

Promising primers for detection of phytoplasma causing coconut lethal yellowing disease in Mozambique

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Abstract Coconut is one of the main cash crop in Mozambique, which occupied the second position after Tanzania in coconut production in Africa. Coconut production was drastically affected by the occurrence of a devastating Coconut Lethal Yellowing Disease (CLYD) epidemics, which reduced significantly the coconut yields. CLYD symptoms triggered upon phytoplasma infection, i.e. premature fruit dropping, necrosis of the inflorescence and progressive yellowing of the leaves, are used to identify infected trees. However, the diagnostic based uniquely on symptoms is not conclusive to confirm infection, and needs to be confirmed by molecular methods. In this study, three previously described reference primers for phytoplasma detection were tested on infected samples collected in Mozambique. Since those primers gave incongruent results, 20 new primer pairs targeting the 16S rDNA region, were newly designed. To evaluate their performance in detecting coconut infecting phytoplasma, 108 samples were tested and selected positive samples

confirmed by sequencing. Our results showed a new primer pair more accurate and reliable compared to the reference pairs for CLYD detection in Mozambique. Moreover, the new primer pair was able to detect a new putative phytoplasma variant in Mozambique. Therefore, this study makes an important contribution to CLYD phytoplasma molecular diagnostics and its causative agent, giving insights that may be applied to the study of CLYD phytoplasma infection systems.

Keywords CLYD · Molecular diagnostic · Phytoplasma · Mozambique

Introduction

Coconut Lethal Yellowing Disease (CLYD) is a highly destructive disease associated with phytoplasmas (Weintraub and Beanland 2006). To date, the vector of CLYD phytoplasma is not yet demonstrated, however a recent study conducted in Mozambique reported the *Diostrombus mkurangai*, of the Derbidae family, as carrier of phytoplasma in CLYD affected areas, suggesting the possible role of the insect in the maintenance and possibly in the transmission of phytoplasma to plants (Bila et al. 2017). Beside possible transmission by means of vectors, vegetative transmission of infected plants is also significant in phytoplasma disease onset (Lee et al. 2000; Bertaccini et al. 2014). The infection can appear around at least 2 weeks after inoculation (Dickinson 2010; Bonnot et al. 2010), the symptoms

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develops between 3 to 6 months and are characterized by premature fruit dropping, necrosis of the inflorescence and progressive yellowing of the leaves starting from the basal ones (Bonnot et al. 2010).

Phytoplasmas are wall-less Gram-positive eubacteria belonging to the Mollicutes class with a low G + C content, related to *Bacillus* and *Clostridium* spp., (Gundersen et al. 1994; Bai et al. 2006; Abdollahi et al. 2012). Currently, phytoplasmas are classified as a monophyletic group in the *Acholeplasmatales* order (Bai et al. 2006; Contaldo et al. 2012). These microorganisms cannot be grown artificially, suggesting that, differently from mycoplasma, their metabolism is completely dependent on the hosts (Lee et al. 2000; Kube et al. 2008). Moreover, the analysis of the 16S rDNA region revealed great genetic diversity within the phytoplasmas, often reflecting geographical distribution of the strain (Lee et al. 2000; Llauger et al. 2002; Husain et al. 2002). These evidences led to define a new genus denominated “*Candidatus* Phytoplasma”, which includes the CLYD infecting phytoplasma (Bai et al. 2006).

In Mozambique, CLYD was initially reported in 1958 in Cabo Delgado province; later in 1972 it spread to Zambezia province, and, up to the 2000, the disease was reported with low intensity in all affected provinces (Nampula, Zambezia and Cabo Delgado) (Eden-Green 2006). At the beginning of the year 2000, CLYD reached epidemic proportions in Mozambique, with the majority of CLYD cases concentrated in Provinces located north of the Zambeze River, where a mortality rate of 100% was recorded in coconut plantations in two districts (Monjana et al. 2010; Bonnot et al. 2010). Since then, CLYD continued affecting drastically the coconut production in Mozambique with significant reduction in crop production, thus representing a major economic drawback for a country that, until 2006, was the second major coconut producer in Africa after Tanzania (Eden-Green 2006; Monjana et al. 2010).

