

## Molecular Characterization of a Phytoplasma Associated with Coconut Yellow Decline (CYD) in Malaysia

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**Abstract Problem statement:** Molecular methods have been used to detect phytoplasma in Malayan Red Dwarf (MRD) ecotype of coconut for the first time in Malaysia. Detect phytoplasma from coconut showing yellowing symptoms by nested PCR and Classify phytoplasma associated with disease of coconut palms, based on analysis of 16S rRNA gene operon sequences and virtual RFLP. **Approach:** Twenty MRD symptomatic palms were tested. Leaf spear, inflorescence and wood shavings from trunks of coconut palms showing yellowing symptoms were harvested from Serdang located in Selangor state. PCR assays and sequence analysis were carried out. **Results:** Nested PCR with primer pairs R16F2n/R16R2 and fU5/rU3 resulted in amplification of products of approximately 1.2 kb and 890 bp respectively, from 8 out of 20 MRD symptomatic palms tested. Sequence analysis of the 16S rDNA PCR products determined that the phytoplasma strain associated with Coconut Yellow Decline (CYD) in MRD ecotype belongs to the 'Candidatus Phytoplasma cynodontis' (16SrXIV) group of phytoplasmas. In addition, nested R16F2n/R16R2 PCR products from 6 spear leaves and 2 inflorescences from MRD palms showed high sequence similarity to the 16S rRNA gene from coconut chloroplasts, with a similar size (approximately 1.3 kb) and a further 5 R16F2n/R16R2 PCR products from MRD inflorescences showed high sequence similarities to *Bacillus* spp. 16S rRNA gene sequences. **Conclusion:** These results indicate that sequencing is a reliable method for the detection. Furthermore, trunk borings are the most reliable source of DNA for phytoplasma detection in coconuts using 16S rRNA gene primers, since there is less co-amplification of PCR products from other organisms when compared to spear leaves and inflorescences, nor from the spear leaves and inflorescences of MRD palms when primers fU5/rU3 were used in nested PCR.

**Key words:** Coconut palm, phytoplasma, nested PCR, cloning, sequencing

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### INTRODUCTION

Coconut palm (*Cocos nucifera* L.) is a versatile tree, popularly known as 'King of Palms' and 'Kalpavriksha', which translates as "tree that gives all that is necessary for living." Considered the most useful tree in the world, the coconut palm not only provides sustainable income to millions who are directly and indirectly dependant on it, but also provides highly nutritious food, drink, clothing, shelter, heirloom history, financial security, alleviate poverty and export earnings<sup>[48]</sup>.

Many historians believe that the origin of coconut was in the Asia-Pacific region, more specifically the Malayan Archipelago<sup>[32]</sup>. The large number of coconut

palms shows general yellowing symptoms of fronds. General yellowing and decline of plants are often considered as the symptoms of the presence of phytoplasmas, which are the causal agents of plants yellows diseases<sup>[23]</sup> and the symptoms of diseased palms show similarities to coconut lethal yellowing. Lethal Yellowing (LY) caused by phytoplasmas is a highly destructive disease on coconut and has been reported in the Caribbean, Florida and Mexico<sup>[38-40]</sup>. The first symptoms are yellowing of fronds followed by blackening of emerging inflorescences, premature nutfall and usually nut production effectively stops. Similar lethal-yellowing-like diseases caused by phytoplasmas have also devastated palms in both west and east Africa<sup>[17]</sup>.

The phytoplasmas are wall-less prokaryotes that live as obligate parasites and are amongst the smallest and simplest self-replicating organisms known. Phytoplasmas normally inhabit the phloem of plants at low concentrations and have an uneven distribution in woody plants especially in monocots. These characteristics make their detection and identification difficult<sup>[46]</sup>, but molecular diagnostics and in particular the use of PCR, have been developed as the main tools for confirming the presence of phytoplasmas and for assigning them to taxonomic groups<sup>[16,17,53]</sup>. On the basis of disease symptoms, the disease in Malaysia has been named coconut yellow decline and a preliminary report on this disease has been published<sup>[34,35]</sup>. Coconut Yellow Decline (CYD) phytoplasma belongs to the 16SrXIV Bermuda grass white leaf group is widespread disease in many coconut-growing areas of Malaysia and wherever the Malayan coconut ecotypes are grown. This current study was undertaken to determine the etiology of coconut yellow decline in Malaysia in greater detail.

