Received: 17 December 2022 | Revised: 30 April 2023 | Accepted: 3 July 2023

DOI: 10.1111/aab.12854

ORIGINAL ARTICLE

Annals of Applied Biology
An international journal of the aab

WILEY

Molecular identification of '*Candidatus* Phytoplasma palmicola' associated with coconut lethal yellowing in Equatorial Guinea

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Funding information
H2020 European Institute of Innovation and Technology, Grant/Award Number: 727459

Abstract

During the past two decades, a high mortality of coconut palms was observed in the coastal areas of Equatorial Guinea. Reportedly, the palm population has been reduced by 60%–70%, and coconut production has decreased accordingly. To identify the cause of the mortality, a survey was carried out in April 2021 in various localities of the coconut belt. Molecular analyses carried out on 16S rRNA and *secA* genes detected phytoplasma presence in the majority of the samples. Sequencing and BLAST search of the 16S rRNA gene sequences showed >99% identity of the detected phytoplasmas to '*Candidatus* Phytoplasma palmicola'. The RFLP analyses of 16S ribosomal gene using *Tru*1I and *Taq*I enzymes led to assign these phytoplasmas to subgroup 16SrXXII-A. In all samples that tested positive, including one from a hybrid coconut palm and two from oil palm the same phytoplasma was identified. The phylogenetic analyses of 16S rRNA and *secA* genes confirmed respectively 99.98%–100% and 97.94%–100% identity to '*Ca.* P. palmicola'. RFLP analyses using *Mbo*II enzyme on the *secA* gene amplicon differentiated the phytoplasma found in Equatorial Guinea from those present in Ghana and Ivory Coast. The Equatorial Guinean phytoplasma strain resulted to be identical to the strains from Mozambique, confirming the presence of a geographic differentiation among phytoplasma strains in the coastal areas of Western and Central Africa. The identified phytoplasma is different from the '*Ca.* P. palmicola' strains found in Ghana and Ivory Coast and represents the first identification a 16SrXXII-A strain in Equatorial Guinea and in Central Africa. Strict monitoring and surveillance procedures for early detection of the pathogen are strongly recommended to reduce its impact and further spread in the country and permit the recovery of coconut plantations.

KEYWORDS
epidemiology, lethal yellowing, molecular identification, palm decline, phytoplasmas

1 | INTRODUCTION

Although production has decreased significantly since the end of the 60s, coconut palm is still an important agricultural commodity for Equatorial Guinea, not only for internal consumption but also for export, representing the third main export product after cocoa and coffee (Banco Africano de Desarrollo, 2018; MAGBOMA and FAO, 2012). The cultivation of this crop is subsidised by the government to increase the production and contribute to the country's economic growth, the generation of foreign exchange and the creation of employment. The coconut production is concentrated along the coastal belt of the continental part of the country, stretching all the way between the borders with Cameroon and Gabon. It consists mainly of self-perpetuating natural stands of the West African tall palm, rather than fully managed plantations. During the last two decades, a lethal yellowing-like disease has been heavily affecting the coconut belt. No official records are available, but according to the local coconut producers and technical officers from the Ministry of Agriculture, the disease first appeared in the 90s around the locality of Rio Campo, close to the border with Cameroon, and with time it extended southward. Since its appearance, the population of coconut palms in that area has reportedly been reduced by 60%–70%, and production has decreased accordingly.

Lethal yellowing (LY) disease in coconut plants was documented in the late 19th century in the Caribbean region, where the first reported occurrence of the disease in epidemic proportions took place in Jamaica during the 1960s, followed by the disease emerging in other countries in the region (Eden-Green, 1997). Similar diseases have also been described in West Africa: Awka wilt in Nigeria, Cape St. Paul wilt in Ghana and Ivory Coast, Kaïncopé in Togo, and Kribi in Cameroon (Dollet et al., 2009; Eden-Green, 1997). Recently, phytoplasmas from diseased coconut palm from Ivory Coast were also obtained as colonies growing in artificial media and were partially characterised at the biochemical level (Contaldo et al., 2019). In East Africa, a lethal disease of palms also occurs in Kenya, Tanzania and Mozambique. Bogia syndrome in Papua New Guinea and Solomon Islands and a lethal wilt in Australia have also been described (Gurr et al., 2016). While the disease symptoms are very similar worldwide, five '*Candidatus* Phytoplasma' species have been detected in palms in the different continents.