Coconut Lethal Yellowing Disease diagnosis in Mozambique was based uniquely on symptomatology inspection, which is a somewhat complex task because the symptoms differ across varieties of coconut palms, and other biotic (*Fusarium* wilt) and abiotic (potassium deficiency) stressing factors induce similar symptoms, hindering an accurate and conclusive diagnosis (Monjana et al. 2010).

To overcome this scenario, a CLYD PCR based diagnostic was carried out using universal 16S rDNA primers (P1/P7) (Deng and Hiruki 1991), and two other

primer sets to detect the West Africa CLYD group (‘*Ca* P. palmicola’-related strains, 16SrXXII-B, detected in Ghana, and ‘*Ca*. P. palmicola’, 16SrXXII-A, detected in Nigeria and Mozambique) (Ghana813/AKSR), and the East Africa group (Tanzania) (RhodeF/R) (Rohde et al. 1993; Tymon et al. 1998). However, after 2012, the three sets of primers began to give unreliable results for CLYD detection in Mozambican samples, suggesting that Mozambique strains may have an inadvertently genetic variability (Harrison et al. 2014).

Thus, the present study aimed at improving the PCR-based detection by designing new primers able to target efficiently and reproducibly a higher number of CLYD infections, including possible new emerging CLYD phytoplasma variants in Mozambique.

Materials and methods

Sample collection

A total of 108 coconut samples were collected in 4 coconut production provinces of Mozambique: 74 in Zambezia, a CLYD endemic area, 7 in Nampula, 8 in Cabo Delgado, where sporadic presence of CLYD was reported, and 19 samples in Inhambane, where cases of putative CLYD were recently reported.

Coconut trees sampled included those exhibiting symptoms such as necrosis of inflorescence, premature fruit drop and yellowing of leaves. Samples were marked as *symptomatic* if showing evident CLYD symptoms, *asymptomatic* if showing no evidence of CLYD symptoms but collected in areas nearby infected plants, and *supposed negative* if collected far from CLYD-affected areas.

Five grams of saw dust were collected from the trunk of the coconut, the second largest area of phytoplasma DNA concentration, and stored in 50 mL tubes containing dried silica at room temperature (Christensen et al. 2004; Oropeza et al. 2011).

DNA extraction

The DNA extraction was made according to Doyle and Doyle (1990). The extracted DNA was quantified by spectrophotometry using Nano Drop™ 1000 (*Thermo Scientific*) and conserved at -20°C until used.

Selection of PCR controls

Five DNA samples from Zambezia and 10 from Nampula were tested by PCR using the universal primers (P1/P7), East (Rhode F/R) and West (G813/ASKR) Africa primer pairs. The positive amplicons were confirmed by direct sequencing and compared by BLASTn in NCBI (<https://www.ncbi.nlm.nih.gov/>). Samples giving sequences identical to CLYD phytoplasma strains by all three primer pairs were used as positive controls. Those samples giving sequences identical to other bacteria, were used as negative control.

Selection of candidate primers and PCR optimization

In order to find a suitable primer pair able to detect a wider range of CLYD *Ca. P.* strains, 20 new primer pairs were designed and named Phyto1F/R to Phyto20F/R (Table 1).

All primers were designed based on phytoplasma rDNA (5S, 16S and 23S) sequences as template using Geneious v.8.1.7 (Kearse et al. 2012), based on sequences of Coconut lethal yellowing phytoplasma isolates MZ-MF, Tanz08–01, and Awka wilt phytoplasma (Accession no. KJ528962, GU952107, Y14175, respectively).

Previously selected positive and negative (2 ng) controls were added to 15 µl of Qiagen PCR Core kit (Qiagen, Germantown, USA) containing 1X buffer, 0.5X of Qsolution, 0.3 mM of dNTP's, 0.3 µM of primers e 0.2 U of Taq Polymerase. The PCR reactions were performed in the Eppendorf thermocycler. After some tuning experiments, optimal parameters for Phyto1 to Phyto16 primer pairs were set: 95 °C for 3 min, followed by 94 °C for 30 s, 59.3 °C for 30 s, 72 °C for 55 s, for 40 cycles and 72 °C for 10 min. PCR conditions for Phyto 17, 18, 19 and 20 primer pairs varied only for the annealing temperature, 55 °C for the first three pairs and 57.5 °C for the latter. All the amplicons were visualized on the TFX-20.M ultraviolet UV transluminator 230 V 50 Hz.