## MATERIALS AND METHODS

### Sources of healthy and diseased coconut palms:

Tissue samples were collected from 20 Malayan Red Dwarf (MRD) coconut palms showing symptoms of the disease referred to as Coconut Yellow Decline (CYD). Coconut palm samples were obtained from disease areas in Selangor State. Symptomless palms from disease-free areas of Kuala Selangor were selected as negative controls. Spear leaves, inflorescences and wood shavings from trunks of MRD ecotypes were used for the preparation of DNA. Wood shavings were obtained by boring a hole 15 cm in length and 1 cm diameter into the palm trunk using a hand drill and were collected into polyethylene plastic bags and stored on ice for transport back to the laboratory. Samples from symptomless tall coconuts were also collected by this method.

Samples of 16SrII chickpea phyllody, 16SrII crotalaria saltiana phyllody, 16SrIV Cape St Paul wilt, 16SrVI brinjal little leaf and 16SrXI napier grass stunt from the University of Nottingham phytoplasma collection<sup>[17]</sup> and 16SrI aster yellows and 16SrXIV bermuda grass white leaf from the University Putra Malaysia Campus, were used for comparative purposes.

**DNA extraction:** DNA was extracted from 3 g of MRD spear leaves and inflorescences employing a phytoplasma enrichment procedure as described by Ahrens and Seemüller<sup>[1]</sup> with some modifications. The MRD tissue was homogenized in 12 mL ice-cold grinding buffer (125 potassium phosphate, 30 mM ascorbic acid, 10 sucrose, 0.15 bovine serum albumin, 2% polyvinylpyrrolidone, pH 7.6) in a polyethylene plastic bag using a hammer. The slurry was then

collected and centrifuged for 5 min at  $1100 \times g$  and the resultant supernatant was re-centrifuged at  $12000 \times g$  for 40 min at  $4^\circ\text{C}$ . The resultant pellet was resuspended in 1.5 mL CTAB buffer (2% w/v cetyltrimethylammonium bromide, 1.4 M NaCl, 0.2% 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-Base, 2% polyvinylpyrrolidone, pH 8.0) and incubated at  $65^\circ\text{C}$  for 30 min. An equal volume of chloroform-isoamyl alcohol (24:1) was added to the lysis, vortexed for 1 min and centrifuged at  $12000 \times g$  for 10 min. This step was repeated twice. DNA was precipitated with a two-third volume of ice-cold isopropanol, incubated at  $-20^\circ\text{C}$  for 20 min and then centrifuged at  $12000 \times g$  for 15 min. The pellet was washed with ice-cold 70% ethanol, dried at room temperature and dissolved in 100  $\mu\text{L}$  of ultrapure sterile water by incubation for 1 h at room temperature.

Total nucleic acid was also extracted from wood shavings of MRD and control coconut palms using the procedure of Zhang *et al.*<sup>[55]</sup> 0.2 g of wood shaving tissue was frozen in liquid nitrogen in a mortar and pestle. 800  $\mu\text{L}$  of CTAB buffer preheated at  $65^\circ\text{C}$  was added to the fine powder and was incubated at  $65^\circ\text{C}$  for 30 min. The lysate was extracted with 2/3 volume of chloroform-isoamyl alcohol and centrifuged at  $13000 \times g$  for 10 min. This step was repeated twice. An equal volume of ice-cold isopropanol was added to the aqueous phase, incubated at  $-20^\circ\text{C}$  for 15 min and then centrifuged for 15 min in  $14000 \times g$ . Pellets were rinsed in 1/3 volume of 70% ethanol, air dried and dissolved in 50  $\mu\text{L}$  ultrapure water.

### Primers and Polymerase Chain Reaction (PCR)

**analysis:** DNA from symptomatic and asymptomatic MRD coconut samples was amplified using direct and nested PCR. The phytoplasma universal primer pairs P1 (5'-AAG AGT TTG ATC CTG GCT CAG GAT T-3') (base 6-30)<sup>[7]</sup> /P7 (5'-CGT CCT TCA TCG GCT CTT-3') (base 68-51)<sup>[42]</sup>, R16F2n (5'-GAA ACG ACT GCT AAG ACT GG-3') (base 149-168)<sup>[11]</sup> / R16R2 (5'-TGA CGG GCG GTG TGT ACA AAC CCC G-3') (base 1397-1373)<sup>[26]</sup> and fU5 (5'-CGG CAA TGG AGG AAA CT-3') (base 369-386)/rU3 (5'-TTC AGC TAC TCT TTG TAA CA-3') (base 1251-1231)<sup>[28]</sup> derived from conserved regions of the 16S rRNA gene sequence were used to amplify phytoplasma ribosomal DNA. Universal primer pair P1/P7 amplify a 1.8 kb fragment that encompasses the entire 16S rRNA gene, the 16S-23S intergenic spacer region and the beginning of the 23S rRNA gene. Primer pairs R16F2n/R16R2 and fU5/rU3 amplify 1239 (137-1376) and 880 (356-1232) bp fragments respectively of the 16S rRNA gene internal to the P1/P7 priming sites. PCR assays were performed in 20 volumes containing 1 of DNA template, 1 of each primer (10 pmol), 0.4 of 10 mM dNTP, 0.6 of 25 mM

MgCl<sub>2</sub>, 2 of 10X Taq polymerase buffer and 0.3 µL (0.5 units) Taq DNA polymerase (Fermentas Inc.).