Responding to a request of the local Ministry of Agriculture, a survey was carried out in various localities of the coconut belt of Equatorial Guinea—as part of an FAO technical assistance project—to verify the aetiology of the coconut decline observed in the area.

2 | MATERIALS AND METHODS

2.1 | Samples collection

The survey was carried out in April 2021 in the localities of Mbini, Kogo, Cabo San Juan and Río Campo, located in the Litoral Province of Equatorial Guinea (Figure 1). Symptoms of lethal yellowing disease stages were identified following the description by Arocha Rosete et al. (2017): yellowing of the older leaves, necrosis of the mature flowers as they emerge from the spathe and premature nut drop (stage 1);

FIGURE 1 Maps showing (on the left) the position of Equatorial Guinea (*Source*: www.google.com/maps/) and (on the right) the localities where the survey was carried out (red circles) (modified from: https://gisgeography.com/equatorial-guinea-map/).

yellowing progresses to younger leaves, with browning of the older leaves, and necrosis of the inflorescences (stage 2); 'skirting' of the older leaves, which hang down around the trunk and fall after several weeks, and eventual necrosis of the apical meristem (Stage 3), leading to the death of the palm (the so-called "telephone pole" stage).

Altogether, 34 samples (Table 1) were collected by boring a single hole into the trunk of each target palm, using a 10 mm diameter drill bit, at a height of 120–150 cm from the ground. The drill bit was cleaned and sterilised in 70% ethanol for 1 min prior to the collection of each sample. Thirty samples were collected from coconut palms of the West African Tall type, 19 showing symptoms corresponding to stages 1, 2 or 3 (Figure 2), four dead and seven showing symptoms not specifically referable to LY. Two samples were collected from plants of a yellow dwarf variety of coconut, called locally "French coconut" and thought to be resistant to LY, while the two last samples were from oil palms (*Elaeis guineensis*) showing skirting of the older leaves (Figure 3). The collected sawdust samples were placed individually into clean plastic bags and within 12 h from collection were partially air-dried in a

TABLE 1 Results of phytoplasma gene amplification in the surveyed coconut growing areas of Equatorial Guinea.

						PCR results on genes	
Code	Sampling date	Locality	Plot	Species	Disease stage[a]	16S rRNA	*secA*
GQ01	15 April 2021	Sipolo	6	*Cocos nucifera*	2	+	+
GQ02	15 April 2021	Sipolo	6	*C. nucifera*	Dead	+/−	−
GQ03	15 April 2021	Sipolo	6	*C. nucifera*	(1)	−	−
GQ04	15 April 2021	Sipolo	6	*C. nucifera*	2	+	+
GQ05	15 April 2021	Sipolo	6	*Elaeis guineensis*	(1)	+	−
GQ06	15 April 2021	Sipolo	5	*C. nucifera*	1	−	+/−
GQ07	15 April 2021	Sipolo	5	*C. nucifera*	2	−	−
GQ08	15 April 2021	Sipolo	3	*C. nucifera*	2	+/−	−
GQ09	15 April 2021	Nume	7	*C. nucifera*	(1)	−	−
GQ10	16 April 2021	Cabo San Juan	1	*C. nucifera*	2	−	−
GQ11	16 April 2021	Cabo San Juan	1	*C. nucifera*	1	−	+/−
GQ12	16 April 2021	Cabo San Juan	1	*C. nucifera*	Dead	−	−
GQ13	16 April 2021	Cabo San Juan	1	*C. nucifera*	1	−	−
GQ14	16 April 2021	Cabo San Juan	1	*C. nucifera*	1	−	−
GQ15	17 April 2021	Rio Campo	4	*C. nucifera* "French"	(2)	−	−
GQ16	17 April 2021	Rio Campo	4	*E. guineensis*	(1)	+	−
GQ17	17 April 2021	Rio Campo	10	*C. nucifera*	3	+	−
GQ18	17 April 2021	Rio Campo	10	*C. nucifera*	2	+	−
GQ19	17 April 2021	Rio Campo	10	*C. nucifera*	Dead	+	−
GQ20	17 April 2021	Rio Campo	10	*C. nucifera*	2	−	−
GQ21	20 April 2021	Ndote	11	*C. nucifera*	No yellowing	−	−
GQ22	20 April 2021	Ndote	11	*C. nucifera*	No yellowing	−	−
GQ23	20 April 2021	Handje	9	*C. nucifera*	3	+	−
GQ24	20 April 2021	Handje	9	*C. nucifera*	Dead	+	+
GQ25	20 April 2021	Handje	9	*C. nucifera*	1	+	−
GQ26	20 April 2021	Handje	9	*C. nucifera*	2	+	
GQ27	20 April 2021	Handje	9	*C. nucifera* "French"	1	+	+
GQ28	20 April 2021	Handje	9	*C. nucifera*	2	+	−
GQ29	20 April 2021	Handje	8	*C. nucifera*	No yellowing	+/−	−
GQ30	20 April 2021	Handje	8	*C. nucifera*	No yellowing	−	−
GQ31	20 April 2021	Handje	8	*C. nucifera*	(1)	−	−
GQ32	20 April 2021	Handje	2	*C. nucifera*	1	+/−	−
GQ33	20 April 2021	Handje	2	*C. nucifera*	1	+/−	−
GQ34	20 April 2021	Handje	2	*C. nucifera*	2	−	−

