

## Detection of the mycoplasma-like organism associated with lethal yellowing disease of palms in Florida by polymerase chain reaction

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DNA amplification by polymerase chain reaction (PCR) was used specifically to detect the mycoplasma-like organism (MLO) associated with lethal yellowing disease of palms in Florida. For PCR, a pair of oligonucleotide primers was synthesized according to partial sequences of a cloned 1.3 kbp fragment of lethal yellowing MLO-specific genomic DNA isolated from a diseased windmill palm (*Trachycarpus fortunei*). A DNA product of about 1 kbp was specifically amplified by PCR in reaction mixtures containing template DNA derived from either heart, inflorescence or leaf tissues of lethal yellowing-affected palms. PCR performed for 35 cycles with as little as 5 pg of DNA template, in some instances, was sufficient consistently to amplify the same lethal yellowing MLO DNA product from hearts of 11 species comprising 30 symptomatic palms. Similar reliable and reproducible detection of the lethal yellowing MLO in palm inflorescence spikelets was also achieved after 35 cycles of PCR. When template DNA for PCR was derived from tissues of the most immature emerging leaf, a 40-cycle reaction was sufficient for consistent foliar detection of the pathogen in all coconut palms including palms with earliest visible symptoms of disease.

### INTRODUCTION

Lethal yellowing disease of the coconut palm (*Cocos nucifera*) has been endemic to parts of the western Caribbean region for at least 100 years (Howard, 1983). During the last three decades, epiphytotics of lethal yellowing prompted by unknown factors have killed millions of susceptible Tall-type coconut palms in both Jamaica (Anonymous, 1986) and southern Florida (McCoy *et al.*, 1983). Today, the once prevalent Jamaica Tall cultivar has been virtually eliminated from both geographic localities.

In areas of southeast Florida severely affected by coconut lethal yellowing, lethal decline diseases of numerous other palm species were also recognized (Thomas, 1979). As with coconut lethal yellowing, mycoplasma-like organisms (MLOs) were implicated as the probable aetiological agents of these additional diseases. This evidence, coupled with the appearance on some palm species of symptoms superficially similar to those of coconut lethal yellowing, indicated that these diseases were probably all caused by the same pathogen (McCoy *et al.*, 1983). The subsequent transmission of lethal yellowing to coconut, Manila (*Veitchia merrillii*) and

Thurston (*Pritchardia thurstonii*) palms by field-collected American palm cixiids (*Myndus crudus*), the suspected principal vector of lethal yellowing in the Americas (Howard *et al.*, 1983), strongly supported this assertion.

Further evidence of the involvement of the lethal yellowing MLO in lethal declines of other palm species was recently obtained with DNA hybridizations using cloned genomic DNA fragments of the pathogen as probes (Harrison *et al.*, 1992). Reliable MLO detection was achieved when DNA samples extracted from immature palm leaf bases (heart tissues) rich in functional phloem were probed. However, most mature organs of infected palms contain few MLOs (Thomas & Norris, 1980). In this respect, lethal yellowing resembles MLO-associated diseases of other woody perennial plant hosts (Hibben *et al.*, 1991; Sinclair *et al.*, 1992). Consistent detection of MLO infections in palms required the use of <sup>32</sup>P-labelled DNA probes to achieve the necessary detection sensitivity (Harrison *et al.*, 1992, 1994). This prerequisite has limited the utility of DNA probes for investigations of lethal yellowing elsewhere in the western Caribbean where the disease is presently most active.

Selective enzymatic amplification of MLO DNA from mixtures with host DNA by polymerase chain reactions (PCR) is a recent approach to studies on MLOs. PCR amplification of MLO 16S ribosomal RNA (rRNA) gene sequences and restriction site analysis (Ahrens & Seemüller, 1992; Lee *et al.*, 1993b; Schneider *et al.*, 1993) or cycle sequencing (Namba *et al.* 1993; Schneider *et al.*, 1993) of the rDNA products have been used to differentiate and group MLOs for taxonomic purposes. MLO-specific PCR has also been developed by exploiting anonymous sequences of cloned MLO DNA as primers (Deng & Hiruki, 1991a; Schaff *et al.*, 1992; Lee *et al.*, 1993a). Furthermore, the sensitivity of detection achievable by MLO-specific PCR substantially exceeds the lower limits reported for DNA probe hybridizations (Schaff *et al.*, 1992) and is sufficient to monitor MLO acquisition by leafhoppers during feeding on infected plants (Vega *et al.*, 1993). Thus, PCR-based techniques could greatly enhance detection of MLOs normally present only in low titres, such as the palm lethal yellowing agent, and possibly facilitate detailed investigations of lethal yellowing disease epidemiology.

We report the development of primers from a genomic DNA sequence of the lethal yellowing MLO isolated from a windmill palm (*Trachycarpus fortunei*) which enable specific detection of the pathogen. The sensitivity and reliability of the lethal yellowing-specific PCR for detecting the pathogen in various tissues of selected palm species was evaluated.