First round amplifications with P1/P7 primers were performed in an iCycler (BioRad) thermocycler using 1 min (2 min for an initial denaturation) at 94, 2 min at 55°C and 3 min at 72°C for 35 cycles and a final extension at 72°C for 10 min<sup>[26]</sup>. one µL of the P1/P7 reaction product was used as the template in nested PCR using primer pairs R16F2n/R16R2 or fU5/rU3 under the same conditions as described for P1/P7. Aliquots of 10 µL of each final reaction mixture were analyzed by 1% agarose gel electrophoresis using TBE (90mM Tris-borate, 2mM EDTA) as the running buffer. Gels were stained in ethidium bromide, visualized by UV transillumination and photographed.

**Cloning and sequencing of PCR products:** The R16F2n/R16R2 primed rDNA PCR products from CYD-infected Malayan Red Dwarf palms were purified from agarose gels using the Qiaquick gel extraction kit (Qiagen). They were cloned using a PCR cloning kit by

ligation into the pCR2.1 vector and transformed into TOP10 *E. coli* competent cells by heat shock following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Plasmids were prepared from transformed bacterial colonies and extracted using the Qiagen plasmid Miniprep kit and sequenced commercially by automated sequencing machines (Medigene Sdn Bhd.).

**Sequence analysis:** Sequence editing and analysis was performed using the DNAsis programs. The phylogenetic analysis of 16S rRNA gene sequences isolated from CYD phytoplasmas in this study and other known phytoplasmas obtained from the Basic Local Alignment Search Tool (BLAST) searches<sup>[2]</sup> at the National Center for Biotechnological Information (NCBI) and *Acholeplasma laidlawii* (Table 1) as an outgroup species, were performed using the program CLUSTAL W (Version 3.1)<sup>[49]</sup>. Phylogenetic and molecular evolutionary analyses were performed with MEGA version 3.1 software<sup>[22]</sup> using the Neighbor-Joining method with default values and 1000 replications for bootstrap analysis.

