Detection and diagnosis of lethal yellowing

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1. Introduction

Lethal yellowing (LY) is one of the most important diseases of the coconut palm (*Cocos nucifera* L.) in the American tropics because it spreads rapidly, kills palms quickly and is incurable at present. Presently, LY is restricted in its distribution to the western Caribbean region (McCoy *et al.*, 1983) where epidemics of disease in recent decades have destroyed millions of palms in Jamaica, Florida and southern México. Further spread into Belize (Eden-Green, 1997) and Honduras (Ashburner *et al.*, 1996) in recent years threatens coconut production in Central America because the Atlantic Tall (also known as Jamaica Tall), the most common coconut ecotype found throughout the Caribbean and along the Atlantic coast of the Americas, is highly susceptible to LY (Howard, 1983).

Although localised outbreaks of LY have probably occurred on certain Caribbean islands since the early 19th century (Martyn, 1945a; 1945b), the disease was not recognised as serious problem until coconut became widely cultivated as a plantation crop in Jamaica. The cause of LY remained an enigma until 1972 when phloem-restricted phytoplasmas (also known as mycoplasma-like organisms or MLOs) were identified by transmission electron microscopy (TEM) (Beackbane *et al.*, 1972; Plavsic-Banjac *et al.*, 1972) as the probable etiological agent. Today, phytoplasmas are now known to be associated with diseases of at least 600 plant species worldwide (McCoy *et al.*, 1989; Lee *et al.*, 1998a). All attempts to culture phytoplasmas in cell-free media have failed thus precluding this technique for pathogen detection and disease diagnosis. Instead, pathogen detection has traditionally relied upon TEM because the small size and variable morphology of these cell wall-less organisms usually prevents their definitive visualisation by standard light microscopy techniques.

Table 1. Listing of palm species known to be susceptible to lethal yellowing disease in Florida.

Scientific name	Common name	Region of origin
Aiphanes lindeniana	----	Caribbean
Allagoptera arenaria	Seashore palm	South America
Arenga engleri ★□	Dwarf sugar palm	Southeast Asia
Borassus flabellifer ★□	Palmyra palm	India
Caryota mitis ★□	Clustering fishtail palm	Southeast Asia
Caryota rumphiana ★□	Giant fishtail palm	Southeast Asia
Chelyocarpus chuco ★□	----	South America
Cocos nucifera ★□	Coconut palm	Western Pacific
Corypha elata	Buri palm	India
Crysophila warsecewiczii ★	Rootspine palm	Central America
Cyphophoenix nucele ★	----	Western Pacific
Dictyosperma album ★□	Princess palm	Madagascar
Dypsis cabadae	Cabada palm	Madagascar
Dypsis decaryi	Triangle palm	Madagascar
Gaussia attenuata	Puerto Rican Gaussia palm	Caribbean
Howea belmoreana	Belmore sentry palm	Western Pacific
Howea forsteriana ★	Kentia or Sentry palm	Western Pacific
Hyophorbe verschafeltii	Spindle palm	Madagascar
Latania lontaroides	Latan palm	Madagascar
Livistona chinensis ★□	Chinese fan palm	China
Livistona rotundifolia ★□	Footstool palm	Southeast Asia
Nannorrhops ritchiana	Mazari palm	Asia Minor
Phoenix canariensis	Canary Island date palm	Canary Islands
Phoenix dactylifera ★□	Edible date palm	North Africa
Phoenix reclinata	Senegal date palm	Africa
Phoenix rupicola ★□	Cliff date palm	India
Phoenix sylvestris	Silver date palm	India
Pritchardia affinis	Kona palm	Hawaii
Pritchardia pacifica	Fiji island fan palm	Western Pacific
Pritchardia remota	----	Hawaiian Islands
Pritchardia thurstonii	Thurston palm	Western Pacific
Ravenea hildebrantii	----	Madagascar
Syagrus schizophylla ★	Arikury palm	South America
Trachycarpus fortunei ★□	Windmill palm	China
Veitchia arecina ★	----	Western Pacific
Veitchia merrillii ★□	Christmas palm	Western Pacific
Veitchia mcdanielsi ★	Sunshine palm	Western Pacific
Veitchia montgomeryana ★	Montgomery's palm	Western Pacific

★□ Identity of the LY phytoplasma associated with this particular palm species has been verified by (□) DNA probe hybridisation and/or by (★) LY-specific PCR.

wing disease in Florida.

