CHAPTER

10

Coconut Lethal Yellowing

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ABSTRACT

Lethal yellowing (LY) is a devastating disease that affects at least 35 species of palms throughout the Caribbean region. Over the last 50 years, its effects have been most conspicuous on coconut (*Cocos nucifera*) palm due to its abundance and importance of this species to the rural economies in the region. The etiological agent of LY is an unculturable phytopathogenic mollicute belonging to the genus Phytoplasma. This small, cell wall-less bacterium parasitizes the phloem of host palms and is transmitted to palms in a circulative-propagative manner by the vector planthopper *Myndus crudus*. Confirmation of preliminary LY diagnoses based on symptoms has traditionally relied upon observation of phytoplasma cells within phloem sieve elements by electron microscopy (EM). Improvements in extraction methods coupled with development of novel DNA-based assays have enabled sensitive detection and specific identification of LY phytoplasma in host tissues, facilitated studies on disease epidemiology and phytoplasma ecology, and provided a means to differentiate and classify these organisms for taxonomic purposes. Evidence provided by DNA hybridization and polymerase chain reaction (PCR) assays indicates that LY phytoplasma exists as a group of near identical strains within the Caribbean region. Collectively, these strains are phylogentically distinct from phytoplasmas associated with lethal yellowing-like diseases of coconut that occur in Africa or southeast Asia.

Key Words: Mollicute, plant disease, phytoplasma, coconut, palms, lethal yellowing

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1. INTRODUCTION

Lethal yellowing (LY) is a fast-spreading, fatal disease that affects coconut (*Cocos nucifera*) and numerous other palm species in the Americas (Harrison *et al.*, 2004; McCoy *et al.*, 1983). LY has been known in parts of the western Caribbean region since the late 19th century (Eden-Green, 1997a). However, epiphytotics of the disease in Jamaica, Florida and Mexico during the decades of the 1970s and 1980s killed millions of the once prevalent 'Jamaica tall' (= 'Atlantic tall' ecotype) coconut palms in these localities causing serious economic hardship for those whose income relies on coconut. Historically, the geographic range of LY has also included the Rio Grande valley of Texas, USA (McCoy *et al.*, 1980), the Bahamas, Cayman Islands, (Eden-Green, 1997a), Cuba (Llauger *et al.*, 2004), Dominican Republic (Harries *et al.*, 2001) and Haiti (Leach, 1946). Spread into previously unaffected regions has continued in recent years. Today, the disease is most active along the Atlantic coasts of Belize (Eden-Green, 1997a), Guatemala (Meija *et al.*, 2004), and Honduras (Ashburner *et al.*, 1996). Recurrent diseases of coconut resembling LY have been recorded elsewhere in the tropics under a variety of names depending on location. These include Awka wilt (Nigeria) (Ekpo and Ojomo, 1990), Cape St. Paul wilt (Ghana) (Dery and Philippe, 1997), Kaïncopé (Togo) (Dabek *et al.*, 1976), and Kribi (Cameroun) (Dollet *et al.*, 1977) in West Africa; lethal disease (Kenya, Tanzania and Mozambique) in East Africa (Mpunami *et al.*, 1999; Schuiling and Mpunami, 1990); Kalimantan wilt (Central Kalimantan), Natuna wilt (Natuna Islands) and Malaysian wilt (Peninsula Malaysia) (Eden-Green, 1997b; Jones *et al.*, 1999; Warokka, 1999) in southeast Asia. Collectively, these diseases are referred to as "lethal yellowing-type diseases" and, along with LY, threaten global coconut production because they kill palms quickly and are incurable (Eden-Green, 1997a; Harries, 1978).

2. SYMPTOMS

On the highly susceptible Atlantic tall ecotype, for which symptomatology has been studied most extensively, primary infection is followed by a prolonged latent (incubation) phase estimated between 210-450 days in duration in mature palms (Heinze *et al.*, 1972; Romney, 1972) and 112-262 days in young, nonbearing palms (Dabek, 1975). About 80 days prior to the appearance of symptoms, growth of affected palms is measurably stimulated. A period of gradual decline ensues and complete inhibition of growth occurs about one month before the end of the incubation phase. The earliest stages of LY are accompanied by numerous biochemical and physiological abnormalities that include marked fluctuations in respiration, total sugars and reducing sugars in roots.

