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Corresponding Author: Dr. Yaima Arocha Rosete, PhD

Corresponding Author's Institution:

First Author: Koffi Eric Kwadjo, Dr

Order of Authors: Koffi Eric Kwadjo, Dr; N'Djiha Isabelle Beugré, MSc; Christopher H. Dietrich, Prof.; Adaba Tano Thierry Kodjo, MSc; Hortense Atta Diallo, Prof; Ndede Yankey, Dr.; Sylvester Dery, Prof.; Michael Wilson, Dr; Jean Louis Konan Konan, Dr; Nicoletta Contaldo, Dr; Samanta Paltrinieri, Dr; Assunta Bertaccini, Prof.; Yaima Arocha Rosete, PhD

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Suggested Reviewers: Nicola Mori Prof.
University of Padova
nicola.mori@unipd.it
Phytoplasma Entomologist

Norma Elena Leyva Lopez Prof.
CIDIIR, Mexico
neleyval@ipn.mx
Specialized on phytoplasma disease epidemiology, diagnosis and control

Rosa La Rosa Prof.
University of Catania
larosar@unict.it
Specialized on phytoplasma epidemiology and diagnosis

Helena Montano Dr
University of Rio de Janeiro
hmontano@ufrrj.br
Specialized on phytoplasma, epidemiology, characterization and diagnosis

Crop Protection Cover Letter

This is a research article entitled 'Identification of a newly described genus and species of the tribe Erythroneurini as a potential vector of the Côte d'Ivoire lethal yellowing phytoplasma in coconut palms sole or in mixed infection with a '*Candidatus* Phytoplasma asteris'-related strain'.

This is the first time that we publish in Crop Protection.

The research described in this paper is novel and has not been published elsewhere nor submitted to any other journal for consideration of publication. Authors declare no conflict of interest.

The list of authors is as follows:

1. Dr. Koffi Eric Kwadjo, *Université Nangui Abrogoua, 02 BP 801 Abidjan 02, Côte d'Ivoire,* kokoferic@gmail.com
2. Ms. N'Djiha Isabelle Beugré, *Station de Recherche "Marc Delorme", Centre National de Recherche Agronomique (CNRA), 07 BP 13, Abidjan, Côte d'Ivoire,* Isabelle.bri@gmail.com
3. Dr. Christopher H. Dietrich, *Illinois Natural History Survey, University of Illinois 1816 S Oak St., Champaign, IL 61820 USA,* chdietri@illinois.edu
4. Mr. Adaba Tano Thierry Kodjo, *Université Nangui Abrogoua, 02 BP 801 Abidjan 02, Côte d'Ivoire,* Thierry.kodjo@yahoo.com
5. Prof. Hortense Atta Diallo, *Université Nangui Abrogoua, 02 BP 801 Abidjan 02, Côte d'Ivoire,* hortensediallo@live.fr
6. Dr. Ndede Yankey, [4]*Council for Scientific Research Program–Oil Palm Research Institute–Coconut Research Program (CSIR-OPRI), Off Well Road Box 254, Sekondi, Western Region, Ghana*, ndedeyankey@yahoo.com

7. Dr. Sylvester Dery, [4]*Council for Scientific Research Program–Oil Palm Research Institute–Coconut Research Program (CSIR-OPRI), Off Well Road Box 254, Sekondi, Western Region, Ghana*, sylvesterdery@yahoo.com

8. Dr. Michael Wilson, *Department of Biodiversity and Systematic Biology, Museum of Wales, Cardiff, CF10 3NP, United Kingdom,* Michael.Wilson@museumwales.ac.uk

9. Prof. Jean Louis Konan Konan, *Station de Recherche "Marc Delorme", Centre National de Recherche Agronomique (CNRA), 07 BP 13, Abidjan, Côte d'Ivoire, konankonanjeanlouis@yahoo.fr*

10. Dr. Nicoletta Contaldo, *Department of Agricultural Sciences,* Alma Mater Studiorum - *University of Bologna viale Fanin 42, 40127 Bologna, Italy, nicoletta.contaldo2@unibo.it*

11. Dr. Samanta Paltrinieri, *Department of Agricultural Sciences,* Alma Mater Studiorum - *University of Bologna viale Fanin 42, 40127 Bologna, Italy, samantapaltrinieri@gmail.com*

12. Prof. Assunta Bertaccini, *Department of Agricultural Sciences,* Alma Mater Studiorum - *University of Bologna viale Fanin 42, 40127 Bologna, Italy, assunta.bertaccini@unibo.it*

13. Dr. Yaima Arocha Rosete, [7]*Sporometrics, 219 Dufferin Street, Suite 20C, Toronto, ON M6K 3J1, Canada. Corresponding author: yarosete@sporometrics.com, Tel: 416-516-1660.*

Identification of a newly described member of the tribe Erythroneurini as a potential vector of the Côte d'Ivoire lethal yellowing phytoplasma in coconut palms sole or in mixed infection with a '*Candidatus* Phytoplasma asteris'-related strain.

Koffi Eric Kwadjo[a], N'Djiha Isabelle Beugré[b], Christopher H. Dietrich[c], Adaba Tano Thierry Kodjo[b], Hortense Atta Diallo[b], Ndede Yankey[d], Sylvester Dery[d], Michael Wilson[e], Jean Louis Konan Konan[c], Nicoletta Contaldo[f], Samanta Paltrinieri[f], Assunta Bertaccini[f], and Yaima Arocha Rosete[g*]

[a]*Université Nangui Abrogoua, 02 BP 801 Abidjan 02, Côte d'Ivoire*

[b]*Station de Recherche "Marc Delorme", Centre National de Recherche Agronomique (CNRA), 07 BP 13, Abidjan, Côte d'Ivoire*

[c]*Illinois Natural History Survey, University of Illinois 1816 S Oak St., Champaign, IL 61820 USA*

[d]*Council for Scientific Research Program–Oil Palm Research Institute–Coconut Research Program (CSIR-OPRI), Off Well Road Box 254, Sekondi, Western Region, Ghana*

[e]*Department of Biodiversity and Systematic Biology, Museum of Wales, Cardiff, CF10 3NP, United Kingdom,*

[f]*Department of Agricultural Sciences,* Alma Mater Studiorum - *University of Bologna viale Fanin 42, 40127 Bologna, Italy*

[g]*Sporometrics, 219 Dufferin Street, Suite 20C, Toronto, ON M6K 3J1, Canada*

**Email: yarosete@sporometrics.com, Tel: 416-516-1660.*

Abstract

Over 300 Hemiptera specimens were collected using sweep nets and hand-made aspirators from coconut palm fronds in six villages of Grand-Lahou. Eight families were identified including Aphrophoridae, Achilidae, Derbidae, Flatidae, Membracidae, Pentatomidae, Tropiduchidae, and Cicadellidae, the latter being the most abundant throughout the surveyed villages. PCR assays with primers targeting the 16S rRNA and the *sec*A translocation protein genes yielded PCR amplicons from 216 out of 296 (73%) of the tested specimens of a newly identified cicadellid leafhopper, *Nedotepa curta* Dmitriev. PCR amplicons were purified, cloned and sequenced. The 16S rDNA and *sec*A sequences from *N. curta* showed a 99% sequence identity with those of the phytoplasma identified in coconut-growing villages of Grand-Lahou, which suggested *N. curta* as a potential vector for the CILY phytoplasma. Phytoplasmas of group 16SrI '*Candidatus* Phytoplasma asteris'-related were identified from phytoplasma-infected coconut palms infected by the Côte d'Ivoire lethal yellowing phytoplasma and *N. curta* specimens from Badadon and Yaokro villages, as well as from the weeds *Dalbergia saxatilis* and *Baphia nitida* from Badadon. Results indicate that mixed infection of both 16SrXXII-B and 16SrI phytoplasmas is occurring in coconut palms affected by CILY in Grand-Lahou, which may impact disease management and control.