## METHODS

### Sources of healthy and diseased palms

Heart tissues consisting mostly of immature leaf bases were obtained from 30 naturally infected 7–12-year-old palms showing mid-stage (yellowing phase) lethal yellowing symptoms (McCoy *et al.*, 1983). With the exception of Manila palms (*V. merrillii*), all others were located on the grounds of the University of Florida's Fort Lauderdale Research and Education Center (FLREC). Palms were felled and samples collected from representatives of the following 10 species: dwarf sugar palm (*Arenga engleri*), giant fishtail palm (*Caryota rumphiana*), *Cocos nucifera* (cultivars Hawaiian Tall, Jamaica Tall, Malayan Dwarf, Panama Tall) and Maypan hybrid coconut (Malayan Dwarf × Panama Tall), hurricane palm (*Dictyosperma*

*album*), spindle palm (*Hyophorbe verschaffeltii*), Chinese fan palm (*Livistona chinensis*), footstool palm (*L. rotundifolia*), true date palm (*Phoenix dactylifera*), silver date palm (*P. sylvestris*), and windmill palm (*T. fortunei*). Five diseased Manila palms each of an undetermined age were provided to us by local homeowners. Young, 1–2-year-old container-grown palms in shadehouses served as sources of healthy tissues for comparison.

Single immature (unemerged) inflorescences with partially necrotic spikelets were removed from solitary, bearing coconut (cultivar Jamaica Tall) palms growing within the vicinity of Miami and Naples, Florida. As both palms also displayed premature nutfall, these combined symptoms were indicative of early-stage (primary phase) lethal yellowing symptoms (McCoy *et al.*, 1983). Similar inflorescences were removed from five symptomatic coconut (cultivar Malayan Dwarf) palms and a Chinese fan palm at the FLREC. Immature, unblemished coconut inflorescences were also excised from a symptomless Malayan Dwarf and an Indian Green Dwarf cultivar for use as sources of apparently healthy tissues.

Non-necrotic leaflets retaining green colour were removed from proximal portions of all leaves of two non-bearing coconut (cultivar Panama Tall) palms with early lethal yellowing foliar symptoms. Leaflets were also sampled from lower portions of the youngest, emerging leaf (spear) on five additional coconut palms. These included two mature, bearing Malayan Dwarfs and a non-bearing Hawaiian Tall with early yellowing phase symptoms; a bearing Malayan Dwarf with primary symptoms; and a non-bearing Jamaica Tall with spear necrosis only.

### Other MLO-associated plant diseases

Plants affected by various other MLO-associated diseases indigenous to Florida were maintained in shadehouses. These included sweet corn (*Zea mays* 'saccharata' cv. Aristogold Guardian) with maize bushy stunt (Davis *et al.*, 1988), Madagascar periwinkle (*Catharanthus roseus*) singly infected with pigeon pea witches' broom (Harrison *et al.*, 1991), or periwinkle witches' broom (McCoy & Thomas, 1980), or periwinkle virescence disease. Periwinkle infected with the following additional MLOs were also kindly provided by other researchers: eastern aster yellows, J. A. Wyman (University of Wisconsin, Madison, WI, USA); western X, peach yellow leafroll strain and prune strain, B. C. Kirkpatrick

(University of California, Davis, CA, USA); Yucatan periwinkle virescence, M. A. Villanueva, (Centro de Investigación Científica de Yucatán, A.C., Merida, Mexico). Coconut inflorescence DNAs extracted from an East African Tall palm in Tanzania with lethal disease, and a West African Tall palm in Nigeria with Awka disease, were both kindly provided by P. Jones (Rothamsted Experimental Station, Harpenden, UK).

### Culturable mollicutes

*Spiroplasma kunkelii* Florida isolate T80 (Davis *et al.*, 1984); *S. citri* California isolate 189 (McCoy *et al.*, 1981); and the *Cocos* spiroplasma isolate N525 (Eden-Green & Waters, 1981), kindly provided by R. E. Whitcomb (USDA ARS, Beltsville, MD, USA), were cultured in C3-G medium (Liao & Chen, 1977). *Acholeplasma axanthum* (S743), *A. oculi* (19L), both NIAID reference strains, and an unidentified *Acholeplasma* sp. (J233), originally from a lethal yellowing-affected coconut palm (Eden-Green & Tully, 1979) were kindly provided by J. G. Tully (Frederick Cancer Facility, Frederick, MD, USA) and cultured in SP-4 medium (Tully *et al.* 1977).

### DNA extractions

Preparations enriched with MLOs were obtained from symptomatic plant tissues by differential centrifugation after tissues were ground in an osmotically augmented buffer (Harrison *et al.* 1991, 1992). Total DNAs were extracted from these preparations as previously described (Harrison *et al.*, 1992). DNAs extracted from comparable tissues of seed-grown or symptomless landscape plants served as experimental controls. Cells from spiroplasma and acholeplasma cultures were collected by centrifugation at 20 000 *g* for 30 min at 4°C and extracted by the procedure of Dellaporta *et al.* (1983).

Small-scale (3 g) extractions of total DNA from freshly harvested coconut leaflet laminae were prepared by the procedure of Doyle & Doyle (1990) except that the DNA extraction buffer also contained 10 g/l PVP-40. All DNA extracts were quantified by fluorometry (TKO-100 minifluorometer, Hoefer Scientific Instruments, San Francisco, CA) and stored at 4°C.