Region of origin
Caribbean
South America
Southeast Asia
India
Southeast Asia
Southeast Asia
South America
Western Pacific
India
Central America
Western Pacific
Madagascar
Madagascar
Madagascar
Caribbean
Western Pacific
Western Pacific
Madagascar
Madagascar
China
Southeast Asia
Asia Minor
Canary Islands
North Africa
Africa
India
India
Hawaii
Western Pacific
Hawaiian Islands
Western Pacific
Madagascar
South America
China
Western Pacific
Western Pacific
Western Pacific
Western Pacific

species has been verified

Phytoplasma profiles vary from saccate to filamentous when examined by TEM. In coconut, nonfilamentous forms average 295 nm in diameter and filamentous forms average 142 nm in diameter and at least 16 μm in length (Waters and Hunt, 1980). Cells are enclosed by a trilaminar unit membrane and contain cytoplasma with DNA fibrils and ribosomes (Thomas and Norris, 1980). In some host plant species (Seemüller, 1976; Sinclair *et al.*, 1992), including coconut, (Schuiling and Mpunami, 1990; Cardeña *et al.*, 1991) intraphloemic accumulations of phytoplasmas have also observed by fluorescence microscopy after treatment of host tissues with DAPI (4'6-diamidino-2-phenyl indole), a reagent that binds to DNA and fluoresces under UV radiation. Although sensitive, an underlying limitation to TEM and fluorescence microscopy is that both detection techniques are nonspecific and as such do not provide any information about phytoplasma identity.

Within the last decade, significant improvements in methods to extract phytoplasmas or their nucleic acids from both plant and insect hosts have been made (Kirkpatrick *et al.*, 1995). Coupled with the use of molecular biological technologies, this has led to development of novel DNA-based assays enabling sensitive detection and identification of phytoplasmas. These assays have also facilitated studies on disease epidemiology and phytoplasma ecology (Lee *et al.*, 1998a) while providing a means to compare and group these organisms for taxonomic purposes (Lee *et al.*, 1998b). Use of these techniques for pathogen detection, identification and diagnosis of LY are discussed.

2. Phytoplasma detection and identification by DNA probe hybridisation

Besides coconut palm, at least 24 other taxa comprising 37 palm species are known hosts of LY disease in Florida (Table 1). Many of these additional species were first implicated as additional hosts on the basis of symptomatology and TEM evidence of phytoplasma infection (Thomas, 1979; Thomas and Norris, 1980). Detection and identification of the LY phytoplasma in symptomatic coconut and 15 other species has since been achieved by DNA dot hybridisation assays using random fragments of phytoplasma genomic DNA cloned from LY-diseased Manila palm (*Veitchia merrillii*) or windmill palm (*Trachycarpus fortunei*) as probes (Harrison *et al.*, 1992; Harrison *et al.*, 1994c; Escamilla *et al.*, 1995). Sample DNAs for confirming phytoplasma identity were extracted from the bases of unpresented leaves surrounding the apical meristem (heart tissues) of palms. Rich in functional phloem, these tissues are a reliable source of phytoplasma in affected palms (Thomas and Norris, 1980). Detectable quantities of phytoplasma

DNA were obtained by extracting the entire heart (100-200 g of tissue) from each palm using a phytoplasma-enrichment method (Harrison *et al.*, 1992). Dot hybridisation analysis of resulting DNA samples, using LY-specific DNA probe LYTC24 (4.4-kb), revealed considerable differences in phytoplasma titers between palm species. For example, titers in giant fishtail palm (*Caryota rumphiana*) and *Chelyocarpus chuco* titer were as much as 10 to12-fold lower than those of other species such as spindle (*Hyophorbe verschafeltii*), footstool (*Livistona rotundifolia*), and cliff date palm (*Phoenix rupicola*) and approximated those typically encountered in coconut palm (Harrison *et al.*, 1992; Harrison and Oropeza, 1997).