Keywords: coconut lethal yellowing, phytoplasma, potential vector, 16SrXXII, Côte d'Ivoire, Nedotepa

1. Introduction

Côte d'Ivoire lethal yellowing (CILY) of coconut palm was first reported in 2013 in Grand-Lahou and since then it has rapidly spread to several coconut-growing villages where over 400 ha have been destroyed and another 7,000 ha are under threat (Arocha-Rosete et al., 2014). Lethal yellowing (LY)-like diseases of palms have been associated with a number of phytoplasmas (Sullivan and Harrison, 2013) worldwide that have killed millions of palms in the last 40 years.

Phytoplasmas are bacteria of the class Mollicutes transmitted by phloem-feeding insect species within the order Hemiptera, particularly Cicadellidae (leafhoppers), but also Cercopidae, Cixiidae, Derbidae, Delphacidae, and Psyllidae (Weintraub and Beanland, 2006). Phytoplasma transmission by hemipteran vectors has previously been shown to be persistent and propagative, and once insect vectors acquire the phytoplasma they remain inoculative for life (Bosco and d'Amelio, 2010). Phytoplasmas are transmitted by a narrow range of hemipteran species (Weintraub and Beanland, 2006), whereas their plant host range is usually broader (Foissac and Wilson, 2010). Only about 1% of known leafhopper species have been shown to be capable of transmitting plant pathogens (Dietrich, 2013), so the number of actual or potential vectors is likely to be much larger than the approximately 200 vector species currently documented.

Despite the widespread occurrence of phytoplasmas in coconuts in Africa, Asia and the Caribbean, many of the insect vectors of LY-like coconut diseases have not been identified. So far, the cixiid *Haplaxius crudus* (Van Duzee) has been the only species reported as vector for the LY phytoplasma in Florida (Howard et al., 2001). The vector for the long known Cape St Paul Wilt Disease (CSPWD) of coconuts in Ghana remains elusive. Although two species, *Diostrombus* sp. (Derbidae) and *Myndodus adiopodoumeensis* (Synave) (Cixiidae), formerly

placed in the genus *Myndus* (*Myndus adiopodoumeensis*) (Ceotto and Bourgoin, 2008) were found to carry the CSPWD phytoplasma, transmission trials were inconclusive (Philippe et al., 2009). An undescribed species of *Cedusa* (Derbidae) has been implicated in transmission of palm phytoplasmas in Jamaica (Brown et al., 2006), but no transmission test was done. In the Cabo Delgado province of Mozambique, some pentatomids of the species *Platacantha lutea* (Westwood) were found to carry the same phytoplasmas as those identified in the diseased coconut on which they were found (Dollet et al., 2011). In Tanzania, *Diostrombus mkurangai* Wilson (Derbidae) and a few specimens of *Meenoplus* spp. (Meenoplidae) were PCR positive for phytoplasmas but experimental transmission was never carried out (Mpunami et al., 2000).

Interestingly, *D. mkurangai* was identified as a potential vector of LY in Mozambique (Bila 2016), where it may also carry the Tanzanian LD phytoplasma type; likewise *D. mkurangai* in Tanzania may possibly harbor '*Candidatus* Phytoplasma palmicola' or related strains. *Patara albida* (Derbidae) was identified as a potential vector for the Texas Decline palm phytoplasma (Brown et al., 2006), and a new species within the derbid genus *Omolicna* was also described as carrier of the same phytoplasma. Two other derbids, *D. mkurangai*, and *Proutista moesta* (Westwood) are implicated in transmission of other palm pathogens in Africa (Howard et al., 2001) and Kerala Wilt disease of coconut in India (Edwin and Mohankumar, 2007). More recently, six taxa belonging to families Derbidae, Lophopidae, Flatidae and Ricaniidae were identified as potential vectors for the Bogia Coconut Syndrome phytoplasma in Papua New Guinea, coupling insect feeding media and LAMP PCR assays (Lu et al., 2016).

LY and LD phytoplasmas affecting coconut and other palm species exhibit wide genetic variation among strains within and from North/Central America and the Caribbean, and Africa (Sullivan and Harrison, 2013). The group 16SrIV appears to be limited to the Americas, the

Caribbean, and Tanzania (Danyo 2011), and is divided into several subgroups that include the 16SrIV-A (Palm LY, Florida), 16SrIV-B (Yucatan LD, Mexico), 16SrIV-C (Tanzania and Kenya LD, 16SrIV-D (Texas Phoenix Decline, TPD, and Mexico *Carludovica palmata* yellows, CPY) (Harrison et al., 2002), and 16SrIV-F (*Washingtonia robusta*, Florida) (Harrison et al., 2008).

The CILY phytoplasma was recently classified as a member of group 16SrXXII, subgroup – B '*Ca.* P. palmicola – related strains' (Harrison et al., 2014) that comprises the CSPWD phytoplasma strain from Ghana, which destroyed the Ghanaian coconut industry in the last 20 years (Danyo, 2011). Within the same group, the subgroup 16SrXXII –A was officially named as the new taxon identified in Mozambique '*Ca.* P. palmicola' that also includes the lethal decline (LD) strain from Nigeria (Harrison et al., 2014). Bila et al., (2015) identified three phytoplasma strains associated with the LY in Mozambique, which included the '*Ca*. P. palmicola' (16SrXXII-A), the Tanzanian LD strain (16SrIV-C), and a '*Ca.* P. pini' – related strain (16SrXXI-A); this latter was found in co-infection with a '*Ca*. P. palmicola' strain.

This paper reports the results of surveys conducted in Grand-Lahou to characterize the Hemiptera entomofauna of the coconut farms affected by CILY, and to identify the potential insect vector(s) for the CILY phytoplasma, and to determine any possible occurrence of phytoplasma mixed infection. Total DNA samples from coconut palms previously surveyed from CILY-affected villages in Grand-Lahou, and weeds present in the coconut farms were PCR- and sequence- assessed with universal primers targeting ribosomal (16S rRNA) and non-ribosomal (*sec*A) genes. A description of the main morphological traits of the recently described typhlocybine, *Nedotepa curta* Dmitriev, in Grand-Lahou, Côte d'Ivoire, is also provided.