Table 1: Phytoplasma 16S rDNA sequences used as references in this study

Acronym	Phytoplasma or association disease	16S rRNA group	Gen Bank accession no.	Reference
<i>Ca. P. asteris</i>	<i>Candidatus</i> phytoplasma asteris	16SrI	M30790	Lee <i>et al.</i> <sup>[25]</sup>
<i>Ca. P. aurantifolia</i>	<i>Candidatus</i> phytoplasma aurantifolia	16SrII	U15442	Zreik <i>et al.</i> <sup>[56]</sup>
WX	Western X	16SrIII	L04682	1999 (Gen Bank submission)
CLY	Coconut lethal yellowing phytoplasma	16SrIV	AF498307	Harrison <i>et al.</i> <sup>[13]</sup>
<i>Ca. P. ulmi</i>	<i>Candidatus</i> Phytoplasma ulmi	16SrV	AY197655	Lee <i>et al.</i> <sup>[27]</sup>
<i>Ca. P. trifolii</i>	<i>Candidatus</i> Phytoplasma trifolii	16SrVI	AY390261	Hiruki and Wang <sup>[15]</sup> ,
<i>Ca. P. fraxini</i>	<i>Candidatus</i> Phytoplasma fraxini	16SVII	AF092209	Griffiths <i>et al.</i> <sup>[10]</sup>
LWB	Loofah witches-broom phytoplasma	16SrVIII	AF353090	2001 (Gen Bank submission)h
<i>Ca. P. phoenicium</i>	<i>Candidatus</i> phytoplasma Phoenicium	16SrIX	AF515636	Verdin <i>et al.</i> <sup>[51]</sup>
<i>Ca. P. mali</i>	<i>Candidatus</i> phytoplasma mali	16SrX	AJ542541	Seemüller and Schneider <sup>[45]</sup>
<i>Ca. P. oryzae</i>	<i>Candidatus</i> phytoplasma oryzae	16SrXI	AB052873	Jung <i>et al.</i> <sup>[21]</sup>
<i>Ca. P. australiense</i>	<i>Candidatus</i> phytoplasma australiense	16SrXII	L76865	Davis <i>et al.</i> <sup>[6]</sup>
<i>Ca. P. japonicum</i>	<i>Candidatus</i> phytoplasma japonicum	16SrXII	AB010425	Sawayanagi <i>et al.</i> <sup>[41]</sup>
MPV	Mexican periwinkle virescence	16SrXIII	AF248960	2000 (Gen Bank submission)
<i>Ca. P. cynodontis</i>	<i>Candidatus</i> phytoplasma cynodontis	16SrXIV	AJ550984	Marcone <i>et al.</i> <sup>[27]</sup>
MBGWL	Malaysia bermuda grass white leaf	16SrXIV	EU294011	2007 (Gen Bank submission)
TBGWL	Thailand bermuda grass white leaf	16SrXIV	AF248961	2000 (Gen Bank submission)
ITBGWL	Italy bermuda grass white leaf	16SrXIV	AJ550984	Marcone <i>et al.</i> <sup>[30]</sup>
INBGWL	India bermuda grass white leaf	16SrXIV	EU032485	Snehi <i>et al.</i> <sup>[47]</sup>
IRBGWL	Iran bermuda grass white leaf	16SrXIV	EF444485	2007 (Gen Bank submission)
CYDMRD	Coconut yellow decline strain MRD	16SrXIV	EU328159	In this study
CYDMT	Coconut yellow decline strain Malayan Tall	16SrXIV	EU636906	2007 (Gen Bank submission)
<i>Ca. P. brasiliense</i>	<i>Candidatus</i> phytoplasma brasiliense	16SrXV	AF147708	Montano <i>et al.</i> <sup>[33]</sup>
<i>Ca. P. graminis</i>	<i>Candidatus</i> phytoplasma graminis	16SrXVI	AY725228	Arocha <i>et al.</i> <sup>[3]</sup>
<i>Ca. P. Caricae</i>	<i>Candidatus</i> phytoplasma caricae	16SrXVII	AY725234	Arocha <i>et al.</i> <sup>[3]</sup>
<i>Ca. P. Americanum</i>	<i>Candidatus</i> phytoplasma americanum	16SrXVIII	DQ174122	Lee <i>et al.</i> <sup>[24]</sup>
<i>Ca. P. castaneae</i>	<i>Candidatus</i> phytoplasma castaneae	16SrXIX	AB054986	Jung <i>et al.</i> <sup>[20]</sup>
<i>Ca. P. rhamni</i>	<i>Candidatus</i> phytoplasma rhamni	16SrXX	X76431	Marcone <i>et al.</i> <sup>[29]</sup>
<i>Ca. P. pini</i>	<i>Candidatus</i> phytoplasma pini	16SrXXI	AJ632155	Schneider <i>et al.</i> <sup>[43]</sup>
Ph.sp	<i>Phytoplasma</i> sp. strain LDN	16SrXXII	Y14175	Tymon <i>et al.</i> <sup>[50]</sup>
BVGYP	Buckland valley grapevine yellows phytoplasma	16SrXXIII	AY083605	Constable <i>et al.</i> <sup>[5]</sup>
S BSP	Sorghum bunchy shoot phytoplasma	16SrXXIV	AF509322	Blanche <i>et al.</i> <sup>[4]</sup>
WTWBP	Weeping tea witches'-broom phytoplasma	16SrXXV	AF521672	2002 (Gen Bank submission)
ScP1	Sugar cane phytoplasma D3T1	16SrXXVI	AJ539179	2003 (Gen Bank submission)
ScP2	Sugar cane phytoplasma D3T2	16SrXXVII	AJ539180	2003 (Gen Bank submission)
DP	Derbid phytoplasma	16SrXXVIII	AY744945	2004 (Gen Bank submission)
<i>A. laidlawii</i>	<i>Acholeplasma laidlawii</i>	-	M23932	Weisburg <i>et al.</i> <sup>[54]</sup>

**In silico restriction enzyme digestions and virtual gel plotting:** The MRD CYD phytoplasma sequence was obtained in this study and 16S rRNA gene R16F2n/R16R2 fragments from *Ca. P. asteris* (16SrI) [M30790], Coconut lethal yellowing (16SrIV) [AF498307], *Ca. P. oryzae* (16SRXI) [AB052873], *Ca. P. cynodontis* (16SrXIV) [AJ550984] and Lethal decline Nigeria (16SrXXII) [Y14175] were exported to the *in silico* restriction analysis and virtual gel plotting program pDRAW32, developed by AcaClone Software (<http://www.acaclone.com>). Each aligned DNA fragment was digested *in silico* with *AluI*, *HhaI*, *HinfI*, *Sau3AI* (*MboI*), *MseI*, *RsaI* and *TaqI* restriction enzymes. After *in silico* restriction digestion, a virtual 3.0% agarose gel electrophoresis image with minimum 50 bp was plotted automatically to the computer screen.