Evaluation of the new candidate primers

Primers were evaluated against 30 DNA samples (Set 1) selected from a screening study of CLYD phytoplasma in endemic Mozambique regions, previously tested by PCR and known to be positive for G813/ASKR and

RohdeF/R primers in former experiments. Those primers that were able to detect the presence of phytoplasma in all 30 Set 1 samples were selected to be tested against 33 *symptomatic* samples (Set 2) showing clear CLYD symptoms. Furthermore, 45 additional DNA samples, of which 38 as *asymptomatic* and 7 *supposed negative* were tested.

Sequence analysis

To confirm if the amplicons were related to CLYD phytoplasma, a BLASTn search of 17 amplicons obtained with the best performing and newly designed primers was carried out against the NCBI database (<https://www.ncbi.nlm.nih.gov/>). Previously, raw sequences were cleaned and trimmed to eliminate poor quality reads with the CLC Genomic Workbench version 7.0.2 (<https://www.qiagenbioinformatics.com>) and aligned to CLYD phytoplasma reference sequences present in GenBank using ClustalW 2.0 (Larkin et al. 2007).

Results

Selection of PCR controls

Fifteen phytoplasma DNA samples were tested by PCR using primer pairs (P1/P7, G813/ASKR and RohdeF/R), with the following outcome: 8 samples were positive when amplified with the P1/P7 pair, 4 samples with the G813/ASKR pair and 4 samples with the RohdeF/R pair.

Out of the 8 amplicons obtained by the P1/P7 primer pair, 4 showed 98–99% sequence identity to CLYD phytoplasma strain M-182 (Accession no. EU549768), while 2 had 100% identity to CLYD phytoplasma isolate Tanz08–01, from Tanzania (Accession no. GU952107). Two (2) samples were not related to *Ca. P.* and showed 98–99% sequence identity to *Bacillus megaterium* QM B1551 (Accession no. CP001983). Seven remaining amplicons were too short and showed no sequence identity with any sequence present in NCBI.

Two amplicon sequences obtained by the RhodeF/R primer pair showed 100% identity to the phytoplasma isolate Tanz08–01 (Accession no. GU952107), while 2 samples showed no sequence homology to any sequence in NCBI. Four amplicon sequences obtained by the G813/ASKR primer pair had 98–99% identity

Table 1 Candidate primers descriptions

Primer region	Primer number	Sequence (5' -> 3')	Product length
16S rDNA	1	CGCTGGCGGCGTGCCTAATA CCCCACCATTACGTGCTGGCAA	1098
16S rDNA	2	AAGCGTGGGGAGCAAACAGG TGGTTTGACGGGCGGTGTGT	639
16S–23S rDNA	3	GCACGAAAAGCGTGGGGAGCA CCCCACCTTCCGGTAGGGAT	763
16S–23S rDNA	4	GCAACGCCGCGTGAACGATG CCCACCATTACGTGCTGGCAA	735
16S rDNA	5	GTGCCAGCAGCCGCGTAAT CACGACACGAGCTGACGACAA	554
16S–23S rDNA	6	CGAGCGCAACCCCTGTGCTT TCGTGGTTTGACGGGCGGTG	316
16S–23S rDNA	7	AACGAGCGCAACCCCTGTCG CGTGGTTTGACGGGCGGTGT	317
16S–23S rDNA	8	AACGCCGCGTGAACGACGAA AACGACAGGGGTTGCGCTCG	711
16S rDNA	9	GCACGAAAAGCGTGGGGAGCA CGACAGGGGTTGCGCTCGTT	350
16S rDNA	10	ACGCTGGCGGCGTGCCTAAT CGACAGGGGTTGCGCTCGTT	1071
16S–23S + 5S rDNA	11	ATTAACGCTGGCGGCGTGTCT AACGACAGGGGTTGCGCTCG	1077
16S–23S + 5S rDNA	12	TTAACGCTGGCGGCGTGTCT TCGTGGTTTGACGGGCGGTG	1369
16S–23S rDNA	13	GATTAACGCTGGCGGCGTGC CGTGGTTTGACGGGCGGTGT	1371
16S–23S rDNA	14	CGAGCAACGCCGCGTGAATG GACCCAGCAAGCCGCCTACG	348
16S–23S rDNA	15	CGTAGGCGGCTTGCTGGGTC TCGTGGTTTGACGGGCGGTG	683
16S–23S rDNA	16	GCACGAAAAGCGTGGGGAGCA CGTGGTTTGACGGGCGGTGT	646
16S–23S rDNA	17	GCCACATTGGGACTGAGACA ACCGAGTTTAACCCCGACAC	533
16S–23S rDNA	18	ACAGCCACATTGGGACTGAG GTTTACGGCGTGGACTACCA	506
16S–23S rDNA	19	CTGACGCTGATGCACGAAAG TTTGCCGAAAACTCGCGTTT	524
16S–23S rDNA	20	ACGCTGGCGGCGTGCCTAAT AGGAGGTGATCCATCCCCA	1494