### DNA manipulations

Lethal yellowing MLO DNA for molecular

cloning was obtained by repeated caesium chloride-bisbenzimidazole buoyant density gradient centrifugation (Kollar *et al.*, 1990) of total DNAs extracted from heart tissues of a lethal yellowing-diseased windmill palm. About 400 ng of the gradient-enriched lethal yellowing MLO DNA was partially digested with *Hind*III (Promega Biological Research Products, Madison, WI, USA) for 1 h at 37°C. Resulting fragments were ligated with dephosphorylated *pUC19* using a 10:1 insert:vector ratio and cloned in *Escherichia coli* DH5- $\alpha$  cells (Gibco BRL Life Technologies, Gaithersburg, MD, USA). Recombinant plasmid DNA was extracted by alkaline lysis from small-scale Luria-Bertani (LB) broth cultures of selected recombinant colonies as described by Sambrook *et al.* (1989). About 200 ng of each DNA preparation was blotted onto nylon membranes (Nytran, Schleicher and Schuell Inc., Keene, NH, USA) following the protocol of Lee & Davis (1988). Replicate blots were air dried and then baked at 80°C for 30 min prior to hybridizations.

Recombinant plasmids containing lethal yellowing MLO DNA inserts were tentatively identified by moderately stringent differential dot hybridizations using *Hind*III-digested healthy coconut palm DNA or gradient-enriched lethal yellowing MLO DNA as probes. Blots were hybridized with probes using previously reported conditions (Harrison *et al.*, 1992) then sealed in plastic wrap and exposed to Konica PB7, X-ray film (Konica Medical Corp., Wayne, NJ, USA) with an intensifier screen (Lightning Plus, DuPont, Newark, DE, USA) for 5 days at -75°C.

To identify pathogen-specific MLO DNA inserts, blots of recombinant plasmid DNA were stripped of initial probes by boiling each membrane in 0.1  $\times$  SSC, 5 mg/l SDS (Sambrook *et al.*, 1989) and then reprobbed at moderate stringency with [<sup>32</sup>P] dATP labelled, gradient-enriched DNA of the western X (prune strain) MLO or with total DNAs extracted from healthy periwinkle. Blots were stripped again and reprobbed with gradient-enriched DNA of the pigeon pea witches' broom MLO.

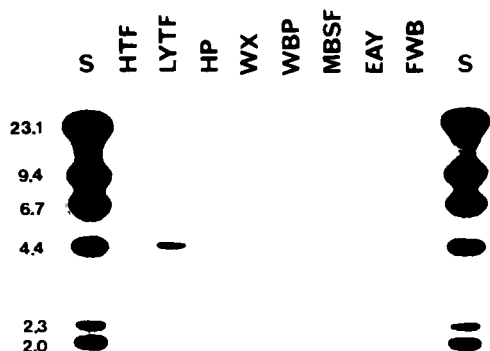
Cloned DNA inserts from eight recombinant plasmids which hybridized only to enriched lethal yellowing MLO DNA were labelled with [<sup>32</sup>P] dATP by using random primers (random primed DNA labelling kit, Boehringer Mannheim Biochemicals, Indianapolis, IN, USA). Inserts were used individually to probe dot blots of various undigested plant and mollicute

DNA and Southern blots of *Hind*III-digested healthy plant DNAs or DNAs from plants with various MLO-associated diseases. For dot blots, each sample was applied to nylon membranes as a series of twofold dilutions beginning with 2  $\mu$ g. For Southern blots, 1  $\mu$ g of each sample DNA was digested with *Hind*III for a minimum of 4 h at 37°C and electrophoresed in 7.5 mg/ml agarose (Low EEO grade, Fisher Scientific, Pittsburgh, PA, USA) gels using 1  $\times$  TAE (40 mM Tris-acetate, 1 mM EDTA) as running buffer. DNA was blotted from gels onto nylon membranes by a modification of Southern's method (Sambrook *et al.*, 1989).

#### Oligonucleotide primers and PCR conditions

Probe LYTC24, a 4.5 kbp fragment of LY MLO DNA, which hybridized only with DNA of lethal yellowing-diseased palms, was digested with *Xba*I. One of the two resulting subfragments (TC24-A, 1.3 kbp) was ligated with *Hind*III-*Xba*I-digested *pUC*19 and cloned, as before. Partial sequencing of subclone TC24-A was achieved by using M13/*pUC* primers and standard dideoxy nucleotide termination reactions (Sambrook *et al.*, 1989). A pair of oligonucleotide primers was synthesized on the basis of the sequence data. Both sequencing and primer synthesis were performed by the DNA sequencing and synthesis Core laboratories at the University of Florida's Interdisciplinary Center for Biotechnology Research.