2. Materials and Methods

2.1 Plant and Entomofauna sampling in coconut groves affected by CILY in Grand-Lahou

Over 300 specimens of Hemiptera were collected with a sweep net and hand-made aspirator from the undersides of coconut leaves exhibiting CILY symptoms from stages 1, 2 and 3 during surveys conducted in six villages of Grand-Lahou from March 2015 to September 2016 (Arocha Rosete et al., 2017). Trunk borings from three coconut palms representing each disease stage in each village, and one symptomless palm were obtained as previously described (Arocha Rosete et al., 2017). Hemiptera specimens were also collected from two weed species *Dalbergia saxatilis* Hook. f. (Leguminosae – Papilionoideae), and *Baphia nitida* Lodd. (Fabaceae) from the village of Badadon. Leaf samples of the weed species were also collected.

Specimens collected were transported to the Entomology Laboratory of the University of Nangui Abrogoua in 1.5 mL microtubes in coolers with ice packs. Once in the lab, insect specimens were sorted and sent out for morphology-based confirmation of the taxonomic identification (genus and species) to Dr. Michael Wilson, Museum of Wales, United Kingdom; and Dr. Christopher Dietrich, University of Illinois, USA. Voucher specimens of identified insects are deposited in the National Museum of Wales and the Illinois Natural History Survey, Champaign.

2.2 Nested polymerase chain reaction (nPCR)

For all PCR reactions, 50 ng of total DNA extracted (FastDNA Spin Kit, MP Biomedicals) was added to a 25 µL PCR reaction (PCR ready-to-go-beads, GE Healthcare, United Kingdom) containing 0.4 µM of each primer. Universal primers P1 (Deng and Hiruki,

1991) and P7 (Schneider et al., 1995) nested with CSPWD phytoplasma primers G813F/AwkaSR (Thompson et al., 1994) were used to amplify the partial 16S rRNA, intergenic spacer and 23S gene of the CILY phytoplasma. One microliter of the 40-fold diluted P1/P7 PCR products was used in the PCR reaction. The R16F2n/R2 (Gundersen and Lee, 1996) and fU5/rU3 (Lorenz et al., 1995) fragments were amplified through nested PCR using the primer pairs R16mF1/R1 (Gundersen and Lee, 1996) and P1/P7, respectively, for the direct PCR reactions. The non-ribosomal secretion protein (*sec*A) gene was also amplified with the primer pair SecAfor1/SecArev3. The direct PCR product was diluted 30-fold and used as a DNA template for PCR with primers SecAfor5/SecArev2 (Dickinson and Hodgetts, 2013). Total DNA extracts used as positive controls were coconut palms confirmed as CSPWD phytoplasma-infected from Ghana representing disease stages 1, 2 and 3 (provided by Dr. Ndede Yankey), and CILY phytoplasma-infected from Grand-Lahou (Badadon, Braffedon, Adjadon, Yaokro and Doudougbazou) (Arocha Rosete et al., 2017). PCR cycling and annealing temperatures were as previously described (Arocha Rosete et al., 2017; Lorenz et al., 1995).

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

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

G813/AwkaSR, R16F2n/R2, fU5/rU3 and secA amplicons 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 bi-directionally using

157 M13F/M13R primers (Centre for the Analysis of Genome Evolution and Function, CAGEF,
158 University of Toronto). The consensus 16S rDNA and secA sequences were deposited in
159 GenBank and compared by BLAST (Altschul et al., 1990) with available phytoplasma
160 sequences. Sequences obtained were aligned and phylogenetic trees were constructed using the
161 neighbour-joining method with MEGA version 4.0 (Kumar et al., 2004) with default values and
162 1,000 replicates for bootstrap analysis.

163 R16F2n/R2 sequences were analysed with *i*PhyClassifier (Zhao et al., 2009) for
164 preliminary identification of the phytoplasma detected in the insect samples based on *in silico*
165 restriction profiles. Ten microliters of the G813/AwkaSR secA and fU5/rU3 PCR amplicons
166 were digested with *Rsa*I, *Hae*III, *Alu*I, *Mbo*II, and *Taq*I restriction endonucleases (New England
167 Biolabs, Canada), following manufacturer's recommendations. RFLP profiles were visualized in
168 a 3 % agarose or 6.7 % polyacrylamide gel stained with SYBR^{R} Safe DNA Gel Stain
169 (Invitrogen, USA) in a gel documenter (Alpha Innotech, USA).

170

171 **3. Results**

172 Surveys were conducted in the villages of Amanikro, Adjadon, Badadon, Braffedon,
173 Yaokro, and Doudougbazou, located at the south littoral of Grand-Lahou. Results revealed the
174 presence of eight major Hemiptera families: Aphrophoridae, Achilidae, Derbidae, Flatidae,
175 Membracidae, Pentatomidae, Tropiduchidae, and Cicadellidae (Table 1). Specimens from the
176 families Cicadellidae and Derbidae were the most abundantly collected. Specimens of Derbidae
177 included *Kamendaka albomaculata* (Muir), *Phenice stellulata* (Boheman), *Diostrombus dilatatus*
178 (Westwood), and *Proutista fritillaris* (Boheman). The family Cicadellidae was represented by a

179 recently described genus and species of the tribe Erythroneurini, *Nedotepa curta* Dmitriev
180 (Cicadellidae*:* Typhlocybinae: Erythroneurini) (Dmitriev, 2016).

181

182 **Table 1**. Hemiptera families collected and tested by PCR specific for the CILY phytoplasma
183 (G813/AwkaSR primers) in six villages of Grand-Lahou. NC: not collected.

Family / Village	Badadon	Braffedon	Doudougbazou	Amanikro	Adjadon	Yaokro	**Total**
	No. specimens nPCR positive / No. specimens collected						
Cicadellidae (*N. curta*)	**91/103**	**69/99**	**15/22**	**0/4**	**17/26**	**24/42**	**216/296**
Derbidae	**0/6**	**0/12**	NC	NC	NC	**2/2**	**2/20**
Tropiduchidae	**0/1**	NC	NC	NC	NC	NC	**0/1**
Membracidae	NC	NC	NC	**0/16**	NC	NC	**0/16**
Pentatomidae	NC	**0/4**	**0/2**	NC	NC	NC	**0/6**
Flatidae	NC	NC	**0/14**	NC	**0/12**	NC	**0/26**
Aphrophoridae	NC	**0/3**	NC	NC	NC	NC	**0/3**
Achilidae	NC	NC	NC	**0/3**	NC	NC	**0/3**
Total	**91/103**	**69/118**	**15/38**	**0/20**	**17/38**	**26/44**	**218/361**

184

185 Specimens of *N. curta* were the only species of leafhopper collected on coconut palm and
186 were present in all villages: Badadon (103), Braffedon (99), Yaokro (42), Adjadon (26),
187 Doudougbazou (17) and Amanikro (4). This leafhopper was the most abundant hemipteran insect
188 on coconut palm overall. Derbidae was the second most collected family in the villages of

Braffedon (12), Badadon (6) and Yaokro (2); while Flatidae was the third most common in Doudougbazou (14) and Adjadon (12) followed by Membracidae (16) limited to Amanikro. Achilidae (3), Aphrophoridae (3) and Tropiduchidae (1) were the least represented families restricted to Amanikro, Braffedon and Badadon, respectively.