Decreased respiration and increased root necrosis occur before any noticeable symptoms appear on above ground portions of palms. The onset of visible symptoms also coincides with alterations in phloem flux rates, changes in water relations due to irreversible suppression of leaf stomatal conductance and a reduction in photosynthetic capacity of leaves marked by decreases in photosynthetic pigments, growth regulators and activity of enzymes of the carbon reduction cycle (Dabek and Hunt, 1976; Islas-Flores, 1999; León *et al.*, 1996; Martinez *et al.*, 2000; Maust *et al.*, 2003; Oropeza *et al.*, 1995).

Visible symptoms of LY disease begin with a premature shedding of most or all fruit regardless of developmental stage. Aborted fruit usually develop a brownish black calyx-end rot reducing seed viablity (Fig. 1). The next symptom is inflorescence necrosis which is most readily observed on newly mature inflorescences as they emerge from the ensheathing spathe. Normally light yellow to creamy white in color, inflorescences instead appear partially to totally blackened. As disease progresses, additional

Fig. 1. Chronology of symptoms of lethal yellowing on coconut palm. A, premature nufall; B, inflorescence necrosis; C, yellowing of oldermost leaves; D, yellowing successively younger leaves and spear necrosis; E, advanced yellowing and loss of the entire crown.

emergent and unemerged infloresences show more extensive necrosis and may be totally discolored. Such symptom intensification results in death of most male flowers and an associated lack of fruit set. Leaf yellowing usually begins once necrosis has developed on two or more inflorescences and is markedly more rapid than that associated with normal leaf senescence. Beginning with the older lowermost leaves, yellowing advances upward to involve successively younger leaves nearest the center of the crown. Occasionally a mid-crown leaf will discolor (flag leaf) early in this sequence although this is an inconsistent symptom. Stomates of yellowed leaves remain closed thus leaves remain turgid throughout the day. Affected leaves eventually turn brown, dessicate and often hang down forming a skirt around the trunk for several days before falling to the ground. A putrid basal soft rot of the newest leaf (spear) occurs once foliar yellowing has reached an advanced stage. Spear leaf collapse and an associated rot of the underlying apical meristem or bud invariably occurs after which the remaining crown perishes and eventually topples away leaving a bare trunk standing. Infected palms usually die within three to six months after the onset of visible symptoms (Harrison and Jones, 2003; McCoy *et al.*, 1983).

Diagnosis of LY based on field observations of affected palms may be complicated by various factors. For example, nonbearing palms lack the initial fruit and flower symptoms. Foliar discoloration also varies among coconut ecotypes and hybrids. Generally, on most tall-type coconuts, leaves turn a distinctive yellow before dying whereas on dwarf ecotypes and some hybrids with green foliage, leaves do not yellow, instead they turn reddish to grayish brown. Occasionally, leaflets on the green form of Malayan dwarf may be become noticeably flaccid and fold around the mid-vein thereby imparting an overall wilted appearance to the palm crown (Harrison and Jones, 2004).

Premature loss of fruit and inflorescence necrosis are early stage symptoms common to all palm species affected by LY. However, differences among species in the stage at which spear leaf collapse occurs have been noted (McCoy *et al.*, 1983). For edible date palm (*Phoenix dactylifera*), death of the spear usually precedes foliar discoloration whereas for Christmas palm (*Adonidia merrillii*) and related *Veitchia* sp., the spear remains unaffected until most or all other leaves have died. Among other susceptible palm hosts, two general patterns of leaf discoloration are recognized. Leaves turn yellow before dying in species such as fishtail palms (*Caryota* sp.), palmyra palm (*Borassus flabellifer*), gebang palm (*Corypha elata*), fan palms (*Livistona* sp. and *Pritchardia* sp.), princess palm (*Dictyosperma album*), windmill palm (*Trachycarpus fortunei*) and round leaf palm (*Chelyocarpus chuco*). On most other susceptible species, leaves turn varying shades of brown rather than yellow. For species such as *A. merrillii, B. flabellifer,*

Caryota sp. and *Phoenix* sp., leaf discoloration begins along the leaflet margins as a brownish stain. Within days, affected leaves yellow or turn brown and wither. Some petioles may buckle and leaves collapse but persist on the palm while younger leaves remain green and upright (Fig. 2).