For PCR, sample DNAs for use as template were diluted to 25 ng/ $\mu$ l with sterile distilled water. Amplifications were performed in 50  $\mu$ l final reaction volumes each containing 50 ng of sample DNA template, 50 ng of each primer, 125  $\mu$ M of each dNTP, 1.5 U of AmpliTaq DNA polymerase (AS) with recommended PCR buffer (Perkin Elmer Cetus, Norwalk, CT, USA) and overlaid with mineral oil. PCR was performed for 35 or 40 cycles in a thermal block cycler (Model 110S, Coy Laboratory Products Inc., Ann Arbor, MI, USA) using the following parameters: 30 s (90 s for first cycle) denaturation step at 94°C, annealing at 53°C for 50 s and primer extension at 72°C for 80 s. Reaction mixtures containing healthy plant DNA or sterile distilled water substituted for template DNA served as negative controls in each experiment. Following all amplifications, 10  $\mu$ l of each reaction mixture was analysed by electrophoresis in a 10 mg/ml agarose gel. PCR products in gels were stained with ethidium bromide, visualized by UV transillumination and photographed.



LYTC 24

Fig. 1. Southern blot hybridization of probe LYTC24, a 4.5 kbp chromosomal fragment of the palm lethal yellowing (LY) mycoplasma-like organism (MLO) to *Hind*III-digested DNA derived from healthy plants and plants with various MLO-associated diseases. DNA from: HTF, healthy *Trachycarpus fortunei* (windmill palm); LYTF, LY-diseased windmill palm; HP, healthy *Catharanthus roseus* (periwinkle); WX, periwinkle infected with western X (prune strain); WBP, periwinkle with pigeon pea witches' broom; MBSF, sweet corn (*Zea mays* 'saccharata' cv. Aristogold Guardian) with Florida maize bushy stunt; EAY, periwinkle with eastern aster yellows; FWB, Florida periwinkle witches' broom. S, lambda DNA/*Hind*III fragments.

#### RESULTS

Of the 94 recombinant plasmids initially evaluated by differential dot hybridizations, 79 were judged to contain cloned MLO DNA inserts. Forty-two of these subsequently hybridized with probes consisting of either gradient-enriched western X MLO or pigeon pea witches' broom MLO DNAs, indicating inserts to be MLO-specific but not lethal yellowing MLO-specific (data not shown). Cloned inserts, ranging in size from 0.8 to 4.5 kbp, were excised from eight of the remaining 37 recombinant plasmids and used as probes to confirm their disease specificity. One of these, clone LYTC24, consisting of a 4.5 kbp fragment of lethal yellowing MLO chromosomal DNA, detected DNA derived only from lethal yellowing-affected palms during both moderately stringent dot (data not shown) and Southern (Fig. 1) hybridizations.

Signals resulting from dot hybridizations between probe LYTC24 and DNA derived from heart tissues of lethal yellowing-diseased

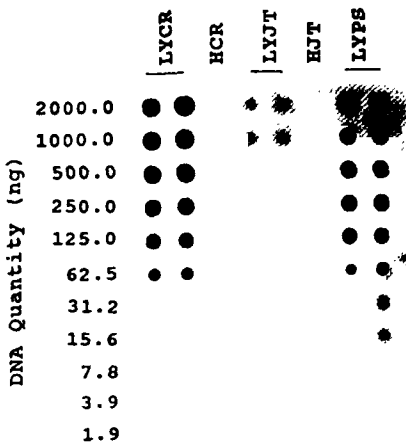


Fig. 2. Dot hybridization of probe LYTC24 (4.5 kbp) to DNA extracted from heart tissues of three palm species with mid-stage foliar symptoms indicative of lethal yellowing (LY) disease. LYCR, *Caryota rumphiana* (giant fishtail palm); HCR, healthy *C. rumphiana*; LYJT, *Cocos nucifera* (coconut palm cultivar Jamaica Tall); HJT, healthy *C. nucifera* cultivar Jamaica Tall; LYPS, *Phoenix sylvestris* (silver date palm).

palms differed in intensity according to the particular palm species. Signals observed for ornamental palm species *C. rumphiana* and *P. sylvestris*, were at least 10- to 12-fold stronger than those routinely encountered for Tall-type *C. nucifera* cultivars. Representative hybridization signals are illustrated in Fig. 2. Following both dot and Southern hybridizations, a minimum of 115 h of autoradiography was necessary in order to resolve clearly probe signals. These weak hybridizations indicated that probe LYTC24 hybridized to a low copy genomic MLO DNA sequence, or reflected an overall low concentration of MLO in lethal yellowing-diseased palms, or both.

Restriction site analysis of probe LYTC24 revealed a single internal *Xba*I site yielding two fragments of about 1.3 kbp and 3.2 kbp, respectively. Partial enzymatic sequencing of the smaller subcloned fragment, LYTC24-A, yielded sequence data with an overall A + T composition of about 82% (Fig. 3). Two 25-mer oligonucleotide sequences designated LYF-1 and LYR-1, with an estimated  $T_m$  of 58.3 and 58.7°C, respectively, were synthesized on the basis of the