Dmitriev (2016) provided a morphological description and detailed illustrations of *Nedotepa curta* based on specimens collected from coconut palm in the Western Region of Ghana. The specimens collected in the present study represent the first records of this species from Côte d'Ivoire. Specimens from Côte d'Ivoire appear to be morphologically identical to specimens of the type series from Ghana (cf. Fig. 1 to illustrations in Dmitriev (2016).

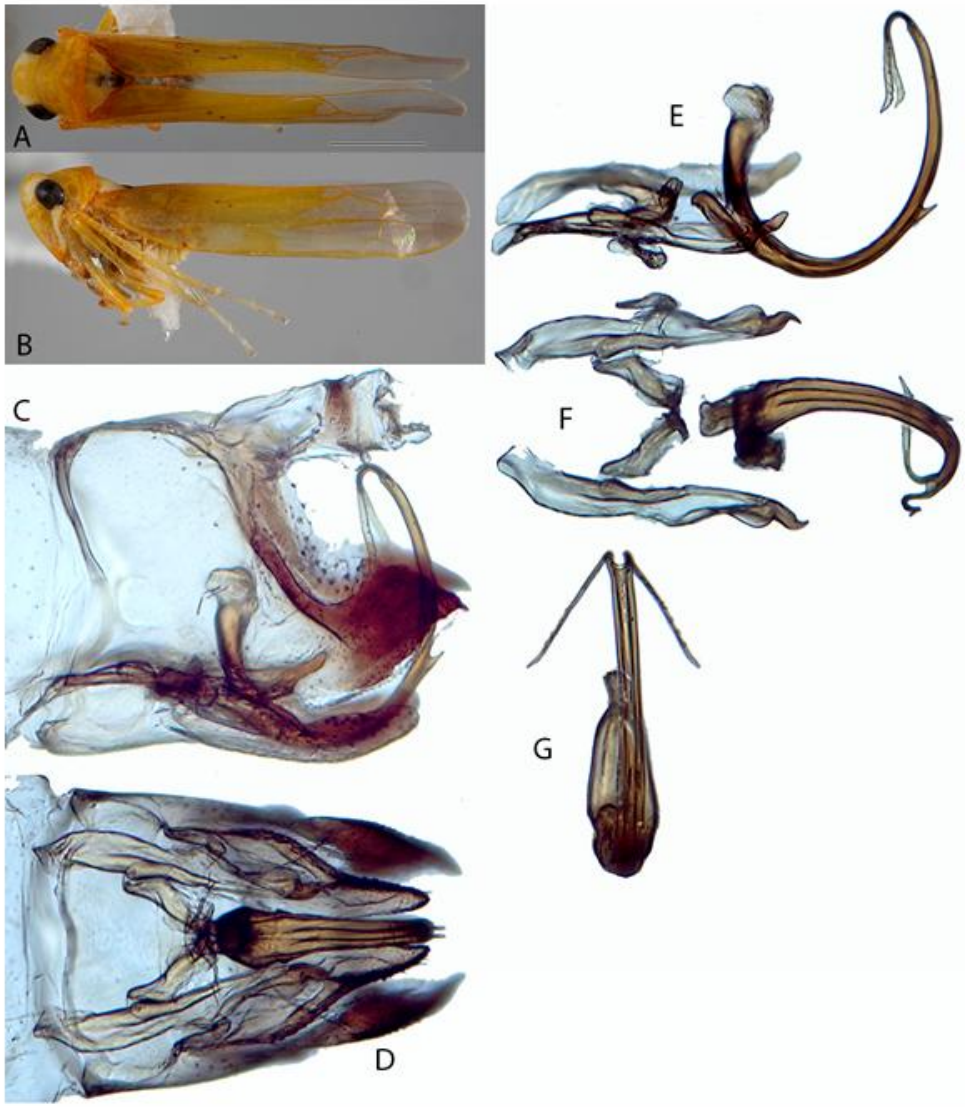


Fig 1. *Nedotepa curta* Dmitriev: A-B, adult female, dorsal and lateral habitus (scale = 1 mm); C-D, male genital capsule, lateral and ventral views; E-F, male genitalia (aedeagus, styles and connective), lateral and ventral views; G, aedeagus, posterior view.

This species may be recognized by the following combination of morphological features: length including wings 3.5-4.0 mm; body slender, elongate; color bright yellow with lateral margins of head and scutellum white and apex of scutellum dark brown; head slightly narrower than pronotum, ocelli absent, crown convex with anterior and posterior margins parallel in dorsal view, coronal suture absent; pronotum and mesonotum strongly convex in lateral view; forewing with inner apical cell oblique basally; male abdominal apodemes vestigial; pygofer with prominent dorsal membranous lobe near base, dorsal margin angulately emarginate, apex acutely angled and darkly sclerotized; subgenital plate triangular with setae strongly reduced in size; style linear with preapical lobe narrow; connective U-shaped, without stem or median anterior lobe; aedeagus slender, curved dorsad, with small posterior spine near midlength and pair of long apical processes extended ventrolaterad; female ovipositor very short with blades vestigial.

This species resembles some other tropical African members of the tribe Erythroneurini in body proportions, coloration, and wing venation. In the form of the male genitalia *Nedotepa* is perhaps most similar to the widespread African genera *Molopopterus* Jacobi and *Nsimbala* Dworakowska, but may be separated from the former by the lack of ocelli and lack of a median anterior lobe on the male connective, and from the latter by the lack of a coronal suture.

The strongly reduced ovipositor of the coconut palm feeding species is unique and presumably related to the unusual oviposition behavior of the females, which lay eggs on the surface of the leaves instead of inserting them into plant tissue. All other species of Erythroneurini for which females have been studied have the ovipositor well developed and similar to those of other Typhlocybinae.

A total of two hundred and ninety six specimens of *N. curta* were collected and two hundred sixteen (216/296) were positive for the CILY phytoplasma (73 %) by PCR with P1/P7

followed by G813/AwkaSR primers (Table 1); while the secA PCR yielded amplicons for 191/296 (64.5 %) specimens. Consensus sequences from representative *N. curta* specimens of each location were deposited in GenBank corresponding to amplicons of G813/AwkaSR (Fig 3) and fU5/rU3 (Fig 6). R16F2n/R2 consensus sequences were also deposited in GenBank and shown in Fig 6. Only two specimens of *Proutista fritillaris* (Derbidae) were collected in Yaokro, both tested positive for the CILY phytoplasma by PCR, their G813/AwkaSR sequences were deposited in GenBank (Ac. ns. KY11134, KY11135).