3. CAUSAL ORGANISM

A phytoplasma is the accepted cause of LY based on its consistent detection in diseased but not healthy palms by electron microscopy (EM) (Plavsic-Banjac *et al.*, 1972, Thomas, 1979), a remission of symptoms on palms in response to tetracycline but not penicillin antibiotic therapy (McCoy *et al.*, 1982), and lack of involvement of any other pathogen. Phytoplasmas are cell wall-less, endocellular bacteria morphologically resembling members of the *Mollicutes*. They are bead-like, filamentous or multi-branched in appearance when observed by EM (Thomas, 1979; Thomas and Norris, 1980) and colonize phloem tissues of host plants as well as tissues and organs, including salivary glands, of their respective insect vectors which transmit these diseases. Estimates of their genome sizes vary from 530 to 1,350 kb (Marcone *et al.*, 1999) and the G+C content of phytoplasma DNA is 23 to 29.5 Mol% (Kollar and Seemüller, 1989); values that are within the known range for *Mollicutes*. To date, sustained *in vitro* culture outside of their plant or insect hosts has not been demonstrated for any phytoplasma. Without confirmation of identity through cultural techniques, phytoplasmas were formerly designated as mycoplasma-like organisms (MLOs) due to their morphological and ultrastructural similarities with mycoplasmas. However, presence of a characteristic oligonucleotide sequence (CAAGAYBATKATGTKTAGCYGGDCT) in the 16S rRNA gene, coupled with functional UGA codons in the ribosomal protein genes, has determined that phytoplasmas clearly represent a distinct monophyletic clade within the *Mollicutes* for which the the genus name '*Candidatus* Phytoplasma' gen. nov. has been recently adopted (IRPCM, 2004).

Sensitive and informative molecular diagnostics have largely replaced EM as the preferred method for detecting phytoplasmas in plants, especially woody perennial hosts such as palms, in which they usually occur in low abundance (Harrison *et al.*, 1995; 1999). Amplification of phytoplasma DNA from host tissues by polymerase chain reaction (PCR) assays and restriction fragment length polymorphism (RFLP) analysis of PCR products has been widely adopted for identification and classification of phytoplasmas (Lee *et al.*, 2000). The LY agent represented one of 14 major groups (16Sr groups) of phytoplasmas delineated by RFLP analysis of PCR-amplified 16S ribosomal RNA (rRNA) gene sequences and was assigned to RFLP group 16SrIV (coconut lethal yellows group) in a classification scheme developed
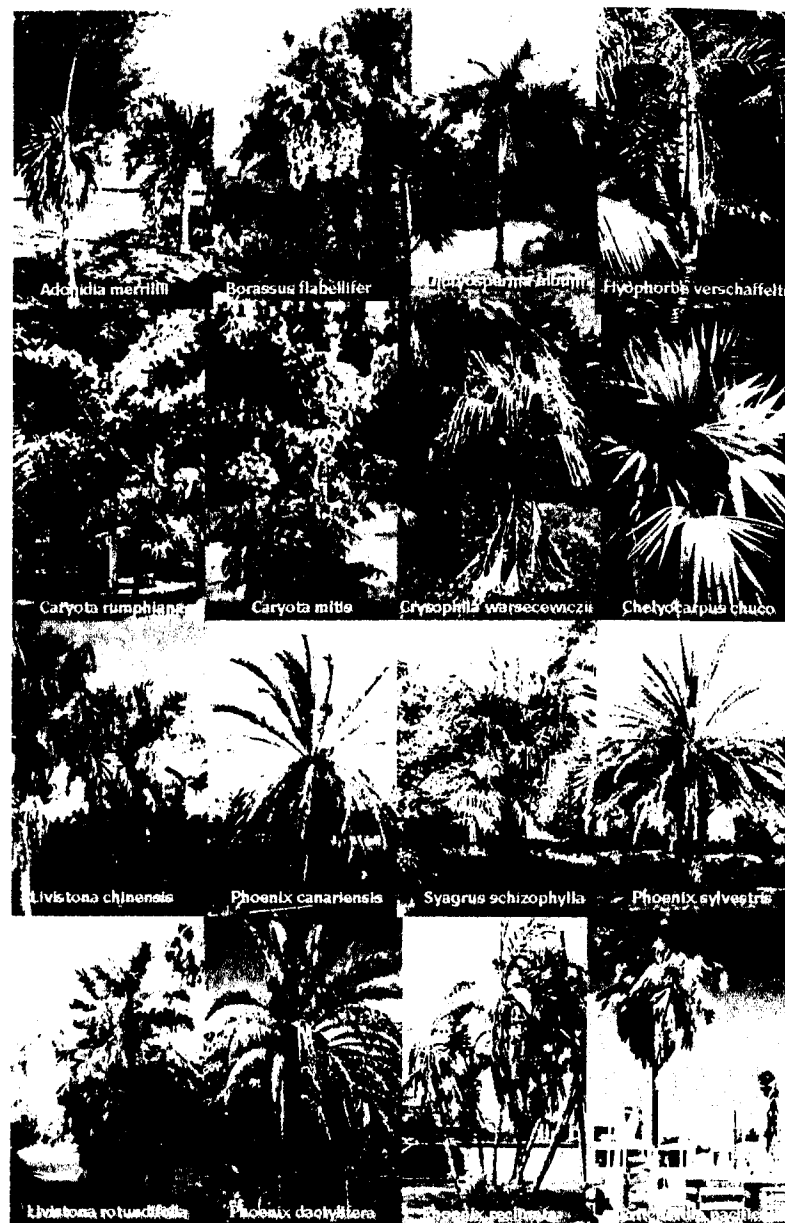
Fig. 2. Variations in appearance of lethal yellowing disease symptoms on ornamental palm species.