[a]Description of disease stage as in materials and methods. Numbers within brackets indicate that the symptoms were either very light, or not typical of LY; +, positive; +/− faint band, −, negative.

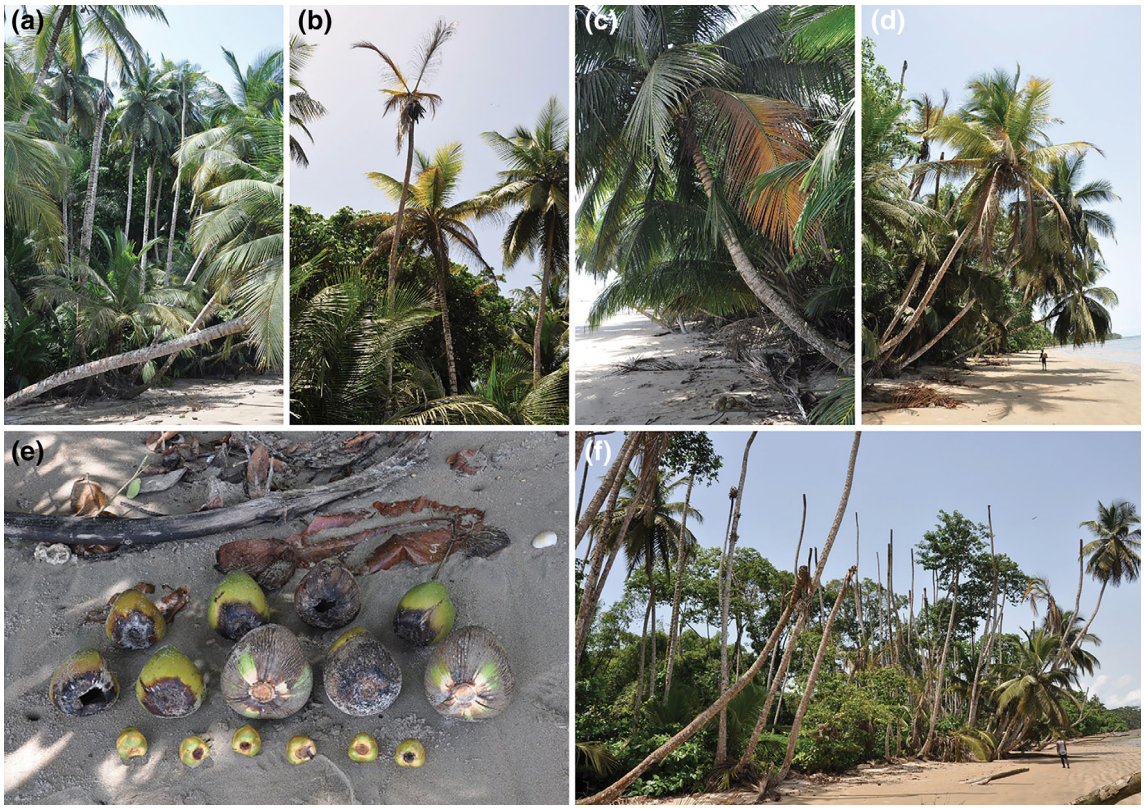

FIGURE 2 Symptoms of lethal yellowing in some of the locations surveyed: (a) stage 3, (b) stages 2 and 3, (c) stage 1, (d) stages 2 and 3, (e) aborted and early-dropped coconuts, many showing calyx-end rot, stage 1, (f) coconut palms in the "telephone pole" phase, stage 3″.

FIGURE 3 "Skirting" in oil palm.

climatised room, to prevent the development of contaminating bacteria or fungi. Samples were then stored at around 4°C, until processing for phytoplasma detection and identification.

2.2 | Phytoplasma detection and identification

DNA extraction was performed on 1 g of sawdust from the trunk borings using a CTAB based method (Angelini et al., 2001; Doyle & Doyle, 1987). The PCR amplifications were carried out using 1 μL of the extracted DNAs diluted 1: 30 in sterile distilled water. For the '*Ca*. Phytoplasma' species detection, the primers P1/P7 (Deng & Hiruki, 1991; Schneider et al., 1995), 3F/3R (Manimekalai et al., 2010), R16(I)F1/R1 (Lee et al., 1994) and M1/M2 (=758f/1232r) (Gibb et al., 1995) were employed in direct and nested-PCR assays, using reported cycling conditions (Arocha Rosete et al., 2017). The phytoplasma *secA* gene was amplified with the