Both virtual and actual G813/AwkaSR (Fig. 2) and secA (Fig 4) RFLP profiles were identical for the CILY phytoplasma strains identified from coconut palms and *N. curta*, and those from the CSPWD controls from Ghana. Phylogenetic trees based on the G813/AwkaSR (Fig 3) and secA (Fig 5) were in agreement with the RFLP profiling by clustering CILY phytoplasma strains from the coconut palms and the *N. curta* specimens within the group 16SrXXII-B '*Ca.* P. palmicola' – related strains (Fig 4). No G813/AwkaSR or secA amplicons were obtained for any of the other Hemiptera specimens captured from CILY-affected coconut palms in Grand-Lahou.

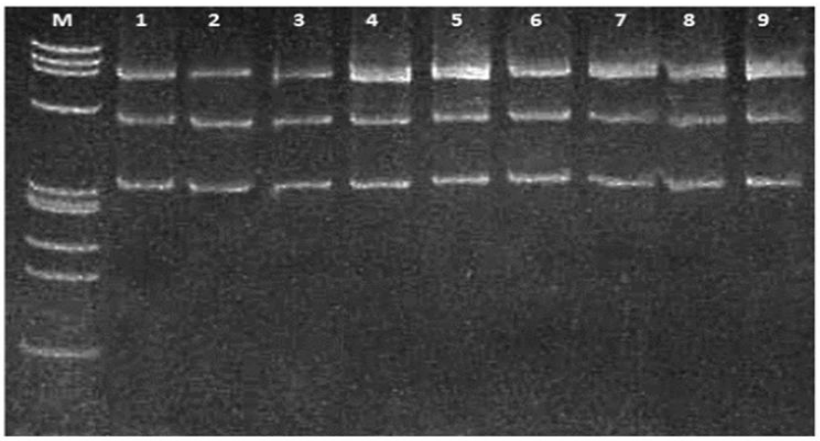
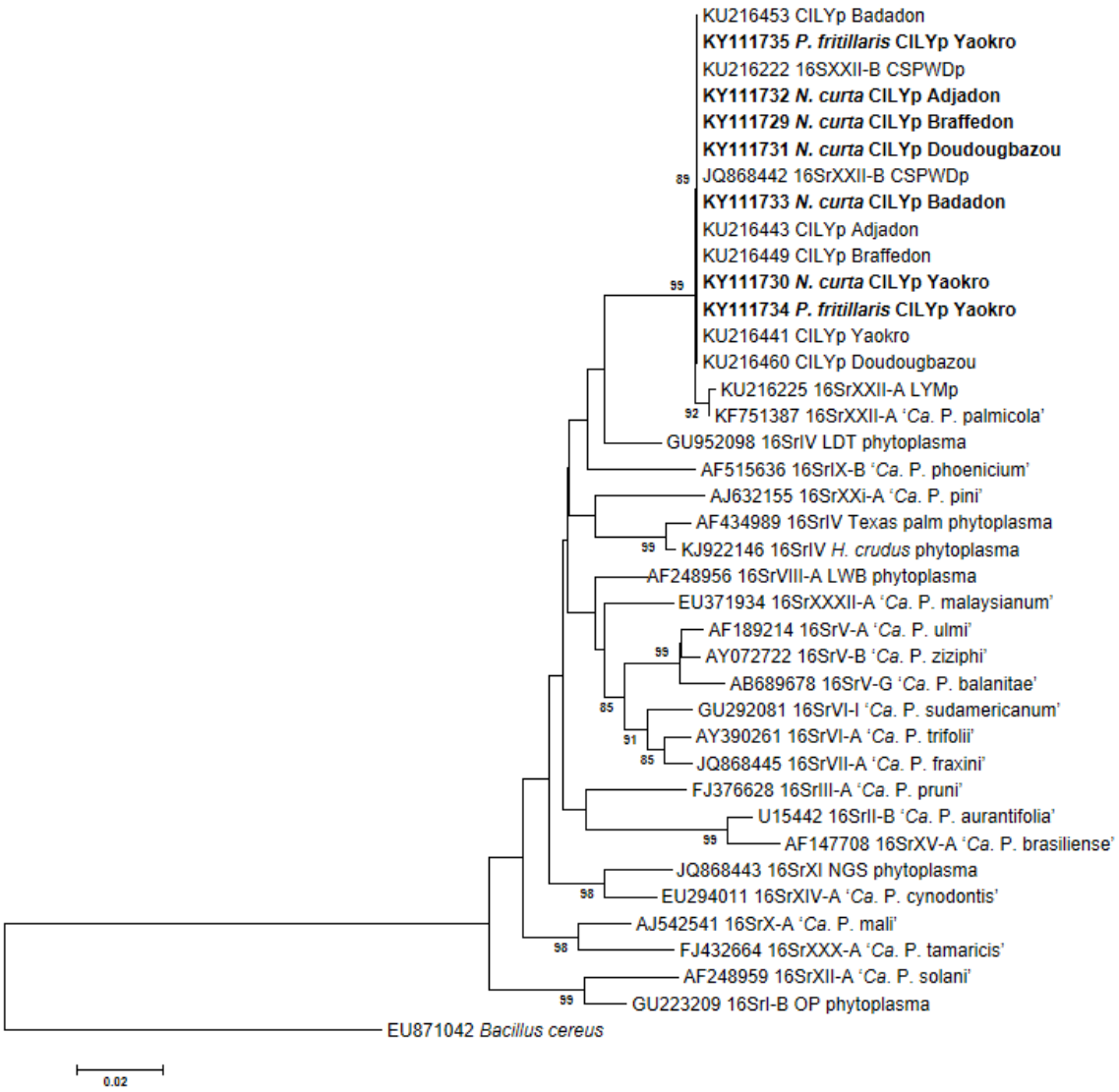


Fig 2. *Rsa*I RFLP patterns in polyacrylamide 6.7% gels of G813/AwkaSR amplicons from *N. curta* and coconut palms from Côte d'Ivoire and Ghana. Lanes 1, 2, 3, 4, 5: *N. curta* (Badadon, Braffedon, Adjadon, Yaokro, Doudougbazou); Lane 6: Ghana CSPWD phytoplasma (disease stage 2); Lanes 7, 8, 9: CILY phytoplasma (palms from Badadon, Braffedon, Adjadon). M: marker phiX174 *Hae*III digested with fragment sizes in base pairs from top to bottom of 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118, and 72.



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254 Fig 3. Phylogenetic tree based on the G813/AswkaSR sequences of the CILY phytoplasma
255 identified from *N. curta* and coconut palms from Grand-Lahou. CILYp: CILY phytoplasma;
256 CSPWDp: CSPWD phytoplasma; LYMp: LYM phytoplasma; LWB: Loofah Witches' Broom;
257 LDT: Lethal Decline Tanzania; *H. crudus*: *Haplaxius crudus*; NGS: Napier Grass Stunt; OP:
258 Onion Proliferation. '*Ca*. P.': '*Candidatus* Phytoplasma' species. Bootstrap values greater than
259 70 % are specified above the nodes. *B. cereus* was used as outgroup to root the tree.