by Lee *et al.* (1993; 1998).

Phytoplasma genomes contain two rRNA operon copies (Schneider and Seemüller, 1994) and heterogeneity between 16S rRNA gene copies has been demonstrated in some phytoplasmas (Davis *et al.*, 2003; Jomantiene *et al.*, 2002; Liefting *et al.*, 1996). This genetic trait exists in LY phytoplasma populations resident in Florida, Mexico and Honduras but is uniformly absent from populations affecting coconut in Jamaica (Harrison *et al.*, 2002a). Phytoplasma strains closely related to but distinguishable from the LY agent have also been identified in recent years. Several of these strains have classified by rDNA RFLP typing and, on this basis, assigned to group 16SrIV as subgroup members (Lee *et al.*, 1998). Presently, group 16SrIV comprises the following subgroup strains: 16SrIV-A, coconut lethal yellowing (LY); 16SrIV-B, Yucatan coconut lethal decline (LDY); 16SrIV-C, Tanzanian coconut lethal decline (LDT); 16SrIV-D, *Carludovica palmata* leaf yellowing (CPY) (Cordova *et al.*, 2000) and Texas Phoenix decline (TPD) (Harrison *et al.*, 2002b).

Phytoplasma groups and subgroups resolved by rDNA RFLP typing have been largely supported by classifications based on phylogenetic analysis of 16S ribosomal RNA genes (Gundersen *et al.*, 1994; Lee *et al.*, 2000). The latter approach has since been adopted as a basis for a formal taxonomy of phytoplasmas (IRPCM, 2004). Within the phytoplasma clade, as many as 32 distinct subclades (putative species) of phytoplasmas have been resolved (Seemüller *et al.*, 2002) for which species names under the provisional status of *'Candidatus'* are currently being assigned (IRPCM, 2004). The LY agent, proposed as the type strain of subclade vii (Gundersen *et al.*, 1994) for which the name *'Ca.* Phytoplasma palmae' has since been suggested (IRPCM, 2004), consists of two distinct lineages (Fig. 3). One lineage is composed solely of 16SrIV-A subgroup strains that induce symptoms typical of LY disease on coconut, other palm species (McCoy *et al.*, 1983) and the arborescent monocot, *Pandanus utilis* (Pandanaceae) (Thomas and Donselman, 1979). A second lineage is composed of strains that induce symptoms atypical of LY (i.e. lethal decline (YLD) and leaf yellowing (CLfY) syndromes) on coconut in southern Mexico (Harrison, 1997; Harrison *et al.*, 2002b), lethal decline (TPD) of Canary Island date palm (*Phoenix canariensis*) in Corpus Christi, southern Texas (Harrison *et al.*, 2002c), leaf yellowing and decline (CPY) of the arborescent monocot *C. palmata* (Cyclanthaceae) in southern Mexico (Cordova *et al.*, 2002) and lethal decline (KPD) of Kentia palm (*Howea forsteriana*) in southern Florida.