primer pair SecAfor1/SecArev3 and the amplification products were used for nested PCR with primers SecAfor5/SecArev2 with the cycling conditions reported (Dickinson & Hodgetts, 2013). The PCR mix in both cases comprised a total volume of 25 μL with 1 μL of template DNA, 0.5 μL of each primer at 20 pmol, 12.5 μL of 2× MyTaq Red Mix (Meridian Bioscience, USA) and 10.5 μL H_2O. Results were visualised in agarose gels 1% in TE buffer, stained with ethidium bromide under UV. Phytoplasma positive controls from micropropagated collection (Bertaccini, 2023; Table 2)

TABLE 2 List of phytoplasmas in periwinkle from collection used as controls for ribosomal subgroup differentiation in RFLP analyses.

Strain	Acronym	'*Candidatus* Phytoplasma'	Ribosomal group/subgroup	Country
Aster yellows strain 2192	AY2192	'*Ca*. P. asteris'	16SrI-B	Germany
Potato purple top	PPT		16SrI-C	France
Cleome witches' broom	CWB		16SrII-A	Thailand
Faba bean phyllody	FBC		16SrII-C	Sudan
Green Valley X disease	GVX	'*Ca*. P. pruni'	16SrIII-A	USA
Alder yellows	ALY		16SrV-C	Italy
Potato witches' broom	PWB	'*Ca*. P. trifolii'	16SrVI-A	USA
Ash yellows	ASHY1	'*Ca*. P. fraxini'	16SrVII-A	USA
Pichris echioides yellows	PEY		16SrIX-C	Italy
Plum leptonecrosis	LNP	'*Ca*. P. prunorum'	16SrX-B	Italy
Flower stunting	BVK		16SrXI-C	Germany
Molière disease	MOL	'*Ca*. P. solani'	16SrXII-A	France
Suriname virescence	SUV	'*Ca*. P. brasiliense'	16SrXV-A	Brazil