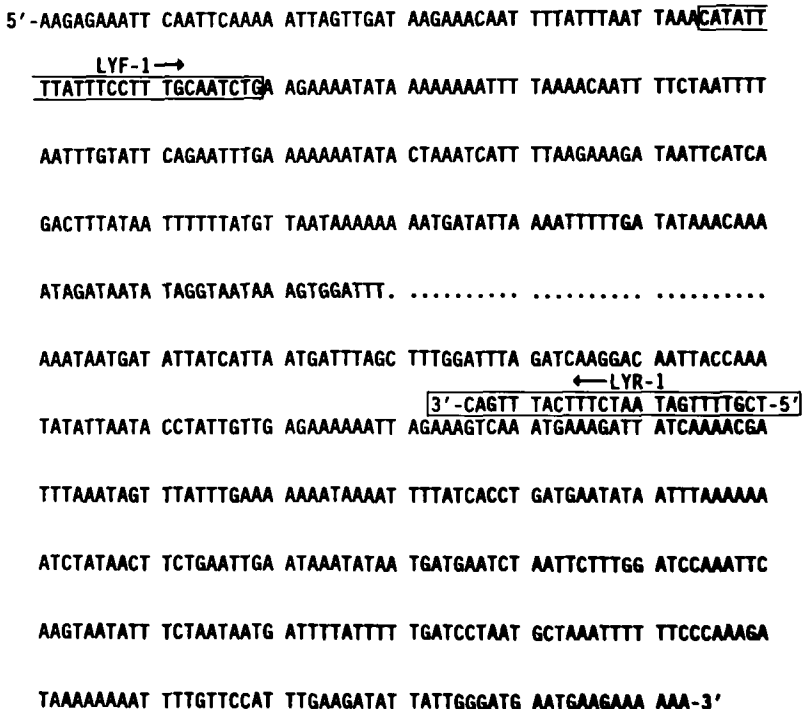
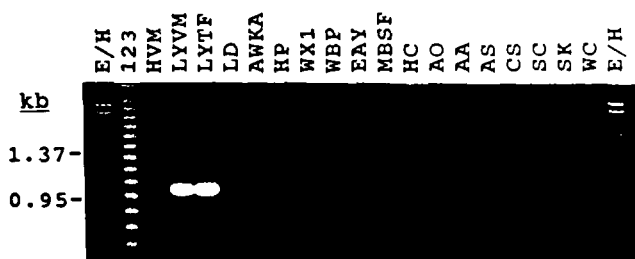


Fig. 3. Partial sequence of subclone LYTC24-A, a 1.3-kbp *Hind*III *Xba*I fragment of lethal yellowing (LY) mycoplasma-like organism chromosomal DNA. The position of primers LYF-1 and LYR-1 used for polymerase chain reactions are indicated in boxes.



**Fig. 4.** Agarose gel electrophoresis of PCR products from healthy plants, plants with various mycoplasma-like organism (MLO)-associated diseases and from culturable mollicutes. PCR products are a result of a 35-cycle reaction using a primer pair derived from a cloned 1.3 kbp fragment of LY (LY) MLO chromosomal DNA. PCR template DNAs were derived from: HVM, healthy *Veitchia merrillii* (Manila palm); LYVM, LY-diseased Manila palm; LYTF, LY-diseased *Trachycarpus fortunei* (windmill palm); LD, lethal disease-affected *Cocos nucifera* cultivar East African Tall from Tanzania; AWKA, Awka disease-affected *C. nucifera* cultivar West African Tall from Nigeria; HP, healthy *Catharanthus roseus* (periwinkle); WX1, periwinkle infected with western X (peach yellow leafroll strain); WBP, periwinkle with pigeon pea witches' broom; EAY, periwinkle with eastern aster yellows; MBSF, sweet corn (*Zea mays saccharata* cv. Aristogold Guardian) with Florida maize bushy stunt; HC, healthy corn; AO, *Acholeplasma oculi*; AA, *A. axanthum*; AS, an uncharacterized *Acholeplasma* sp. (J233) from coconut palm; CS, *Cocos* spiroplasma; SC, *Spiroplasma citri*; SK, *Spiroplasma kunkelii*; WC, water control; E/H, lambda DNA *EcoRI*–*HindIII* fragments; 123, BRL 123 bp ladder.