Similar studies on lethal yellowing-type diseases of coconut in Africa have since clarified relationships among causative phytoplasmas while revealing them to be phylogenetically distinct from the LY agent (Harrison *et al.*, 1994a; Mpunami *et al.*, 1997; Tymon *et al.*, 1997;1998). Strains associated

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                                                           ┌────────────── Kentia decline-Florida-B
                                                    ┌──────┤
                                                    │      └──── Carludovica palmata leaf yellowing-Mexico-C14
                                              ┌─────┤
                                              │     └──── Carludovica palmata leaf yellowing-Mexico-C13
                                              ├───────────── Coconut leaf yellowing (MA3)-Mexico
                                          Texas Phoenix decline-Texas
                                              ├──────────────── Kentia decline-Florida-A
                                              │    ┌───── Coconut leaf yellowing (Oax65)-Mexico
                                              │    ├───── Coconut leaf yellowing (MA5)-Mexico
                                    ┌─────────┤    ├ Coconut lethal decline-Dominican RepublicR
                                    │         │    └ Coconut lethal decline-Mexico
                          ┌─────────┤         └ Coconut leaf yellowing (Oax56)-Mexico
                          │         │    ┌────────── Coconut leaf yellowing (Alv1)-Mexico
                          │         └────┤
              ────────────┤              └ Coconut leaf yellowing (Alv2)-Mexico
                          │              ┌ Coconut LY-Jamaica
                          └──────────────┤  ┌── Coconut LY-Cuba
                                         └──┤  ┌─ Coconut LY-Honduras-A
                                            └──┤  ┌─ Coconut LY-Florida-C5
                                               └──┤ ┌ Ccoconut LY-Mexico
                                                  └─┤ Coconut LY-Belize
                                                    │ Coconut LY-Honduras-B
                                                    │ Pandanus decline-Florida
                                                    └ Coconut LY-Florida-C2
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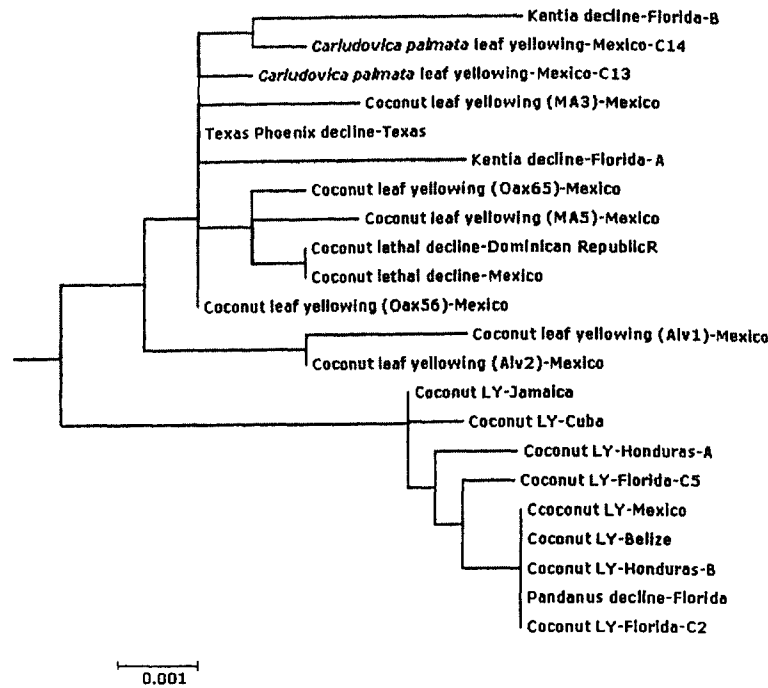
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Fig. 3. The coconut lethal yellowing subclade constructed by phylogenetic analysis of phytoplasma of 16S rRNA genes using the Neighbor-Joining method
*Disease names, phytoplasma strain acronyms and GenBank accession numbers of available phytoplasma 16S rRNA gene sequences are given.
Scale bar represents a phylogenetic distance of 0.1%.

with coconut lethal disease (LYD) in Tanzania and Kenya were found to be coidentical and distinct from LYD phytoplasma in Mozambique (Mpunami *et al.*, 1999). The latter proved to be most closely related to West African strains that are associated with Cape St. Paul wilt (CSPW) in Ghana and Awka or bronze leaf wilt of coconut in Nigeria.