and strains of '*Ca*. P. palmicola' from Ghana, Ivory Coast and Mozambique (Arocha Rosete et al., 2017) were amplified separately to avoid cross contamination. Negative controls in PCR reactions were samples devoid of nucleic acid template and/or containing sterile distilled water. The '*Ca*. Phytoplasma' species identification was achieved by direct 16S ribosomal gene amplicons sequencing in both senses using the 3F and 3R primers at Macrogene (The Netherlands). RFLP analyses with *Tru*1I, *Hae*III and *Taq*I (Fermentas, Vilnius, Lithuania) were carried out in a polyacrylamide gel 6.7% in TAE buffer and visualised as described for the phytoplasma ribosomal group/subgroup attribution (Lee et al., 1998). Further RFLP analyses on the *secA* gene amplicons with *Mbo*II (Fermentas, Vilnius, Lithuania) restriction enzyme and direct sequencing with SecAfor5 and SecArev2 primers were carried out to confirm the phytoplasma identification. After alignment the sequences were used in phylogenetic studies to identify their clustering with '*Candidatus* Phytoplasma' species (Bertaccini et al., 2022). The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach, and then selecting the topology with superior log likelihood value. Evolutionary analyses were conducted in MEGA 7 (Kumar et al., 2016). The 16S rRNA and *secA* phytoplasma genes sequences obtained from Equatorial Guinea were deposited in NCBI GenBank.

3 | RESULTS

The molecular analyses carried out on 16S rRNA and *secA* genes allowed to detect the phytoplasma presence in 20 out of the 34 palms tested (Table 1), from plants at all stages of the disease, including two of the dead plants. The 16S ribosomal gene amplification in nested PCR provided the highest number of positive samples (18/34), while for two samples the amplification was only obtained on the *secA* gene (Table 1). Sequencing and BLAST search of the 16S rRNA and partial spacer region gene sequences obtained (GenBank accession numbers OP912899 and OP912900), evidenced that they were identical and had 100% identity to '*Ca*. P. palmicola' strain LYDM-178 (GenBank accession number KF751387) from Mozambique. Phylogenetic analyses confirmed the close clustering of the detected phytoplasma with '*Ca*. P. palmicola' strains from Mozambique and Nigeria (Figure 4a). The RFLP analyses using *Tru*1I, *Taq*I and *Hea*III enzymes showed that the phytoplasmas found in all samples (including those from *E. guineensis* and the two "French" hybrid coconuts) had restriction profiles identical to each other and to those enclosed in subgroup 16SrXXII-A (Figures 5 and 6a). Moreover, both *Mbo*II RFLP profiles and phylogenetic analyses on the *secA* gene sequences (GenBank accession numbers OP952942 and OP952943) distinguished the phytoplasmas from Ghana and Ivory Coast from those from Mozambique and Equatorial Guinea, confirming that those from the latter two countries have identical profiles and therefore a closer genetic relationship (Figures 4b and 6b). The phylogenetic analysis of the two genes confirms the clustering of the strains from Equatorial Guinea with the '*Ca*. P. palmicola' strains from Mozambique and Nigeria (strain LND, GenBank accession number KJ462070), while the '*Ca*. P. palmicola' strains in 16SrXXII-B subgroup and the '*Ca*. P. cocostanzaniae' are clearly grouped in separate branches (Figure 4b). The alignment and comparison of 415 nucleotides in the *secA* amplified gene sequence showed the presence of 7 SNPs in strain LND compared to the Equatorial Guinea strains while the three strains from Mozambique LYM, MLD and MozQ showed 1–3 SNPs (data not shown).

4 | DISCUSSION

No data are available on the incidence of this LY disease in the area surveyed, nor on the loss of production it caused, because no