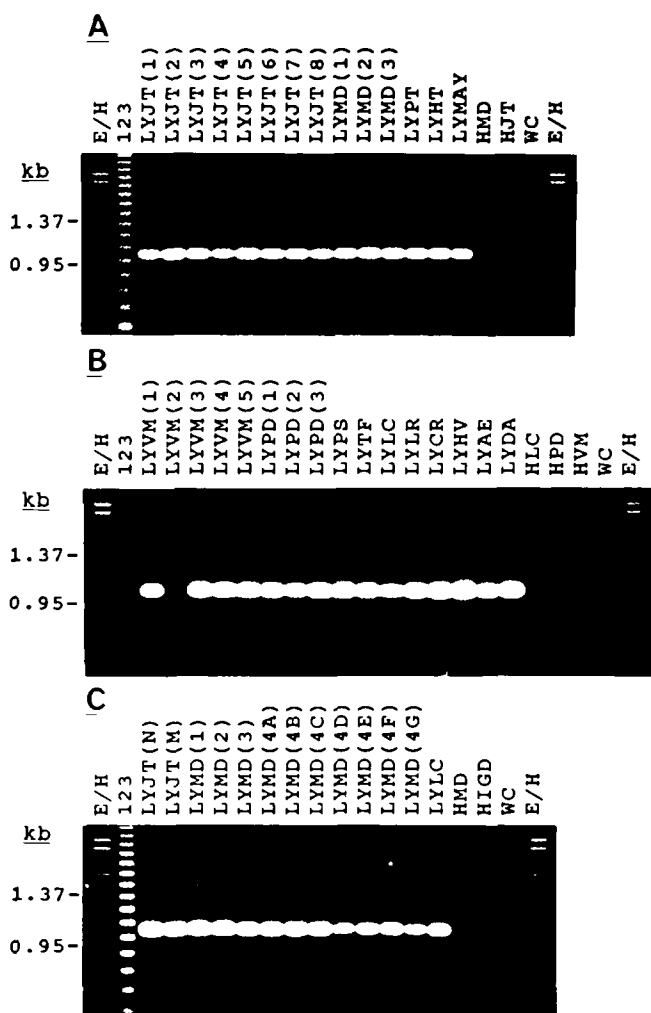
sequence data. When used during initial PCR, this primer pair permitted amplification of a prominent DNA product of about 1 kbp from reaction mixtures containing template DNA derived from heart tissues of both lethal yellowing-diseased Manila and windmill palms (Fig. 4). The size of the DNA product was consistent with its predicted size according to the distance between primer sequences within the original lethal yellowing MLO chromosomal DNA fragment. No product was amplified by PCR from similar mixtures containing template DNA extracted from either healthy Manila palm or from coconut palms with African MLO-associated lethal decline diseases. No DNA product was evident either when template DNA was derived from various other MLO-associated diseases or from culturable achleoplasmas and spiroplasmas.

The reliability of the chosen primer sequences to amplify lethal yellowing MLO DNA was established by PCR analysis of template DNAs from heart tissues of 30 different symptomatic palms. After 35 cycles, amplification of a 1 kbp DNA product was again repeatedly achieved from all palm specimens, which included 14 individuals and five cultivars of coconut palm (Fig. 5A) as well as 10 additional ornamental species comprising 16 palms (Fig. 5B). Amplifications of a similar size product were also obtained when the template for PCR consisted of

DNA from immature, partially necrotic inflorescences of palms exhibiting either primary or early yellowing phase lethal yellowing symptoms (Fig. 5C). No PCR products were amplified from DNAs of healthy palm hearts or from inflorescence DNAs of other symptomless coconut palms.

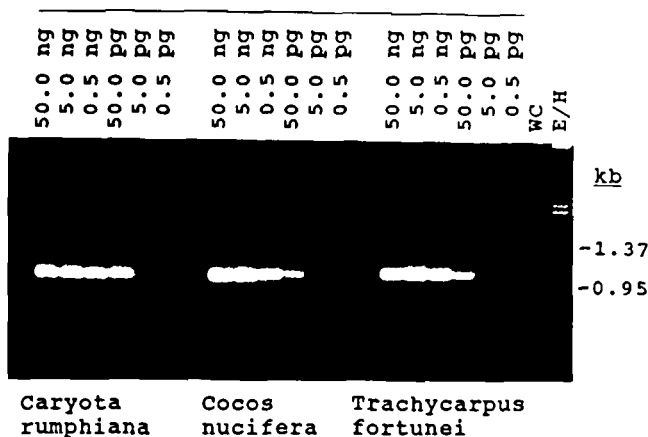
Use of decreasing quantities of DNA extracted from hearts of lethal yellowing-diseased giant fishtail, coconut cultivar Jamaica Tall, and windmill palm for PCR provided a measure of the relative sensitivity of this technique for detection of the lethal yellowing MLO in different palm hosts. After 35 cycles of PCR and agarose gel electrophoresis of 10 µl of each final reaction mixture, amplification of MLO DNA was clearly evident in mixtures which initially contained as little as 5 pg of DNA from each of the three palm species (Fig. 6).

A 40-cycle reaction was required to detect lethal yellowing MLO DNA in 12 of 35 leaflet DNA samples derived from seven symptomatic coconut palms. In two non-bearing coconut palms (cultivar Panama Tall) examined in detail (Fig. 7A, B), MLOs were undetectable in basal leaflets from all but the youngest leaves. When sampling was limited to the spear leaf only, positive detection of the pathogen was achieved for all five additional palms tested (Fig. 7B).