4. HOST RANGE AND EPIDEMIOLOGY

In Florida where a great diversity of palms are grown as valued landscape ornamentals, at least 35 palm species besides coconut are known to be affected by LY (Table 1).

For most of these species, there is insufficient information to adequately evaluate their relative susceptibility. The few exceptions include *A. merrilli,*

Table 1. Listing of palm species suceptible to lethal yellowing disease in Florida

Scientific name	Taxonomic authority	Common name	Region of origin
Adonidia merrillii	(Becc.) Becc.	Christmas or Manila palm	Western Pacific
Aiphanes lindeniana	(H. Wendl.) H. Wendl.	Ruffle palm	Caribbean
Allagoptera arenaria	(Gomes) Kuntze	Seashore palm	South America
Arenga engleri	Becc.	Dwarf sugar palm	Southeast Asia
Borassus flabellifer	L.	Palmyra palm	India
Caryota mitis	Lour.	Clustering fishtail palm	Southeast Asia
Caryota rumphiana	Mart.	Giant fishtail palm	Southeast Asia
Chelyocarpus chuco	(Mart.) H. E. Moore	Round leaf palm	South America
Cocos nucifera	L.	Coconut palm	Western Pacific
Corypha taliera	Roxb.	Buri palm	India
Crysophila warsecewiczii	(H. Wendl.) H. E. Bartlett	Rootspine palm	Central America
Cyphophoenix nucele	H. E. Moore	Lifou palm	Western Pacific
Dictyosperma album	(Bory) H. Wendl. & Drude ex Scheff.	Princess palm	Madagascar
Dypsis cabadae	(Moore) Beentje & J. Dransf.	Cabada palm	Madagascar
Dypsis decaryi	(Jum.) Beentje & J. Dransf.	Triangle palm	Madagascar
Gaussia attenuata	(O. F. Cook) Becc.	Puerto Rican Gaussia palm	Caribbean region
Howea belmoreana	(C. Moore & F. Muell.) Becc.	Belmore sentry palm	Western Pacific
Howea forsteriana	(C. Moore & F. Muell.) Becc.	Kentia palm	Western Pacific
Hyophorbe verschaffeltii	H. A. Wendl.	Spindle palm	Madagascar
Latania lontaroides	(Gaert.) H. E. Moore	Latan palm	Madagascar
Livistona chinensis	(Jacq.) R. Br. ex Martius	Chinese fan palm	China
Livistona rotundifolia	(Lam.) Mart.	Footstool palm	Southeast Asia
Nannorrhops ritchiana	(Griff.) Aitch.	Mazari palm	Asia Minor
Phoenix canariensis	Chabaud	Canary Island date palm	Canary Islands
Phoenix dactylifera	L.	Edible date palm	North Africa
Phoenix reclinata	Jacq.	Senegal date palm	Africa
Phoenix rupicola	T. Anders.	Cliff date palm	India
Phoenix sylvestris	(L.) Roxb.	Silver date palm	India
Pritchardia affinis	Becc.	Kona palm	Hawaii
Pritchardia pacifica	Seeman & Wendl.	Fiji island fan palm	Western Pacific
Pritchardia remota	Becc.	Remota loulu palm	Hawaiian Islands
Pritchardia thurstonii	F. Muell. & Drude	Thurston palm	Western Pacific
Ravenea hildebrantii	Bouche ex Wendl.	Hildebrandt's palm	Madagascar
Syagrus schizophylla	(Mart.) Glassman	Arikury palm	South America
Trachycarpus fortunei	(Hook.) H. Wendl.	Windmill palm	China
Veitchia arecina	Becc.	Montgomery's Palm	Western Pacific

P. dactylifera and *Pritcharida* sp. which have sustained sufficient losses over time that they are not recommended for widespread landscape use.