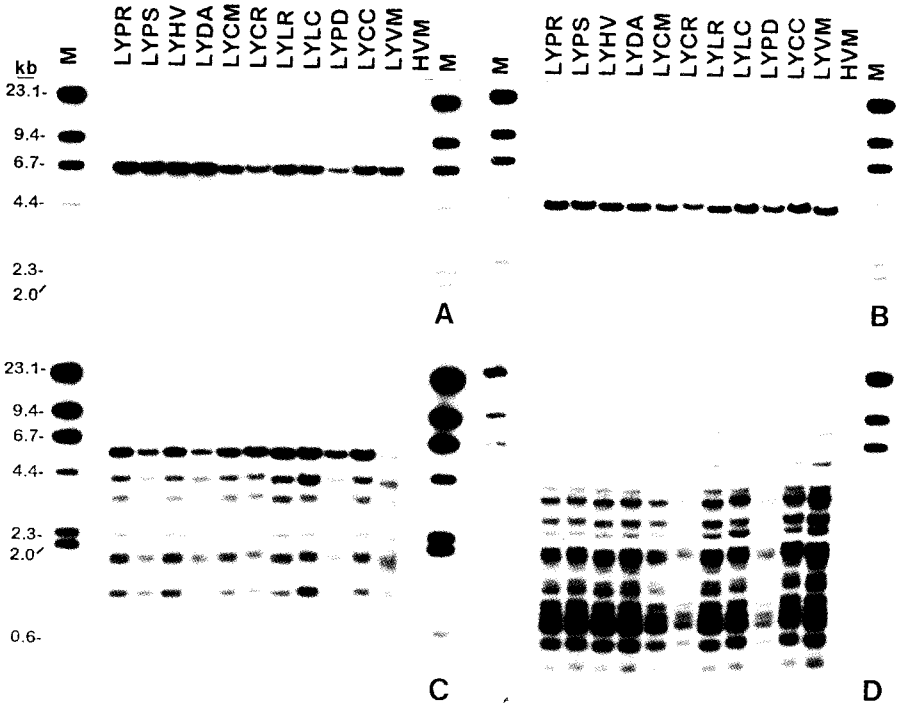

Figure 1. Southern blot analysis of phytoplasma strains associated with various palm species with symptoms indicative of lethal yellowing disease. Replicated blots of DNA derived from immature leaf bases of palms were digested with the restriction endonuclease *Hin*dIII and then screened with [^{32}P]dATP-labeled LY-specific genomic DNA probes: **(A)** LYT-D32 (6.6 kb), **(B)** LYT-C36 (3.3 kb), **(C)** LYT-C13 (5.8 kb) or **(D)** LYT-C19 (1.2 kb).

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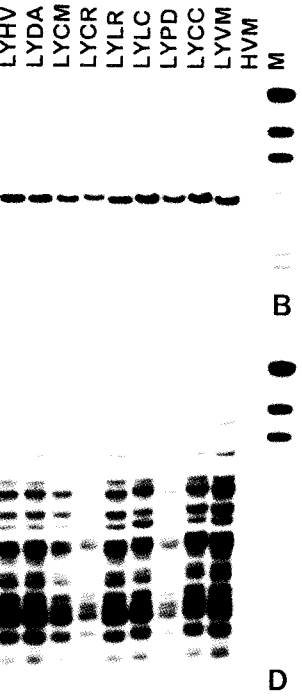

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e *Hin*dIII and then screened with
(6.6 kb), (**B**) LYT-C36 (3.3 kb), (**C**)

Southern hybridisation analysis of phytoplasma DNA restriction profiles with cloned probes (genotyping) can provide a measure of genetic variability among closely related phytoplasma strains (Lee *et al.*, 1992). Genotyping of LY phytoplasma isolates infecting 11 palm species in Florida was similarily accomplished with a selection of 12 genomic DNA probes. Probe hybridisation's to profiles of *Hin*dIII-digested DNAs blots generated either simple patterns indicative of single fragment hybridisation's or complex patterns as a result of multiple fragment hybridisation's (Fig. 1). However, patterns observed for phytoplasma strains associated with all palm species were identical regardless of the particular probe used. Thus, despite the diversity of palm species examined, no evidence of genetic variability among strains associated with these species was obtained. As these data conclusively demonstrated the causal phytoplasmas of lethal declines of ornamental palm species to be synonymous, the observed homogeneity of probe hybridisation patterns also supports the concept that the LY phytoplasma exists as a group of closely related, possibly identical strains, at least in southern Florida.