260

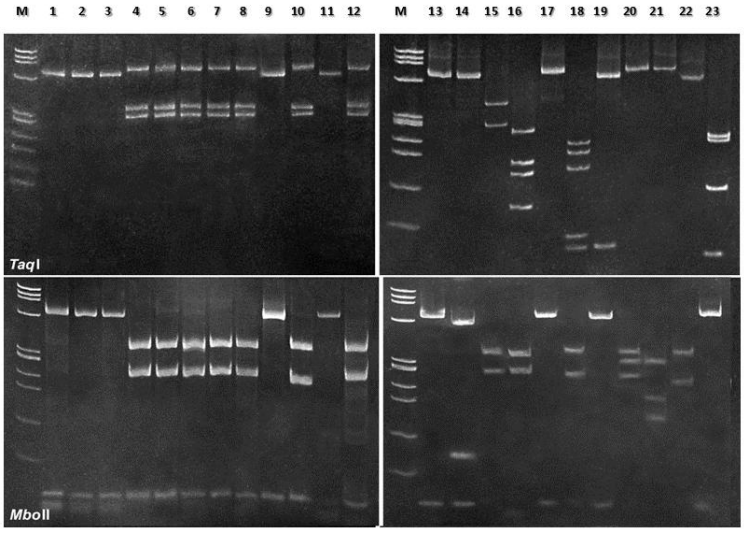


Fig 4. *Taq*I and *Mbo*II RFLP patterns in polyacrylamide 6.7% gels of secA amplicons of phytoplasmas detected in *N. curta,* coconut palms from Côte d'Ivoire and Ghana, and weeds *D. saxatalis* and *B. nitida*. Lanes 1: palm from Yaokro; 2, 3: *N. curta* (Badadon and Yaokro); 4, 5, 6: palms from Badadon, Braffedon and Yaokro; 7, 8: *N. curta* (Badadon and Braffedon); 9: *D. saxatalis* (Badadon); 10: *N. curta* (Yaokro); 11: *B. nitida* (Badadon); 12: Ghana CSPWD phytoplasma (palms with disease stage 2); 13: PRIVA, primula virescence aster yellows (16SrI-B); 14: A-AY, apricot aster yellows (16SrI-F); 15: FBP, faba bean phyllody (16SrII-C); 16: CX, X-disease of peach (16SrIII-A - '*Ca.* P. pruni'); 17: ULW, elm witches' broom (16SrV-A – '*Ca*. P. ulmi'); 18: CP1, clover proliferation (16SrVI-A – '*Ca*. P. trifolii'); 19: ASHY, ash yellows (16SrVII-A – '*Ca*. P. fraxini'); 20: ESFY, European stone fruit yellows (16SrX-B – '*Ca*. P. prunorum'); 21: PD, pear decline (16SrX-C – '*Ca*. P. pyri'); 22: AP, apple proliferation (16SrX-A – '*Ca*. P. mali'); 23: MOL, "Molière" disease (16SrXII-A – '*Ca*. P. solani'); M: marker phiX174 *Hae*III digested with fragment sizes in base pairs from top to bottom of 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118, and 72.

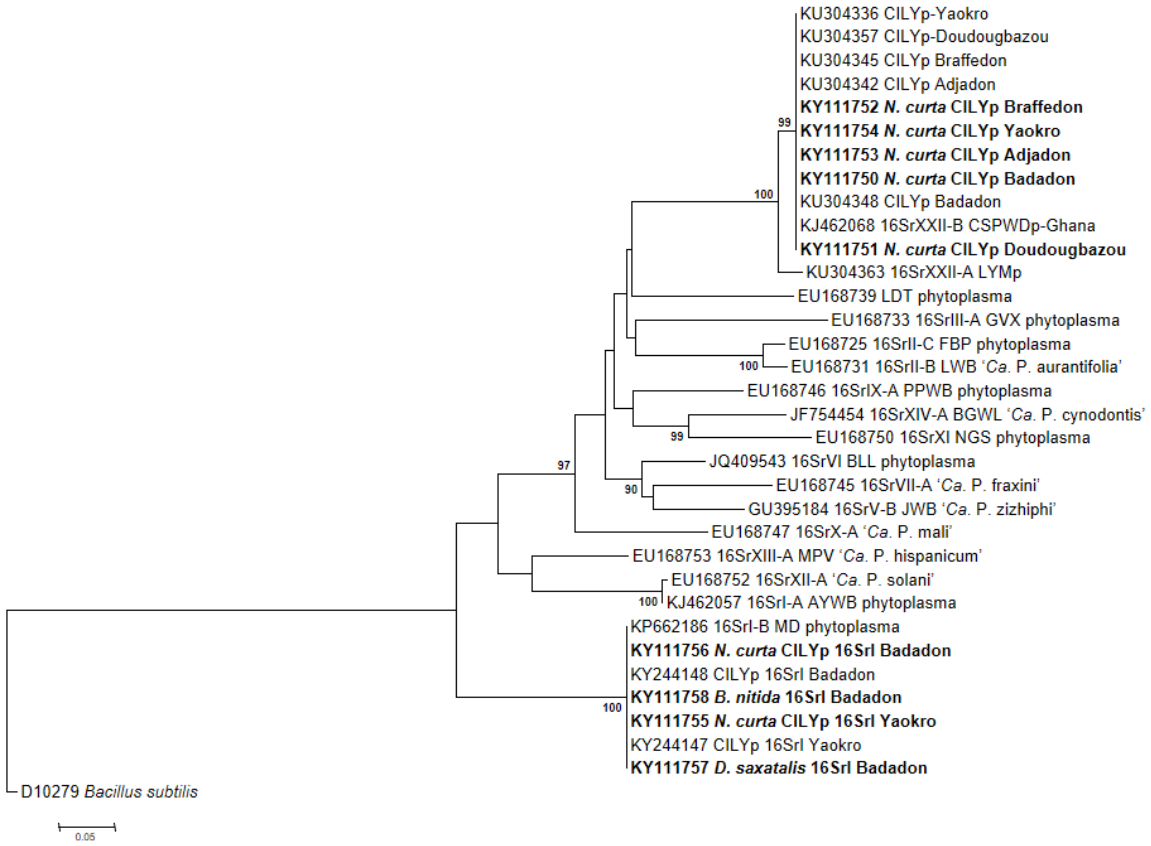
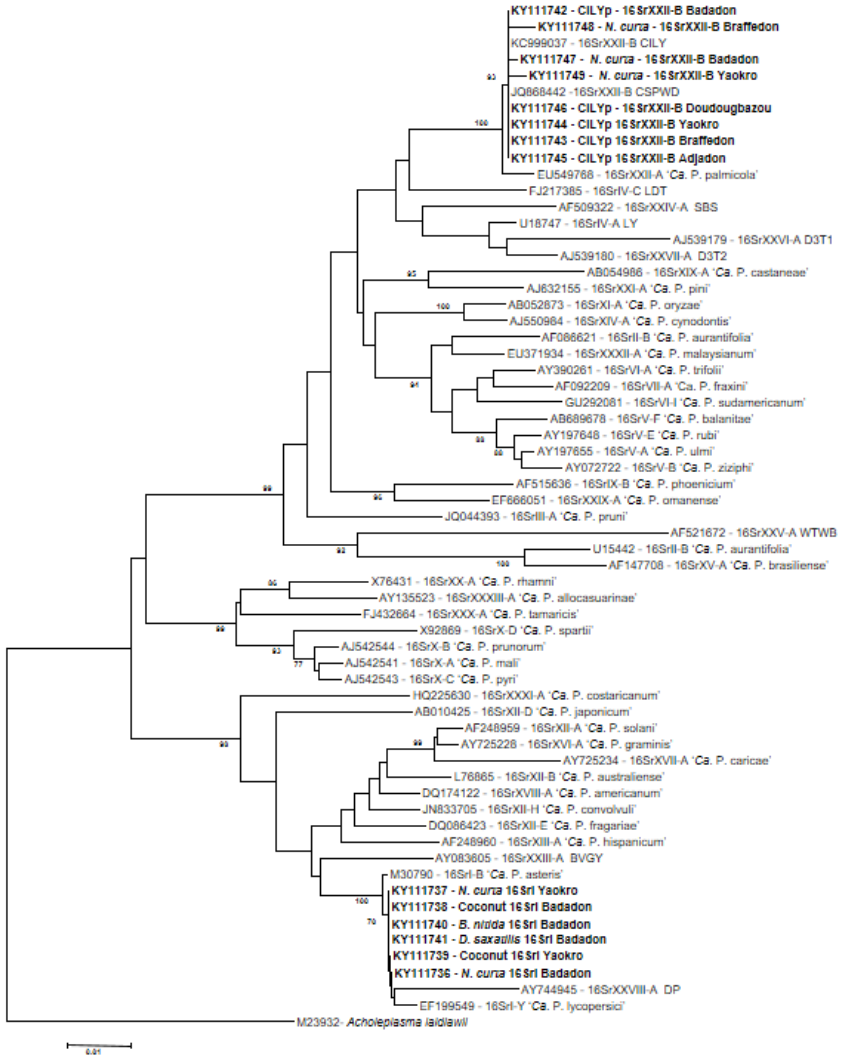