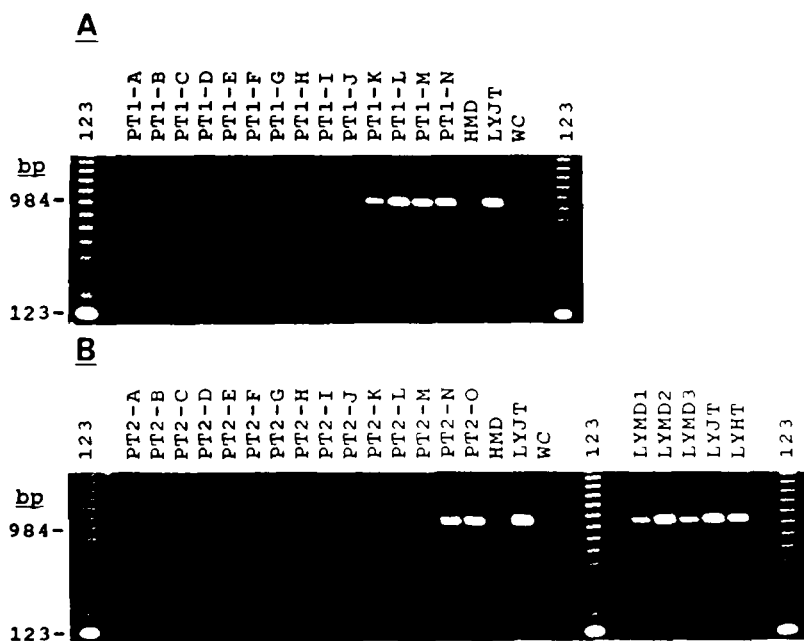


**Fig. 5.** PCR analysis of DNA extracted from tissues of healthy container-grown palms, symptomless landscape palms and landscape palms with mid-stage foliar symptoms indicative of lethal yellowing (LY) disease. After 35 cycles of amplification, one-fifth of each reaction mixture was analysed by 10 mg ml<sup>-1</sup> agarose gel electrophoresis. Template DNA for PCR was derived from heart tissues of healthy and LY-symptomatic *Cocos nucifera* (coconut palm) cultivars (A), or heart tissues of other palm species (B), or immature palm inflorescences (C). (A) LY-diseased *C. nucifera* cultivars: LYJT (1-8), eight Jamaica Tall palms, LYMD (1-3), three Malayan Dwarf palms, LYPT, Panama Tall, LYHT, Hawaiian Tall, LYMY, Maypan hybrid (Malayan Dwarf × Panama Tall), HMD, and HJT, healthy Malayan Dwarf and Jamaica Tall, respectively. (B) LY-affected palm species: LYVM (1-5), five *Veitchia merrillii* (Manila) palms, LYPD (1-3), three *Phoenix dactylifera* (true date) palms, LYPS, *P. sylvestris* (silver date), LYTF, *Trachycarpus fortunei* (windmill palm), LYLC, *Livistona chinensis* (Chinese fan palm), LYLR, *L. rotundifolia* (footstool palm), LYCR, *Carvota rumphiana* (giant lishtal palm), LYHV, *Hyophorbe verschaffeltii* (spindle palm), LYAE, *Arenga engleri* (dwarf sugar palm), LYDA *Dactyosperma album* (princess palm). HLC, HPD, HVM, healthy Chinese fan, true date and Manila palms, respectively. (C) Single inflorescences from LY-symptomatic coconut cultivars: LYJT(N), Jamaica Tall (Naples, FL), LYJT(M), Jamaica Tall (Miami, FL), LYMD (1-3), three Malayan Dwarf palms, LYMD(4A-G), seven successively younger inflorescences from a symptomatic Malayan Dwarf, LYLC, inflorescence from *L. chinensis*. Immature inflorescences from symptomless (apparently healthy) *C. nucifera* cultivars: HMD, Malayan Dwarf, HIGD, Indian Green Dwarf cultivars, WC, water control, E/H, lambda DNA *EcoRI*-*HindIII* fragments, 123, BRL 123bp ladder.

Quantity of template DNA used for PCR



**Fig. 6.** Detection of the lethal yellowing (LY) mycoplasma-like organism DNA by PCR using pathogen-specific primers and PCR template DNA derived from heart tissues of three lethal yellowing-affected palm species. Following 35 cycles of amplification, one-fifth of each reaction mixture was analysed by 10 mg/ml agarose gel electrophoresis. WC, water control; E/H, lambda DNA *Eco*RI-*Hind*III fragments



**Fig. 7.** Detection of lethal yellowing mycoplasma-like organism DNA by PCR in leaves of *Cocos maitera* (coconut) cultivars with primary or yellowing stage symptoms. Template DNA for PCR was derived from laminae of basal leaflets from successively younger leaves of two Panama Tall (PT) cultivars with early foliar yellowing (A,B) and from the youngest (spear) leaf only of five additional palms (B). PT1, PT2, Panama Tall (A, N or A, O, oldest to youngest leaf, respectively); LYMD1, Malayan Dwarf with primary phase symptoms, LYMD(2-3), Malayan Dwarfs with early yellowing phase symptoms; LYJT, Jamaica Tall with spear necrosis only, LYHT, Hawaiian Tall with early yellowing. HMD, healthy Malayan Dwarf, LYJT, heart tissue of lethal yellowing-diseased Jamaica Tall. WC, water control; 123, BRL 123 bp ladder.

## DISCUSSION

The goal of this work was to develop primers for use in LY MLO-specific PCR. The exceptional sensitivity of PCR offers many advantages for detection of plant pathogens (Henson & French, 1993). Application of this technique to detection and investigation of the lethal yellowing MLO seems particularly appropriate because of the very low titres of the MLO in mature palm tissues. Previous studies of lethal yellowing disease have been complicated by several other factors including an inability to culture the pathogen *in vitro*; the ability of the disease to kill palms quickly, and an absence of a means to perpetuate efficiently the disease. Palms remain as the sole source of the lethal yellowing MLO for study since no alternative plant hosts have been identified. Similarly, both presence and multiplication of the lethal yellowing MLO in its putative insect vector, *M. crudus*, and experimental transmission of the MLO to other plants have yet to be demonstrated.