3. Phytoplasma detection and identification by PCR

Use of the polymerase chain reaction (PCR) to amplify DNA sequences has provided a more sensitive means than DNA hybridisation assays for phytoplasma detection and identification. Primer pairs PCR derived from conserved regions of the ribosomal RNA (rRNA) operon permit amplification of 16S rRNA gene sequences (Deng and Hiruki, 1991; Lee *et al.*, 1993; Gundersen and Lee, 1996) as well as the 16-23S rRNA spacer region (16-23S SR) (Smart *et al.*, 1996) of phytoplasmas in a universal manner. However, the utility of these primers is constrained by the fact that non-phytoplasma target sequences may be coamplified from DNAs of certain plant species, thus preventing unequivocal confirmation of phytoplasma infections in these species. Also, PCR products generated from phytoplasmas with these primer pairs are usually all very similar in size and as such reveal little about phytoplasma identity (Fig. 2).

Further characterization of PCR products is necessary for phytoplasma identification. Restriction fragment profiles resolved by agarose or polyacrylamide gel electrophoresis (PAGE) after digestion of rDNA amplification products with various endonuclease enzymes has been widely used (Lee *et al.*, 1998b). Phytoplasma identification by this method is then determined by direct comparison of resulting profiles with those of known phytoplasma standards included on the same gel. Profiles resolved by PAGE after digestion with *Alu*I,

*Hin*fI, *Taq*I or *Tru*9I endonucleases are especially useful for identifying the LY phytoplasma and for distinguishing this pathogen from phytoplasmas associated with African coconut lethal decline diseases (Le. *et al.*, 1993; Harrison *et al.*, 1994b; Tymon *et al.*, 1997). Restriction fragment length polymorphism (RFLP) analysis of rDNA fragment profiles has also provided a basis for grouping phytoplasmas for classification purposes. Based upon RFLP analysis of 16S rRNA gene sequences, 14 major (16Sr) groups and 32 subgroups of phytoplasmas were delineated in one recent classification scheme (Lee *et al*, 1998b). In this scheme, a Florida isolate of the LY phytoplasma was assigned as a sole representative member to 16Sr group IV (coconut lethal yellows group), subgroup A (16SrIV-A).

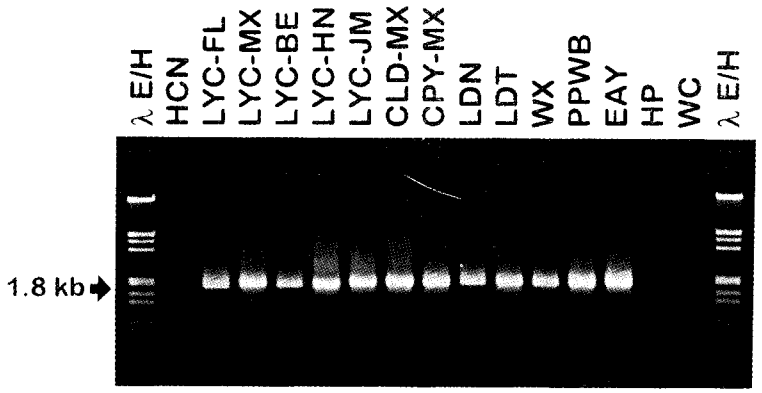

Figure 2. Selective amplification of ribosomal DNA (rDNA) by the polymerase chain reaction (PCR) employing employing phytoplasma universal ribosomal RNA primers P1 and P7. DNA templates for PCR were derived from healthy plants or plants with the following phytoplasma-associated diseases: healthy coconut palm (HCN), lethal yellowing-affected coconut palm from Florida (LYC-FL), Yucatan, Mexico (LYC-MX), Belize (LYC-BE), Honduras (LYC-HN) and Jamaica (LYC-JM); lethal decline-affected coconut from Tabasco, Mexico (CLD-MX), *Carludovica palmata* yellows from Yucatan, Mexico (CPY-MX), Awka disease-affected coconut from Nigeria (LDN); lethal disease-affected coconut from Tanzania (LDT), *Catharanthus roseus* (periwinkle) with western X disease (WX), pigeonpea witches'-broom (PPWB) or eastern aster yellows (EAY); healthy periwinkle (HP), water control (WC). Lambda DNA *Eco*RI-*Hin*dIII molecular size ladder (λ E/H).