Fig 5. Phylogenetic tree based on the secA sequences of the CILY phytoplasma identified from *N. curta*, coconut palms and weeds in Grand-Lahou. CILYp: CILY phytoplasma. CSPWDp: CSPWD phytoplasma. LYMp: LYM phytoplasma. '*Ca*. P.': '*Candidatus* Phytoplasma' species. GVX: Green Valley X disease; LWB: '*Ca*. P. aurantifolia'; LDT: Lethal Decline Tanzania; FBP: Faba Bean Phyllody; NGS: Napier Grass Stunt; PPWB: Pigeon pea Witches' Broom; BLL: Brinjal Little Leaf; AYWB: Aster Yellows Witches' Broom; MD: Mulberry Dwarf. Bootstrap values greater than 70 % are specified above the nodes. *B. subtilis* was used as outgroup to root the tree.



293

294 Fig 6. Phylogenetic tree based on the 16S rRNA gene sequences of the CILY phytoplasma
295 identified from *N. curta*, coconut palms and weeds in Grand-Lahou. CILYp: CILY phytoplasma;
296 CSPWDp: CSPWD phytoplasma; SBS: Sorghum Bunchy Shoot phytoplasma; LDT: Lethal
297 Decline Tanzania; D3T1: Sugarcane phytoplasma D3T1; WTWB: Weeping Tea Witches' Broom
298 phytoplasma; BVGY: Buckland Valley Grapevine Yellows phytoplasma; DP: Derbid
299 phytoplasma; LY: Lethal yellows; LWB: '*Ca*. P. aurantifolia'; '*Ca*. P.': '*Candidatus*
300 Phytoplasma' species. *Acholeplasma laidlawii* was used as outgroup. Bootstrap values greater
301 than 70 % are specified above the nodes.

Out of a total of 54 palms sampled, 52 yielded G813/AwkaSR amplicons confirming the presence of the CILY phytoplasma, The fU5/rU3 and secA phytoplasma sequences from four coconut palms: three from Badadon (one positive and two negative for G813/AwkaSR PCR); and one from Yaokro (positive for G813/AwkaSR PCR); and two *N. curta* specimens (one from Badadon and one from Yaokro), were 99% similar to sequences from '*Ca*. P. asteris'-related strains. Interestingly, samples of *D. saxatalis* and *B. nitida* plants collected in Badadon yielded fU5/rU3 amplicons whose sequences were also 99% similar to those of the 16SrI ('*Ca*. P. asteris') group. The fU5/rU3 consensus sequences deposited in GenBank were from coconut palms (Ac. ns KY111738_Badadon; KY111739_Yaokro); *N. curta* (Ac. ns KY111736_Badadon; KY111737_Yaokro), and the weeds *D. saxatalis* and *B. nitida* (Ac. ns. KY111741 and KY111740, respectively). The secA consensus sequences were deposited in GenBank from coconut palms (Ac. ns. KY244147_Yaokro; KY244148_Badadon), *N. curta* (Ac. ns. KY111756_Badadon; KY111755_Yaokro), and the weeds *D. saxatalis* and *B. nitida* (Ac. ns. KY111757and KY111758, respectively). Both virtual and actual RFLP profiles of the secA sequences of the phytoplasma strains identified from Badadon and Yaokro were identical to those of the 16SrI phytoplasma strains (Fig 4). The grouping of these phytoplasmas within the 16SrI cluster was confirmed through phylogenetic analysis on both secA (Fig. 6) and 16S rRNA (Fig. 6) gene sequences.

4. Discussion

Nedotepa curta, was identified as the potential vector for the CILY phytoplasma in West Africa. This was the most abundant hemipteran collected and the only leafhopper to test positive for presence of the phytoplasma. Both the 16S rDNA and secA sequences of the CILY

phytoplasma from *N. curta* specimens were 99% identical to those of the CILY phytoplasma previously identified (Arocha Rosete et al., 2017) from the villages of Badadon, Braffedon, Adjadon, Doudougbazou, and Yaokro. Moreover, the CILY phytoplasma was detected in 216/296 (73 %) of the *N. curta* specimens captured from CILY-affected and CILY phytoplasma-infected coconut palms from all the villages surveyed. Also, the percentages of detection for the CILY phytoplasma from *N. curta* specimens were the highest for Badadon (the westernmost village) and Braffedon (the easternmost village) (Table 1) and previous studies reported Badadon and Braffedon as the most severely CILY-affected villages and those with the highest percentages of CILY phytoplasma detection (Arocha Rosete et al., 2017). These data suggest that two main separate foci occur in Badadon and Braffedon from which the disease may have been spread to other villages of the south littoral of Grand-Lahou.