to CLYD phytoplasma strain M-182 (Accession no. EU549768).

Based on these results, 4 samples (positive to both P1/P7 and G813/AKSR) were chosen as positive controls for CLYD West Africa group (*Ca* P. Palmicola, 16SrXXII-A), 2 samples (positive to both P1/P7 and RhodeF/R) were chosen as positive controls for CLYD East Africa group and two samples were chosen as negative controls for further PCR experiments.

Selection of candidate primer pair

Eighteen samples known to be positive for the G813/AKSR primer pair and 12 samples known to be positive to the RhodeF/R primer pair in former experiments (Set 1, $n = 30$) were tested with the newly designed 20 candidate Phyto primer pairs. According to the results, 2 primer pairs (Phyto3F/R and Phyto14F/R) were able to amplify all 30 positive samples (Table 2).

Table 2 Evaluation of the ability of 2 candidate primers, Phyto3F/R and Phyto14F/R, to detect positive samples compared to RhodeF/R and G813/AKSR. Sensitivity was calculated as the number of positive detected of Set 1 and Set 2 over the total of true positives ($n = 63$)

	No. of samples	No. of positive			
		Phyto3 F/R	Phyto14 F/R	Rhode F/R	G813/AKSR
Set 1	30	30	30	18	12
Set 2	33	14	33	4	8
Asymptomatic	38	7	20	0	8
Supposed negative	7	0	0	0	0
Sensitivity (%)		70	100	35	32

To evaluate detection efficiency, the Phyto3F/R and Phyto14F/R primer pairs were used to amplify 33 DNA samples obtained from *symptomatic* plants, showing clear CLYD symptoms, which were never tested before by PCR (Set 2) (Table 2).

Among Set 2 samples, the Phyto3F/R primer pair detected as positive 14 out of 33 (42.4%) while the Phyto14F/R primer pair detected as positive 33 out of 33 (100%) (Table 2).

Furthermore, 45 samples, of which 38 were considered from a clinical evaluation as *asymptomatic* and 7 as *supposed negative* were also tested. The Phyto3F/R primer pair detected as positive 7 out of 38 (18.4%) of the *asymptomatic*, and 0 out of 7 of the negative samples while the Phyto 14F/R detected as positive 20 out of 38 (52.6%) of the *asymptomatic* and 0 out of 7 of the negative samples (Table 2).

Results obtained with Phyto3F/R and Phyto14F/R primer pair against all samples were compared with those obtained with G813/AKSR and RhodeF/R primer pairs (Table 2). The sensitivity (number of positive detected / number of true positive) of the primers over a total of 63 true positive (30 of Set 1 + 33 of Set 2) was 32%, 35%, 70 and 100% for G813/AKSR, RhodeF/R, Phyto3F/R and Phyto14F/R primer pairs, respectively (Table 2).