Group or subgroup-specific detection of phytoplasmas by utilising primers for PCR derived from variable regions of the 16S rRNA gene (Davis and Lee, 1993) or the 16-23S SR sequence (Smart *et al.*, 1996) or from other regions of phytoplasma genome (Schaff *et al.*, 1992) have been reported. A similar assay based on rRNA sequences has been recently developed for selective detection of

the LY phytoplasma and closely related strains. Primers 503f (5'-CAGCAGCCGCGGTAATACATA-3') and LY16Sr (5'-CAGCAGCCGCGG TAATACATA-3') derived from the 16S rRNA gene of the LY phytoplasma, selectively amplify a 931 bp rDNA product from the LY phytoplasma strains infecting coconut and Pandanus and from the CLD and CPY phytoplasmas (Fig. 3A). Strains can be differentiated further by *Alu*I digestion of the resulting amplification products (Fig. 3B).

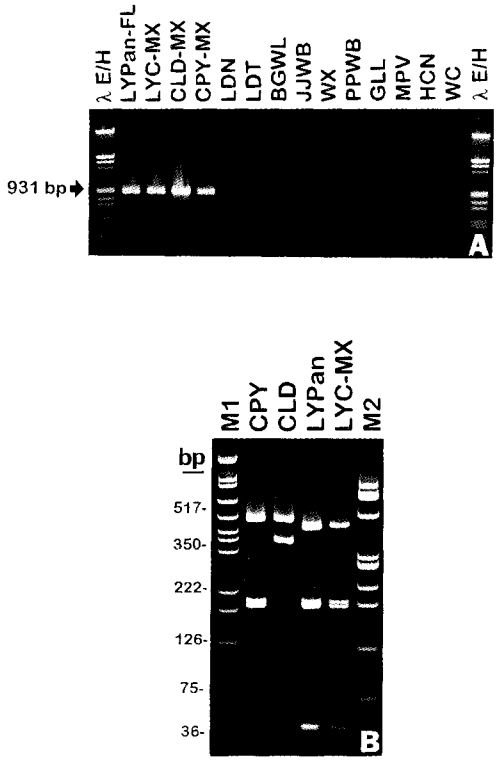

Figure 3. Group-specific amplification of ribosomal DNA by the polymerase chain reaction (PCR) employing a primers derived from the lethal yellowing (LY) phytoplasma 16S rRNA gene (**A**), and differentation of LY group strains by polyacrylamide gel electrophoresis of amplified rDNA products after digestion with restriction endonuclease *Alu*I (**B**). Sample DNAs used as template for PCR were derived from the healthy plants or plants with the following phytoplasma associated diseases: LY-affected *Pandanus utilis* from Florida (LYPan-FL), and coconut palm in Yucatán, México (LYC-MX); Awka disease-affected coconut from Nigeria (LDN); lethal disease-affected coconut from Tanzania (LDT); bermudagrass with white leaf (BGWL) and jujube witches'-broom (JJWB) from China, periwinkle with western X disease (WX) and pigeonpea witches'-broom (PPWB), little leaf of *Gliricidia sepium* from Honduras (GLL), periwinkle virescence from Yucatán, México (MPV). Healthy coconut (HCN) or water (WC) substituted for template DNA served as negative controls. Lambda DNA, *Hin*dIII-*Eco*RI, molecular size ladder (λ E/H); pGem DNA markers (M1) and ϕX174 DNA/*Hae*III markers (M2).