## RESULTS

**Disease symptoms:** The initial symptom observed in Malayan red dwarf coconut palms was yellowing of the coconut foliage. In addition, the lower canopy foliage turned light yellow and eventually light-brown and the symptoms spread rapidly to the younger leaves. Severe chlorosis of the emerging spear leaves, inflorescence necrosis, premature nut fall and gradual collapse of fronds also occurred and there was terminal rot of the growing point of immature palms and palms generally died within 5 months of initial symptom appearance.

**Detection of phytoplasma DNA in coconut palms by PCR:** No amplification products were visible after the first round 35 cycles of amplification from the DNA of CYD infected samples from trunk borings or any other tissues. However, PCR products of approximately 1250 and 900 bp were amplified from 8 of 20 symptomatic MRD DNA samples with primers R16F2n/R16R2 or fU5/rU3 respectively, after PCR products from the first round were subjected to nested PCR (Fig. 1a and b). A PCR product of approximately 1.3 kb was also amplified in nested PCR using primers R16F2n/R16R2 from the spear leaves of 4 symptomatic and 2 of the apparently healthy MRD coconut palms (Fig. 2) and from 2 (one from diseased and one from healthy) inflorescences (results not shown). In addition, a PCR product of approximately 1250 bp was amplified from a further 5 symptomatic MRD inflorescences. No amplification products were produced from trunk borings of asymptomatic MRD palms using any primer combinations, nor from the spear leaves and inflorescences of MRD palms when primers fU5/rU3 were used in nested PCR.

**Sequence analysis of amplified products:** PCR products obtained from the MRD palms were cloned into the vector pCR2.1 and the sequences of the inserts determined using primer pairs R16F2n/R16R2 (1250 bp) and fU5/rU3 (890 bp). Clones from the 8 symptomatic MRD samples that were tested were all identical. Comparisons of these 16S rRNA gene sequences of CYD to other phytoplasma 16S rDNA sequences reported in Gen Bank, using the tool BLAST revealed that CYD belongs to the '*Candidatus* phytoplasma cynodontis' group (16SrXIV) with 99% homology to the rRNA gene sequence of bermuda grass whiteleaf phytoplasma from Thailand (AF248961) and Malaysia (EU294011), members of the 16SrXIV. Phylogenetic analysis confirmed this classification (Fig. 3). The CYD phytoplasma rRNA gene sequences obtained in this study from MRD coconuts were deposited in the Gen Bank database under accession numbers EU328159.

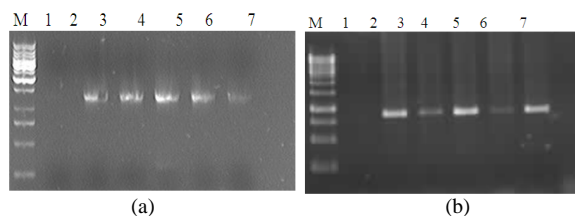


Fig. 1: (a): Amplification of CYD and other phytoplasma rRNA gene products with nested PCR primers R16F2n/R16R2. Lanes (M) 1 kb DNA ladder; (1) Healthy coconut palm (2, 3) Coconut yellow decline infected sample: (4) Bermuda grass white leaf: (5) Aster yellows: (6) Cape St Paul wilt: (7) water control; (b): Amplification of CYD and other phytoplasma rRNA gene products with nested PCR primers fU5/rU3. Lanes (M) 1 kb DNA ladder: (1) healthy coconut palm (trunk sample): (2, 3) Coconut yellow decline infected sample: (4) Bermudagrass white leaf: (5) Cape St Paul wilt: (6) Aster yellows: (7) Water control

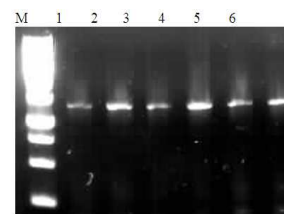


Fig. 2: Amplification of PCR products with nested PCR primers R16F2n/R16R2 from healthy MRD coconut samples. Lanes (M) 1 kb DNA ladder: (1-3) Midribs of spear leaf samples, (4-6) inflorescence samples