Although our results strongly suggest that *N. curta* is a vector of the CILY phytoplasma, we caution that the vector capacity of this species still needs to be proven through transmission tests. *N. curta* was first observed in Ghana (Dmitriev, 2016), but a previous attempt to confirm it as a vector for the CSPWD phytoplasma, which is closely related to the CILY phytoplasma, failed (Philippe et al., 2009). During our study, leafhopper specimens collected from Badadon, Braffedon, Adjadon, Yaokro, Amanikro and Doudougbazou were confirmed, through morphological comparison, as the same species as the palm leafhopper previously reported but, until recently, unnamed (Dmitriev, 2016) from Ghana, and were widespread among the CILY-affected coconut farms of Grand-Lahou.

Nedotepa curta belongs to the highly diverse and globally distributed leafhopper subfamily Typhlocybinae (microleafhoppers). Very few species of Typhlocybinae have previously been shown to be competent vectors of phytoplasma diseases (Galeto et al., 2011). The ability of

typhlocybines to transmit phloem-borne pathogens such as phytoplasmas is thought to be limited, in part, by the apparent preference of most studied species for feeding on leaf parenchyma cell contents (mesophyll) rather than vascular fluids, but some species have been shown to feed, at least occasionally, on xylem and phloem sap (Saguez et al., 2015).

To date, most documented phytoplasma vectors belonging to the Typhlocybinae are members of the tribe Empoascini (Galetto et al., 2011). These include *Empoasca papayae*, proven as vector of the phytoplasma associated with Bunchy Top Symptom of papaya Acosta Perez et al., 2010). Other reports of typhlocybine vectors of phytoplasma diseases include *E. decedens* as a vector of European stone fruit yellows in Italy (Pastore et al., 2004) and potential vector in Lebanon for almond witches' broom (Abou-Jawdah *et al.*,2014); *E. decipiens* in Saudi Arabia for the lime decline disease (Alhudaib et al., 2007), alfalfa witches' broom (Al-Saleh et al., 2014), Ranunculus virescence in Italy (Parrella et al., 2005) and almond witches' broom in Lebanon (Dakhil et al., 2011); and *E. kraemeri* for phytoplasmas affecting citrus species (*C. sinensis* and *C. limon*), coffee (*Coffea arabica*), periwinkle (*Catharanthus roseus*), and tabebuia (*Tabebuia heterophylla*) in Puerto Rico. *Empoasca fabae* and *Erythroneura ziczac* Walsh have been found as carriers of '*Ca*. P. asteris' in Canada (Olivier et al., 2014). Only a single species of the typhlocybine tribe Erythroneurini (which includes. *N. curta*) has, so far, been shown to be capable of infecting plants with a phytoplasma disease in the laboratory - *Tautoneura mori* (Matsumura) - for the mulberry dwarf phytoplasma (Jiang et al., 2005).

Diostrombus and *Proutista* are reported to be common derbids in West Africa (Wilson 1987) and species of these genera have been reported as the potential vectors of LD in Tanzania Mpunami et al., 2000), LY in Mozambique (Bila, 2016), and Kerala Wilt disease in India (Edwin and Mohankumar, 2007), although their transmission capacity has not been yet proven. In our

study only 20 derbid specimens were collected from three villages and, among these, only two specimens of *P. mirabilis* yielded nested PCR amplifications positive for the CILY phytoplasma, and confirmed by sequencing of the G813/AwkaSR PCR product. *P. mirabilis* specimens captured were limited to only one (Yaokro) out of the six villages surveyed, so they were very poorly represented among the hemipteran fauna of the region and seem unlikely to play a major role in the spread of CILY.

The fact that the 16S rDNA sequences of the 16SrI phytoplasma detected in two specimens of *N. curta* from Badadon were 99% identical to those from four CILY-affected coconut palms in Badadon and Yaokro, suggests that *N. curta* may play a role in transmitting both 16SrXXII-B and 16SrI phytoplasmas across the CILY-affected coconut farms. Four out of 54 coconut palms were infected with the 16SrI phytoplasma, among which two of them (one from Badadon and one from Yaokro) were co-infected with the 16SrXXII-B phytoplasma. This indicates that natural mixed phytoplasma infection of the 16SrXXII-B and 16SrI phytoplasmas may occur in coconut groves in Grand-Lahou. A larger sample and further characterization studies would help elucidatie the epidemiological factors related to the occurrence of group 16SrI in coconut farms of Grand-Lahou and the *N. curta* populations.

Mixed phytoplasma infections naturally occur in coconut and other palm species. Bila et al., (2015) identified LY-affected coconut palms in Mozambique co-infected with '*Ca*. P. palmicola' and '*Ca*. P. pini'-related strains. In Malaysia, the popular evergreen foxtail palm *Wodyetia bifurcata* was reported as a host for two different phytoplasmas, 16SrXIV, ('*Ca.* P. cynodontis') group and '*Ca.* P. asteris' (Naderali et al., 2013). A 16SrI phytoplasma was associated with the Al-Wijam disease of date palm (*Phoenix dactylifera*) in Saudi Arabia (Akhudaib et al., 2007), and the lethal wilt of oil palm (*Elaeis guineensis* Jacq.) in Colombia (Alvarez et al., 2014). The

16SrXI (‘*Ca*. P. oryzae’) group (Manimekalai et al., 2010) and ‘*Ca.* P. asteris’ (Naderali et al., 2013) have been indistinctly associated with diseases of arecanut (*Areca catechu*n L.) in India. Therefore, the fact that the group 16SrI phytoplasma was identified from *N. curta* specimens captured from coconut farms affected by CILY in Grand-Lahou is highly significant since this is the phtoplasma group with the widest plant host range and most complex epidemiology (Weintraub and Beanland, 2006).

Epidemiological conditions in Badadon and Yaokro associated with the presence and spread of the 16SrI phytoplasma by the *N. curta* specimens are not clear and require further investigation. On the other hand, since *D. saxatilis* and *B. nitida* harbor a 16SrI phytoplasma strain that potentially affects few other coconut palms in Badadon and Yaokro, these two new alternative plant hosts of the 16SrI phytoplasma may also hasten the spread of CILY disease or worsen its severity. Although PCR detection of the phytoplasma in an insect does not prove the insect's vector capacity unless transmission trials are performed (Bosco and D’Amelio, 2010), our results strongly support the possible role of *N. curta* as vector for the CILY phytoplasma. Transmission cage trials are currently ongoing with *N. curta* populations in pilot farms of Grand-Lahou under different disease pressure levels to prove *N. curta*’s vector capacity and study aspects of its biology.

Conclusions

Although further study is needed to prove the role of *N. curta* as vector of CILY, this work provides strong evidence indicating *N. curta* as a potential vector involved in the spread of CILY throughout CILY-affected coconut farms in Grand-Lahou. Moreover, CILY phytoplasma-infected coconut palms may be co-infected with 16SrI phytoplasma strains, suggesting that, management and control of the coconut lethal yellowing disease in Grand-Lahou may be complicated by a more complex epidemiology.

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