PCR employing pathogen-specific primer pair LYF1/LYR1, derived from a cloned anonymous fragment of LY phytoplasma genomic DNA (Harrison *et al.*, 1994c), has facilitated reliable detection and unequivocal identification of the pathogen in both palms, the arborescent monocot *Pandanus utilis* and vector *Myndus crudus* planthoppers (Harrison *et al.*, 1994a; Harrison and Oropeza, 1997). The sensitivity and specificity of this assay has made possible practical, nondestructive sampling of palms for the purpose of confirming LY diagnoses. For example, in young 4-5 yr old coconut palms with incipient foliar symptoms indicative of LY disease, the pathogen was consistently detected in pinnae samples taken from the spear (youngest leaf). By comparison, positive detections were rarely obtained with pinnae removed from mature leaves of the lower crown (Harrison *et al.*, 1994c; 1995; Harrison and Oropeza, 1997). In a related study, PCR analysis of spear samples from symptomless nonbearing Atlantic Tall coconut palms provided a means to identify phytoplasma infections in several palms between 47-57 days prior to the advent of visible foliar symptoms of LY. Earliest detections of incubating palms during the course of a year long study coincided with the cooler months (November to April) (Harrison *et al.*, 1994a; Harrison and Oropeza, 1997).

Because of the physical difficulty involved in their acquisition, spear leaf sampling for the purpose of confirming LY infection of large symptomatic palms is impractical. Instead PCR analysis of phloem in tissue shavings removed from the lower trunk with an auger or other drill bit appears to be a suitable alternative sampling technique for these palms.

PCR technologies have greatly facilitated sequencing of 16S rRNA genes and other regions of the rRNA operon of phytoplasmas. Sequences have been obtained from amplified rDNA products cloned into vectors or by direct thermal cycle sequencing of PCR products. Phylogenetic analyses of 16S rRNA gene sequences have revealed phytoplasmas to comprise a unique monophyletic group (clade) of organisms within the class *Mollicutes* most closely related to acholeplasmas (Gundersen *et al.*, 1994; Toth *et al.*, 1994) of the anaeroplasma clade (Weisburg *et al.*, 1989). These analyses also delineated as many as 20 major groups (subclades) within the phytoplasma clade (Seemüller *et al.*, 1998) thereby providing important information concerning phytoplasma identity and interrelationships. A formal phylogenetically-based taxonomy of these organisms has been proposed (ICSB, 1993).

The LY phytoplasma (Florida isolate) and coconut lethal decline (LDY) phytoplasma, a distinct, albeit closely related strain from the Yucatán peninsula, México (Harrison and Oropeza, 1997), comprise one (subclade vii) of 11

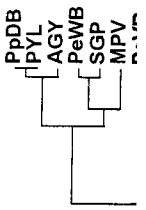

Figure 4. Phylogenetic dendrogram of 16-23S rRNA spacer region sequences of 43 phytoplasmas. The dendrogram was constructed by the unweighted pair group method (UPGMA) in the Phylogeny Inference Package (PHYLIP) version 3.5. Phytoplasma strain acronyms and GenBank nucleotide database sequence accession numbers (from top of dendrogram)

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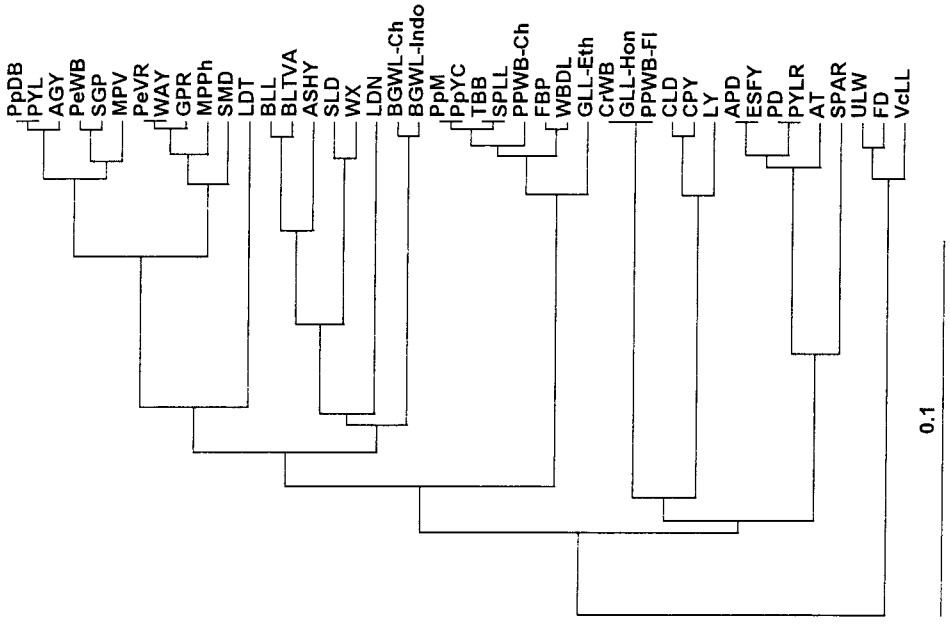