Therefore, the experiments strongly supported that Phyto14F/R (Forward 5'-CGAGCAACGCCGCG TGAATG-3', Reverse 5'-GACCCAGCAAGCCG CCTACG-3', amplicon size 350 bp) was the best candidate to be used for the detection of CLYD phytoplasma in PCR experiments with samples from Mozambique, with a more accurate detection efficiency compared to G813/AKSR and RhodeF/R primer pairs.

To confirm the efficiency of the Phyto14F/R primer, we randomly selected 17 amplicons for sequencing, among which 10 were positive only with the Phyto14F/R pair,

and the other 7 samples were also positive for Ghana813/ASKR ($n = 4$) and RhodeF/R ($n = 3$) (Table 3). The BLASTn analysis of the 17 sequences showed that all 17 amplicons were 98–100% homologous to CLYD phytoplasma (Accession no. EF186805, EU549768 and GU952107) (Table 3). Eleven samples were homologous (93–98% identity for 93–100% sequence length) to the East Africa group phytoplasma (Accession no. AC GU9252107.1), while 6 contained DNA showing 99% sequence identity for 94–100% of sequence length to a phytoplasma strain isolated in Mozambique in 2014 (Accession no. AC KJ528984.1) (Bila et al. 2014). The latter were also positive with the G813/AKSR primer pair, generally detecting the West Africa group 'Ca Phytoplasma palmicola' 16SrXXII and related strains), and the RhodeF/R pair (1) specific to East Africa phytoplasma (Table 3).

The relative position of the three primer pairs was investigated. The resulting map showed that the Phyto14 pair annealed in a region of the phytoplasma 16S rDNA which is more conserved compared to the G813/AKSR and RhodeF/R (Fig. 1). The alignment of 8 representative strains, including representative strains from Mozambique, West and East Africa as well as Caribbean strains, showed a significant polymorphism within the annealing regions of the G813/AKSR and RhodeF/R pairs compared to a single nucleotide mismatch observed in the annealing region of the Phyto14R primer (Fig. 1).

Discussion

High genome variability in phytoplasma was described by Razin (2007), as resulting of repeated regions grouped in potential mobile units (PMUs) presenting

Table 3 Origin and eventual concurrent positivity by G813/AKSR and RohdeF/R amplification of 17 amplicons generated by Phyto14 primer pair. The Accession number of each sequence is indicated. Percent of sequence identity to published

reference sequences are indicated with corresponding Accession numbers: KJ528962, a CLYD sample from Mozambique and GU952107, a CLYD sample from Tanzania (East Africa group)

No.	Province	G813/AKSR	RohdeF/R	Sequence accession no.	% of identity	% of coverage	Accession no.
3	Cabo Delgado	-	+	Poor quality			
11	Quelimane	+	-	MF782645	99	95	KJ528962
14	Quelimane	-	-	MF782644	99	94	KJ528962
15	Quelimane	+	-	MF782643	99	100	KJ528962
16	Quelimane	+	-	MF782642	99	95	KJ528962
17	Inhambane	+	+	MF782641	99	94	KJ528962
19	Quelimane	-	+	Poor quality			
20	Quelimane	-	-	MF782640	93	99	GU952107
21	Quelimane	-	-	MF782639	98	99	GU952107
23	Quelimane	-	-	MF782647	98	99	GU952107
24	Quelimane	-	-	MF782646	94	98	GU952107
25	Quelimane	-	-	MF782648	93	100	GU952107
26	Quelimane	-	+	MF782652	97	98	GU952107
27	Quelimane	-	-	Poor quality			
28	Quelimane	-	-	MF782651	95	97	GU952107
30	Quelimane	-	-	MF782650	97	97	GU952107
31	Quelimane	-	-	MF782649	97	98	GU952107

numerous transposases and insertion sequences. Evidence of high variability of 16S rDNA regions in

phytoplasma was also evidenced by RFLP analyses (Harrison et al. 2014). It is not surprising then, that the