In order to achieve the desired specificity to the PCR we first cloned and identified genomic DNA unique to the lethal yellowing MLO from which sequence information could be exploited for primer design. Detection of MLOs in plant tissues by PCR using primers based upon MLO 16S rRNA sequences has been reported (Deng & Hiruki, 1991b; Ahrens & Seemüller, 1992; Schneider *et al.*, 1993). However, none of these primer sets has thus far enabled pathogen-specific detection, thereby limiting their possible use in searches using PCR for insect and alternative plant hosts of MLOs.

Evaluation of cloned fragments of lethal yellowing MLO DNA by dot hybridizations, using enriched DNA of the western X-MLO and pigeon pea witches' broom MLO as probes, and then by using cloned MLO DNA fragments as probes in reciprocal hybridizations, proved to be a suitable method for identifying lethal yellowing MLO-specific DNA sequences. That we were successful with this approach was attributed to choice of these two particular MLOs for use as both probe and target DNAs. Their selection was based upon recent comparisons of MLO ribosomal RNA gene sequences which indicated close relationships between both MLOs and the lethal yellowing MLO (Ahrens *et al.* 1992; N. A. Harrison, unpublished data).

For the design of pathogen-specific primers, the foremost consideration was to use genomic DNA sequences which would permit amplifica-

tion of a lethal yellowing MLO DNA product of sufficient size (*c.* 1 kbp) that could be conveniently resolved by electrophoresis in standard agarose gels. This was effectively accomplished by selecting a primer pair from sequences of an appropriately sized subclone derived from a larger lethal yellowing MLO-specific probe. The specificity of the chosen primer set was verified during initial PCR in which they failed to amplify any product from target DNAs extracted from other MLOs indigenous to Florida and California, culturable mollicute contaminants of palms, phytopathogenic spiroplasmas, and MLOs associated with African coconut lethal decline diseases. Lack of detection of the last of these Old World coconut pathogens by our PCR complements recent studies which revealed these MLOs to be similar but not identical to the lethal yellowing MLO (Ahrens *et al.*, 1992; Harrison *et al.*, 1994).

Detection of the lethal yellowing MLO was consistent and reproducible for all affected palm species and cultivars examined when template DNAs for PCR were derived from either unemerged inflorescences or hearts. These results agree with earlier ultrastructural observations of MLO distribution in lethal yellowing-diseased palms (Waters & Osborne, 1978; Thomas & Norris, 1980). However, inflorescences are not always available for sampling as immature palms frequently contract lethal yellowing (McCoy *et al.*, 1983). Also, removal of hearts and concomitant death of palms is a particularly unsuitable sampling practice in questionable cases of disease for which diagnostic information provided by PCR has potentially the greatest value. Spear leaves and roots have been reported to contain MLOs once distal portions of these organs showed evidence of necrosis (Waters & Osborne, 1978). The pathogen was rarely observed in mature foliage (Thomas & Norris, 1980) with the exception of flag leaves, in which MLO distribution appeared to be restricted to yellowed portions only (Waters & Osborne, 1978).

In the present study, detection of lethal yellowing MLO infections in predominantly non-symptomatic coconut leaves by PCR confirmed the effectiveness of this technique and indicated that sampling of foliage could provide the necessary practical means for diagnosing lethal yellowing disease in this palm species. The capacity of PCR to detect the lethal yellowing MLO was greatly enhanced by using leaflets of the least mature leaves. Our detection success, in

limited sampling, was 100% for leaflets removed at random from the mid to lower portions of the spear, the most immature leaf. We purposely chose palms exhibiting the earliest visible symptoms indicative of lethal yellowing in order to challenge the detection sensitivity of PCR. Thus, for palms such as the Panama Tall cultivar with early yellowing phase symptoms, necrosis and yellowing were present only in distal portions of one or two of the most mature leaves, leaving basal leaflets unaffected. We were unable to detect the pathogen in these leaflets. However, during a recent investigation of MLO acquisition by phloem-feeding homopterans from coconut palms with mid- to late-stage yellowing symptoms, we frequently detected MLO in yellowed leaflets from mature leaves by PCR (N. A. Harrison, unpublished data). Therefore, our inability to locate the pathogen in mature leaves suggests perhaps an uneven distribution of MLO rather than its absence from these organs.

The sensitivity and convenience of the lethal yellowing MLO-specific PCR should encourage further research on lethal yellowing epidemiology such as detailed studies of vector biology, host resistance and identification of alternative plant hosts which have previously been beyond the reach of the available technology.

## ACKNOWLEDGEMENTS

We are grateful to T. K. Broschat and F. W. Howard, University of Florida, for providing us with samples of healthy and diseased palms. We also thank J. A. Wyman, B. C. Kirkpatrick, M. A. Villanueva and P. Jones for providing additional MLO-infected plants or MLO DNAs and R. F. Whitcomb and J. G. Tully for providing culturable mollicutes. Support for this research was provided by USDA Tropical/Subtropical Agriculture Special Grants Program (grant No. 90-34135-5165 and 92-34135-7281). This paper is published as Florida Agricultural Experiment Station journal series No. R-03392.

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