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subclades of phytoplasmas originally resolved by Gundersen *et al.* (1994). Phytoplasmas associated with LY-like diseases of coconut in eastern Africa (lethal disease, Tanzania) and western Africa (Awka disease, Nigeria; Cape St. Paul wilt, Ghana) were found to be phylogenetically distinct from LY and CLD phytoplasmas and therefore assigned to the new subclades xii and xiv, respectively (Tymon *et al.*, 1998).

Similar analyses of another evolutionary marker, the 16-23S SR, revealed phytoplasma groupings comparable to those obtained by 16S rRNA phylogenetic analyses (Kirkpatrick *et al.*, 1994). Phytoplasma 16-23S SRs are variable in size (ca. 220-260 bp) and considerably smaller than 16S rRNA genes (1.5 kb). As such, the 16-23S SR sequences can be readily obtained in their entirety from PCR-amplified rDNA template by thermal cycle sequencing on a standard size sequencing gel and resolved by using a Silver Sequence DNA sequencing system (Promega) (Wongkaew *et al.*, 1997; Kenyon *et al.*, 1998). By this means, a phytoplasma strain detected in association with a yellows disease of the palm-like *Carludovica palmata* (Cyclanthaceae) in southern México was identified as a new additional member of the lethal yellowing group (Fig. 4).

4. Conclusions

Development of molecular diagnostic assays based upon DNA probe hybridisation and PCR has significantly enhanced detection of phytoplasmas, especially in woody perennial plant hosts such as coconut palm which usually contain low pathogen concentrations. Assays developed initially to detect and characterise phytoplasma strains infecting palms and *Pandanus* (*P. utilis*) (Thomas and Donselman, 1979) in Florida similarly detect strains in tall-type or hybrid coconut palms with typical LY symptoms in Jamaica, Belize, México and Honduras. While low phytoplasma concentrations have prevented definitive genotypic characterization of coconut-infecting strains by DNA probe hybridisation, the collective evidence derived from detections by pathogen-specific PCR and RFLP-typing or sequence analysis of PCR-amplified rDNA, indicates that strains inducing typical LY in Florida and elsewhere in the Caribbean region are synonymous.

Both DNA probe hybridisation and PCR are well suited for assessing large numbers of samples which should facilitate detailed studies on vector biology, plant host range, host resistance and disease indexing schemes. The sensitivity and convenience of PCR make it particularly attractive for these purposes. Recent

improvement in phytoplasma detection by use of nested PCR assays employing rRNA primer pairs has been reported (Lee *et al.*, 1994; Gundersen and Lee, 1996; Marcone *et al.*, 1996). These assays show considerable promise for more reliable identification of incubating palms and should facilitate survey work to monitor disease spread. Successful detection of the LY phytoplasma in native *M. crudus* planthoppers in Florida (Harrison and Oropeza, 1997) has shown that systematic examination of putative vectors of LY in other areas of the Caribbean is possible. Furthermore, the capability of PCR to detect phytoplasma in salivary glands excised from single vector insects (Liefting *et al.*, 1997) may provide a convincing alternative to controlled experimental transmissions for unequivocal determination of vector identity.

The finding of phytoplasmas in declining coconut palm and the cyclanth *C. palmata* which are phylogenetically very similar to the LY agent adds to the diversity of strains comprising the coconut lethal yellows taxonomic group. The fact that both pathogens were identified in the Yucatán peninsula of México in areas previously unaffected by LY, and the involvement of phylogenetically distinct phytoplasmas with coconut diseases in Africa (Tymon *et al.*, 1998) and southeast Asia (P. Jones, pers. comm.) supports the hypothesis that LY is a disease of New World rather than Old World origin.

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