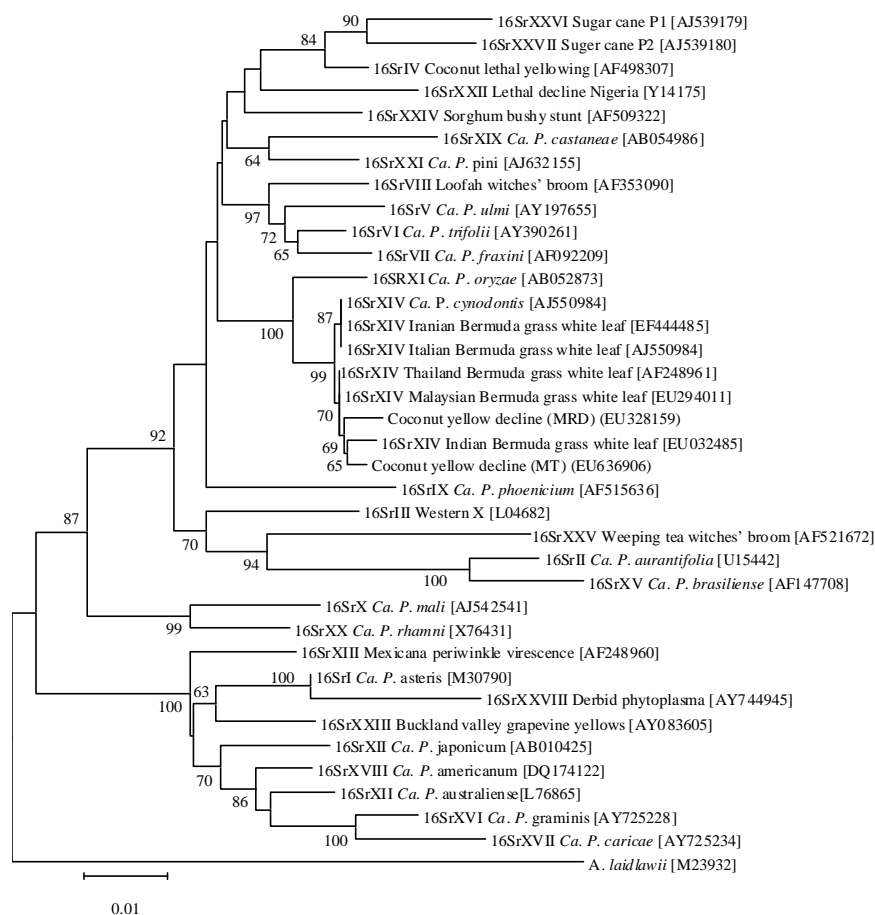


Fig. 3: Dendrograms, constructed by the Neighbor-Joining method, showing the phylogenetic relationships between the Malaysian coconut yellow decline phytoplasmas with 36 phytoplasmas and *A. laidlawii* as out group constructed based on 16S rRNA gene sequences. 16Sr groupings are based on the classification system of Wei *et al.*<sup>[53]</sup>. Gen Bank accession numbers for sequences obtained as part of this project along with previously published sequences are shown in Table 1 along side the names of the phytoplasmas. Bootstrap values greater than 50% (expressed as percentages of 1000 replications) are shown and branch lengths are proportional to the number of inferred character state transformations. Bar substitutions per base

In addition to these phytoplasma sequences, the PCR products from spear leaves and inflorescences from symptomatic and asymptomatic MRD palms were also cloned and sequenced and alignments showed that these were clearly not of phytoplasma origin. Sequences from the R16F2n/R16R2 amplified fragments cloned from the 6 spear leaf and 2 inflorescence 1.3 kb samples from MRD palms showed >99% similarity to 16S rRNA gene sequence from the chloroplast of *Elaeis oleifera* with accession no. EU016925<sup>[18]</sup>. There is no record in the database of the chloroplast 16S rRNA gene from coconuts, but it is presumed that it is the coconut chloroplast rRNA gene that has been amplified in these spear leaf samples. This 16S rRNA gene sequence

which is presumed to come from the chloroplast of *Cocos nucifera* has been deposited at Gen Bank under accession number EU717157.

Comparison between the sequences of R16F2n/R16R2 1250 bp products derived from the 5 symptomatic MRD inflorescence samples and the Gen Bank database indicated >99% similarity with the 16S rRNA gene sequences from *Bacillus* spp. and *B. megaterium*. This presumably indicates the presence of these bacteria in the inflorescences and that the primers are not specific. To determine whether RFLP analysis could be used to distinguish between the R16F2n/R16R2 *Bacillus* PCR product that obtained from the CYD phytoplasma, cloned samples were digested