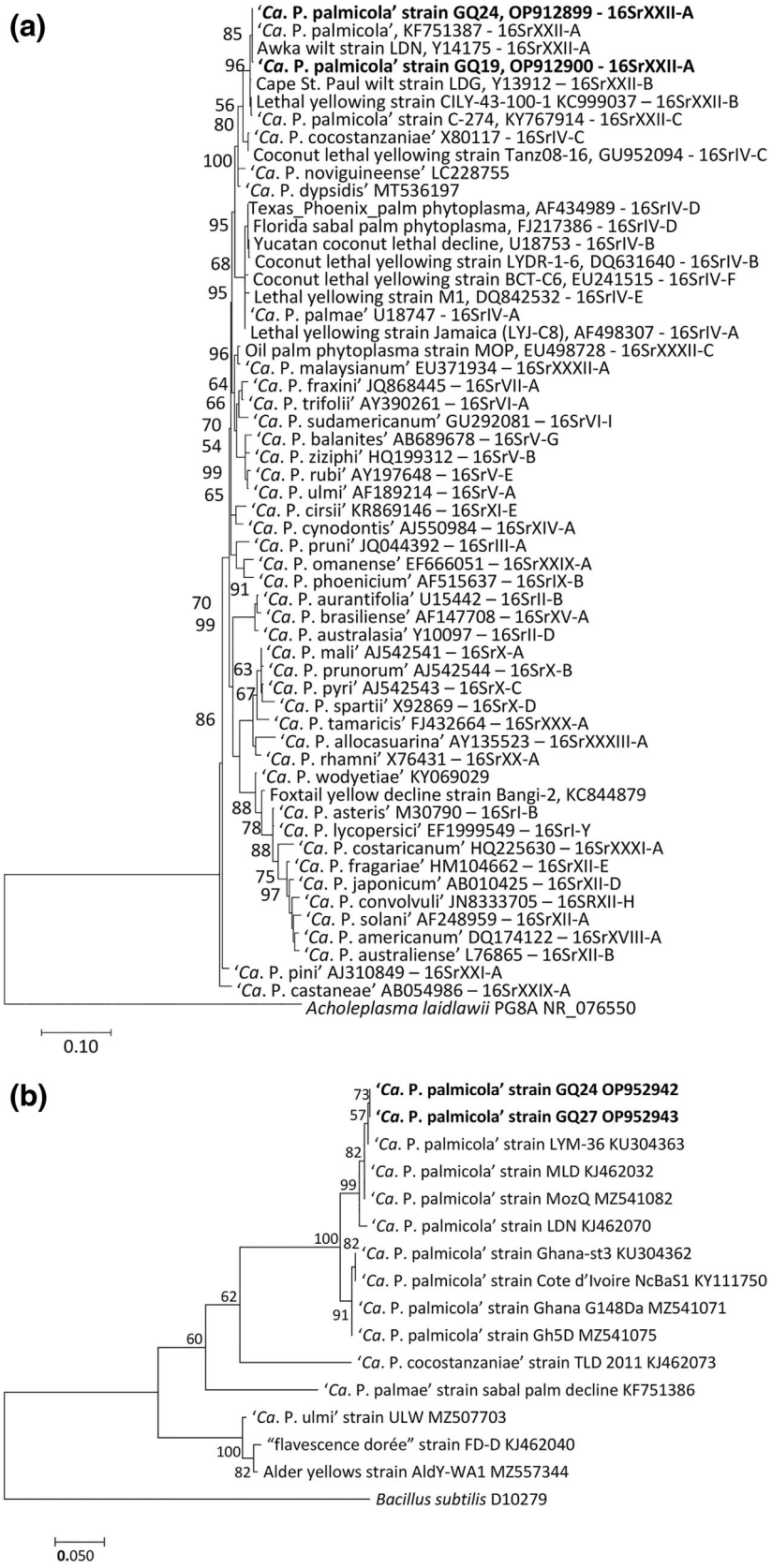

FIGURE 4 Phylogenetic tree where evolutionary history is inferred by using the Maximum Likelihood method. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The sequences from phytoplasma detected in Equatorial Guinea are marked in bold; the GenBank accession numbers are added on the right. In (a) 16S rRNA gene sequences of detected phytoplasmas and of '*Candidatus* Phytoplasma' ('*Ca*. P.') strains in diverse ribosomal groups and subgroups (available information are added on the right of the GenBank accession numbers), *Acholeplasma laidlawii* is used as outgroup to root the tree. In (b) *secA* gene sequences from phytoplasmas closely related to those identified in Equatorial Guinea, *Bacillus subtilis* is used as outgroup to root the tree.

