity and to support differentiation within the CILY phytoplasma strains, and among them and other LY-like phytoplasmas.

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