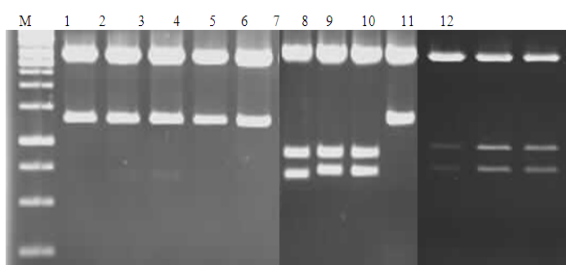


Fig. 4: RFLP analysis using *EcoRI* from cloned R16F2n/R16R2 PCR samples in the pCR2.1 vector. Lanes (M) 1 kb DNA ladder: (1-5) chloroplast rDNA from spear leaves: (6-8) *Bacillus* rDNA from inflorescences: (9) chloroplast rDNA from an inflorescence; (10-12) CYD phytoplasma rDNA from trunk borings. The large band of approx 4 kb is the pCR2.1 plasmid DNA and the smaller bands are from the rDNA PCR products

with *EcoRI*. This enzyme was chosen because it had sites flanking the inserts but no other sites within the vector sequence, so the plasmid derived DNA could be separated from the insert DNA. Results in Fig. 4 show that there is a single *EcoRI* site in both the CYD phytoplasma and the *Bacillus* PCR product which gives rise to similar sized fragments, whilst there is no *EcoRI* site in the chloroplast DNA.

**Virtual RFLP analysis:** When nested-PCR products (1.2 kb) resulting from reamplification of primary P1/P7 products by F2n/R2 were analysed by digestion with *AluI*, *HhaI*, *HinfI*, *Sau3AI* (*MboI*), *MseI*, *RsaI* and *TaqI* restriction endonucleases respectively, collective virtual RFLP patterns of CYD isolates from MRD ecotype were identical and belong to the 16SrXIV group (Fig. 5).

The virtual RFLP patterns of CYD phytoplasma isolates were very similar to Bermuda grass white leaf phytoplasma with all of these restriction endonucleases. Therefore, CYD phytoplasma strains was identified as a member of the Bermuda grass white leaf group (16SrXIV). Furthermore, *HinfI*, *MboI*, *RsaI* and *TaqI* digests revealed similar patterns CYD phytoplasma strains to *Ca. P. Oryzae* (16SrXI). Whereas, *AluI*, *HhaI*, *HinfI*, *Sau3AI* (*MboI*), *MseI*, *RsaI* and *TaqI* digests indicated profiles differentiating CYD phytoplasma strains from coconut lethal yellowing (16SrIV) and lethal decline (16SrXXII).

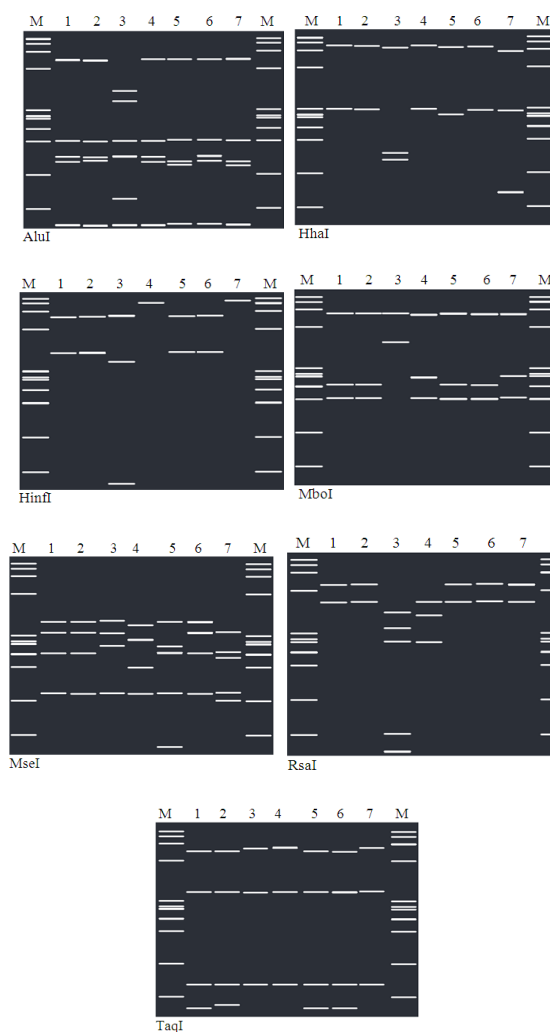


Fig. 5: Virtual RFLP analysis with *AluI*, *HhaI*, *HinfI*, *Sau3AI* (*MboI*), *MseI*, *RsaI* and *TaqI* restriction enzymes. Lanes (1): CYD from MRD ecotype (EU328159) (2): CYD from MT ecotype ((EU636906) (3): *Ca. P. Asteris* (16SrI) [M30790] (4): Coconut lethal yellowing (16SrIV) [AF498307] (5): *Ca. P. oryzae* (16SrXI) [AB052873] (6): *Ca. P. cynodontis* (16SrXIV) [AJ550984] (7): Lethal decline Nigeria (16SrXXII) [Y14175]. M: phX174DNA-*HaeIII* Digest DNA MW-marker