investigation was ever carried out in the country to try and define the nature of the problem and its extent. During this survey, both local coconut producers and technical officers of the Ministry of Agriculture were interviewed, as well as the manager of the state-owned company in charge of exporting coconut into Cameroon. The information they provided led the authors to estimate a 60%–70%

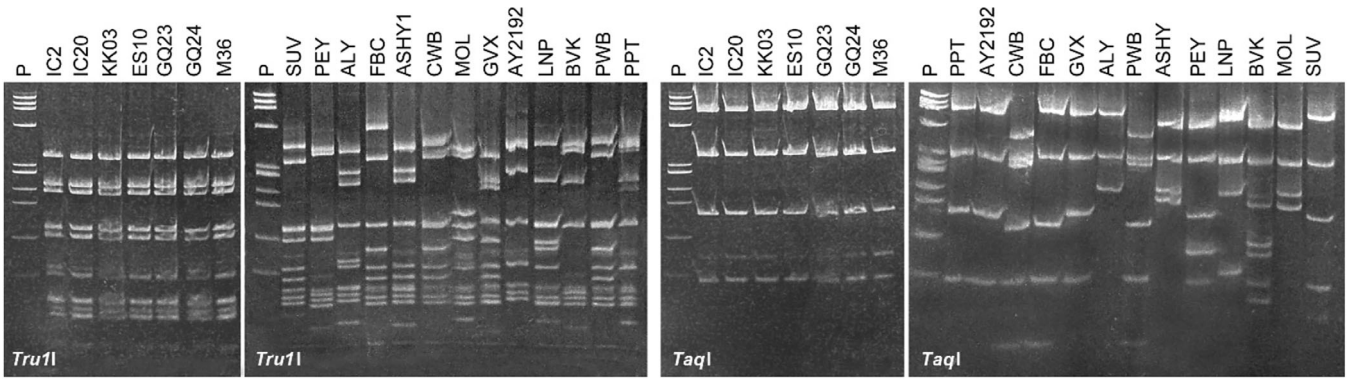

FIGURE 5 RFLP results in 6.7% polyacrylamide gels of 16S rRNA gene amplified sequences with primers 3F/3R of samples in coconut from Equatorial Guinea (GQ23, GQ24), Ivory Coast (IC2, IC20), Ghana (KK03, ES10) and Mozambique (M36). The enzymes used are indicated at the bottom of each figure. P, marker PhiX174 DNA digested with *Hae*III with fragment sizes in base pairs from top to bottom of 1353; 1078; 872; 603; 310; 281; 271; 234; 194; 118 and 72. Acronyms of control samples from different subgroups are described in Table 2.

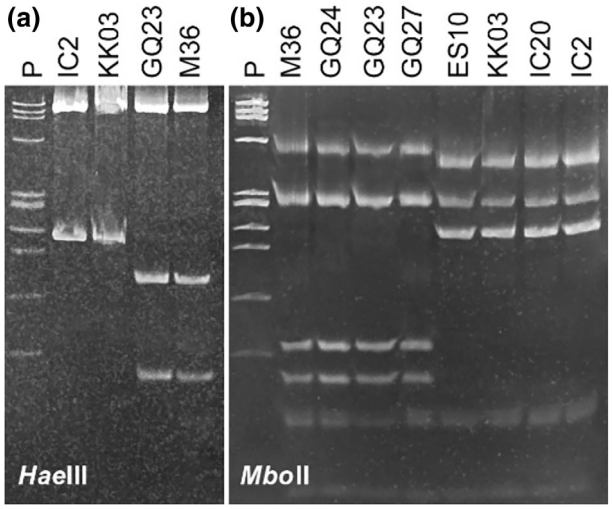

FIGURE 6 RFLP results in 6.7% polyacrylamide gels in (a) of 16S rRNA and in (b) of *secA* genes amplified sequences of samples from coconut from Equatorial Guinea (GQ23, GQ24, GQ27), Ivory Coast (IC2, IC20), Ghana (KK03, ES10) and Mozambique (M36). The enzymes used are indicated at the bottom. P, marker PhiX174 as in Figure 5.

reduction in the palm population, and a similar reduction in the production of coconut.