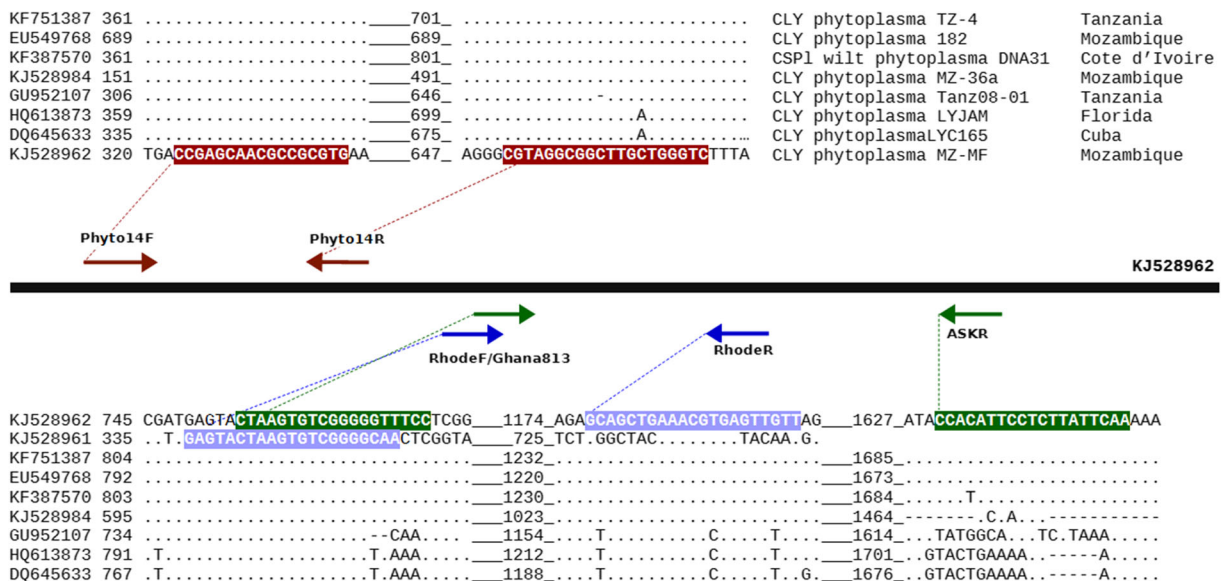


Fig. 1 Diagram showing the relative position of the Phyto14F/R, Ghana813/ASKR and RhodeF/R primer pairs on the 16S rDNA of a CLYD phytoplasma reference strain (Accession no. KJ528962).

Alignment of the annealing regions of 9 representative CLYD phytoplasma published strains is shown

high genetic variability of these organisms, often reflected in their geographic distribution, may hinder the efficacy of a universal diagnostic method based on the amplification of conserved regions, such as a 16S rDNA-based PCR.

Our diagnostic approach based on the PCR was initially designed to use 16S universal primers P1/P7, as well as the other two previously described primer pairs G813/AKSR and RhodeF/R. This procedure worked well until 2012, when clear *symptomatic* palms started to result positive only to universal primers and not to the regional primers, suggesting a possible genetic diversity of the Mozambican phytoplasma strains under study. Around the same time, Harrison reported that the G813/AKSR primer pair was not able to detect phytoplasma in infected palms and reported the existence of a new variant circulating in Mozambique (Harrison et al. 2014). Recently, Bila et al. (2015) confirmed the presence of two strains of phytoplasma in Mozambique ('*Ca. P. palmicola*' related strains 16SrXXII-B and '*Ca. P. pini*' related strains), stressing the difficulties of an effective diagnostic by PCR due to the high grade of variability in 16S rRNA regions with available primers.

Therefore, in the present study we aimed to develop a diagnostic molecular method able to detect phytoplasma strains infecting coconuts in Mozambique. We designed a very effective primer pair, Phyto14F/R, selected for its consistency to detect phytoplasma strains, not only strains of East and West Africa groups, but also those previously gone undetected in Mozambique. The primer pair shows a significant increase in detection sensitivity compared to the G813/AKSR and RhodeF/R primer pairs.

The use of Phyto14F/R needs to be extended to different countries affected by CLYD to evaluate its diagnostic capability within CLYD control strategies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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