## DISCUSSION

Results from PCR analysis confirmed the presence of a phytoplasma, but showed that the CYD phytoplasma is different from those that infect coconut in other parts of the world even though the symptoms

are very similar. Coconut palms are known to be susceptible to the lethal yellowing (16SrIV) group phytoplasmas around the world. Lethal yellowing in the USA<sup>[12]</sup>, Lethal Disease (LD) in Tanzania<sup>[44]</sup>, Cape St. Paul Wilt (CSPW) in Ghana<sup>[19,37]</sup>, Kaincope disease in Togo<sup>[36]</sup>, Kribi disease in Cameroon<sup>[8]</sup> and Awka or bronze leaf wilt (LDN) in Nigeria<sup>[9]</sup> are all classified as 16SrIV phytoplasmas and give similar symptoms of premature fruit drop, floral necrosis leaf discoloration and decline. Subgroup 16SrIV-A phytoplasmas are associated with Lethal Yellowing (LY) of coconut and other palm species in the Americas<sup>[13]</sup> whereas phytoplasmas that induce symptoms similar to LY on coconut in Africa are referred to by other names to reflect strain differences that have previously been identified through 16S rRNA gene analysis. Recently, Wei *et al.*<sup>[53]</sup> have allocated the Nigerian coconut Lethal Decline Group (LDN) to a distinct 16Sr group, 16SrXXII-A and this has been confirmed by the work of Hodgetts *et al.*<sup>[17]</sup>, who showed a high degree of divergence between the different coconut phytoplasmas based on the *secA* gene which supported their separation into at least three distinct 'Ca. Phytoplasma' species that reflect the geographical origins of the strains.

The 16SrXIV phytoplasma found on coconut in Malaysia is from a very different phylogenetic group to the lethal yellowing-like phytoplasmas and is more closely related to the phytoplasmas found in Bermuda grass in Thailand and Malaysia. This situation is similar to the findings for the Kalimantan Wilt (KW) disease of coconuts in Indonesia which belongs to the 16SrXI rice yellow dwarf phytoplasma group<sup>[52]</sup>. Rice and sugarcane in Indonesia is known to be infected with 16SrXI phytoplasmas such as rice yellow dwarf, sugarcane white leaf, sugarcane grassy shoot and Ramu stunt disease and sugarcane is grown in gardens in the area affected by KW disease. It is therefore possible that these phytoplasmas in Indonesia and Malaysia are ones that are adapted to grasses and use coconut as an alternate host, possibly because their vectors, which have not as yet been identified, are able to feed on coconut and these grasses. By contrast, the 16SrIV lethal yellowing-like phytoplasmas seem to be much more specific to coconut and closely related palm species. This may also explain why some of the symptoms associated with the Malaysian and Indonesian coconut phytoplasmas are distinct and different from those that occur in Africa and the Americas.

Two unexpected results were obtained during this study. Firstly, the universal phytoplasma primer pair, R16F2n/R16R2 amplified the 16S rRNA gene from coconut chloroplast derived from spear leaf and inflorescence samples of some asymptomatic coconut

palms. These results indicate that R16F2n/R16R2 can amplify plant DNA. Secondly, *Bacillus* spp., were amplified with these primers from the inflorescences of some asymptomatic coconut palms. The *Bacillus* and chloroplast rDNA sequence sizes were similar to the phytoplasma rDNA sequence size. *Bacillus megaterium* has previously been isolated from the trunk samples of date palms (*Phoenix canariensis* Chabaud) affected by the lethal decline phytoplasma using universal phytoplasma primer pair (P1/P7) in Texas<sup>[14]</sup>. This clearly indicates that care needs to be taken when using these universal phytoplasma primers on samples that contain other Gram positive bacteria, especially as RFLP analysis with some enzymes such as *EcoRI* can give similar sized products. Other restriction enzymes could be used to discriminate between *Bacillus* and phytoplasma and in addition, more specific universal phytoplasma primers are currently being developed<sup>[16,17,31]</sup> and it may be that these will be more suitable for diagnostics from samples such as coconuts which appear to harbor other Gram-positive bacteria in significant populations particularly on the spear leaves and inflorescences.

## CONCLUSION

The results presented herein reveal the molecular characterization of phytoplasma associated with CYD disease of coconut palm. The results presented show that sequencing is a reliable method for the detection, identification and classification of CYD phytoplasmal disease. In order to achieve a basic understanding of phytoplasma pathogenesis and phytoplasma control strategies, sequencing is the powerful and reliable method to identify and classify the phytoplasmas.

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