The coconut palms lethal yellowing of Equatorial Guinea appears to be associated with a phytoplasma strain 16SrXXII-A that is molecularly distinguishable from the '*Ca*. P. palmicola' strains detected in Ghana and Ivory Coast in Western Africa (Arocha Rosete et al., 2017; Yankey et al., 2009). This represents the first identification and molecular characterisation of a 16SrXXII-A strain for Equatorial Guinea and the Central Africa region. A 16SrXXII-A strain had been previously reported from Nigeria, Western Africa (Osagie et al., 2015), but it was not fully characterised. The *secA* gene polymorphism detected in RFLP and sequencing analyses confirms the phytoplasma identification (Yankey et al., 2014) and also shows the ability of this gene in differentiating strains enclosed in the same ribosomal subgroup, but geographically distributed in diverse areas (Nigeria, Equatorial Guinea and Mozambique). The detection of this phytoplasma also in *E. guineensis* is one of the first reports of a '*Ca*. P. palmicola' strain 16SrXXII-A outside *C. nucifera* and suggests the possibility that it may have a wider host range than it has been reported so far. Moreover, the infection of the supposedly resistant "French" coconut variety represents an additional alert related to difficulties involved in the development of an effective strategy for the LY management.

Approximately 40% of the samples tested negative for phytoplasma presence. This may be due to the uneven distribution of the pathogen within the plants, that would have required multiple trunk borings to ensure detection, but also to the fact that the majority of the plants that tested negative had symptoms which were not typical of LY, thus likely due to factors other than phytoplasma infection. Additionally, in some of the samples the quality of the genomic DNA might have been negatively affected (oxidation) by the phenolics released by the wood fragments after collection.

Lethal yellowing in coconut is associated with '*Ca*. P. palmicola' and '*Ca*. P. cocostanzaniae' in Africa, '*Ca*. P. palmae' in America, '*Ca*. P. noviguineense' and '*Ca*. P. dypsidis' in Papua New Guinea and Australia, respectively (Bertaccini et al., 2022; Harrison et al., 2014; Jones et al., 2021; Miyazaki et al., 2018). Other phytoplasma-associated diseases are described in palms in South and South-East Asia, such as Weligama wilt in Sri Lanka and Kerala wilt in India. Different palm species such as oil palm, date palm and areca nut palm are reported in Colombia, Indian subcontinent, Saudi Arabia and Kuwait as infected by '*Ca*. P. asteris', '*Ca*. P. oryzae', '*Ca*. P. malaysianum', '*Ca*. P. wodyetiae' and '*Ca*. P. cynodontis' (Gurr et al., 2016; Hemmati et al., 2020; Yankey et al., 2018). Although the transmission of phytoplasmas among palms has been intensively studied, to date the only insect vector identified is *Haplaxius crudus*, which is known to transmit '*Ca*. P. palmae' and is reported only in the American continent (Ogle & Harries, 2005). For such phytoplasma the possibility of embryo transmission was also recently proved (Oropeza et al., 2017).

The survey results and the already high incidence of '*Ca*. P. palmicola' in the main coconut growing areas of Equatorial Guinea, indicate that the recovery of this, once important sector, urgently requires the deployment of an integrated management strategy, whose first component should be a specific surveillance programme for the early detection and elimination of infected palms.

ACKNOWLEDGEMENTS

This work was partly funded by the European Union's Horizon 2020 research and innovation program under grant agreement No. 727459, project "Insect-borne prokaryote-associated diseases in tropical and subtropical perennial crops", TROPICSAFE.

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How to cite this article: Bertaccini, A., Contaldo, N., Feduzi, G., Andeme, A. M. E., Yankey, E. N., & Rovesti, L. (2023). Molecular identification of '*Candidatus* Phytoplasma palmicola' associated with coconut lethal yellowing in Equatorial Guinea. *Annals of Applied Biology*, *183*(3), 262–270. https://doi.org/10.1111/aab.12854