Most of what is known about spread of LY disease has been learned from studies of its effects on populations of highly susceptible Atlantic tall coconuts. Following an initial disease outbreak, two patterns of disease spread have been observed. One pattern involves a localized center or focus of infection that develops in one or two palms only, followed by the appearance of new cases at random among palms surrounding the initial center, thereby extending local spread. The second is a 'jump-spread' pattern involving distances varying from a few to 70 km or more (Carter, 1964), followed by appearance of new disease foci and establishment of local spread. Jump-spread is characterstic of disease dissemination by an airborne vector insect. Differences in the rates of long distance dispersal of LY at different locations have been noted. In southeasern Florida, spread of the disease from Miami to Palm Beach, a distance of about 128 km occurred within 3 years (McCoy *et al.*, 1983). In Jamaica, however, the disease required about 60 years to move from the west to the east end of the island, a distance of approximately 238 km. In México, LY spread about 900 km westward from the Cozumel-Cancun area, where the disease was first observed in 1979, to the Campeche-Tabasco border required about 15 years (Oropeza and Zizumbo, 1997).

McCoy *et al.* (1976) surveyed disease spread and apparent infection rates during the ephiphytotic of LY in urban areas of Miami-Dade County, Florida in the early 1970s. He noted that after an initial lag phase, the number of diseased palms increased incrementally in a logarithmic fashion. It was estimated that each affected coconut palm in a primary focus of infection served to infect 4.6 new palms during the first 8 months of the developing epiphytotic, and 9.3 new palms within 2 years. Of the estimated original population of 350,000 coconut palms, 0.015% of the palms were already diseased when the survey began; 0.6% had sustained LY by the end of 1972; almost 6% by the autumn of 1973; 50% by the end of 1974; and 75% by the end of 1975. Within localized areas of southeastern Florida, the apparent rates of spread of LY disease were measurably lower among palms situated adjacent to saltwater than palms at inland sites under high cultural maintenance (McCoy *et al.*, 1983), but the reason for this relationship was never adequately determined.

Gradients of LY spread within a coconut grove and between coconut groves, along with the pattern of palm-to-palm spread have also been investigated in Yucatan, Mexico. There it was found that as the proportion of infected palms involved in an outbreak increased, the greater the distance the disease spread from the outbreak and did so as a symmetrical radial gradient (Gongora *et al.*, 2001). For long distance dispersal between groves,

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gradients proved to be asymmetrical and related to prevailing wind direction. Since the prevailing direction is east-west in Yucatan, LY spread was greater to the west than to the east (Mora and Escamilla, 2001). When LY was monitored by visual symptoms alone, palm- to-palm spread was randomly distributed within the first 10 months of study, began to form aggregates after 12 months, and eventually became uniformly distributed throughout the study area (Perez *et al.*, 2000). However, when pathogen detection by molecular diagnostics was used to follow palm-to-palm spread, evidence of aggregate formation was obtained earlier, at a time according to symptoms when LY distribution was still random (Canché, 2002).

There is persuasive evidence implicating the cixiid *Myndus crudus* Van Duzee (Fig. 4) as a vector of LY in Florida. Surveys of insect populations established that *M. crudus* was by far the most abundant potential vector on coconut palms and that populations of this planthopper species were as much as 40 times higher in areas of high LY incidence than in disease-free areas (Howard, 1980). When coconut and other palms in insect-proof cages were exposed to *M. crudus* adults gathered from palms in LY-affected areas, transmission of LY to most test palms occurred within 34 months, whereas similar palms protected from *M. crudus* remained healthy (Howard *et al.*, 1982). Refinements to transmission experiments resulted in more efficient transmission of LY to younger palms (Howard *et al.*, 1984). Distribution of *M. crudus* coincides with the known distribution of LY in the Americas (Howard, 1983) lending further support to its role as a vector. However, numbers of this species on coconut palms at LY-active sites in southern Mexico were found to be several-fold lower than those of many other potential vectors (Escamilla *et al.* 1994). Coupled with the fact that transmission of LY by *M. crudus* has not been confirmed experimentally in LY-affected areas other than Florida, this observation suggests that the involvement of additional vector species cannot be discounted.
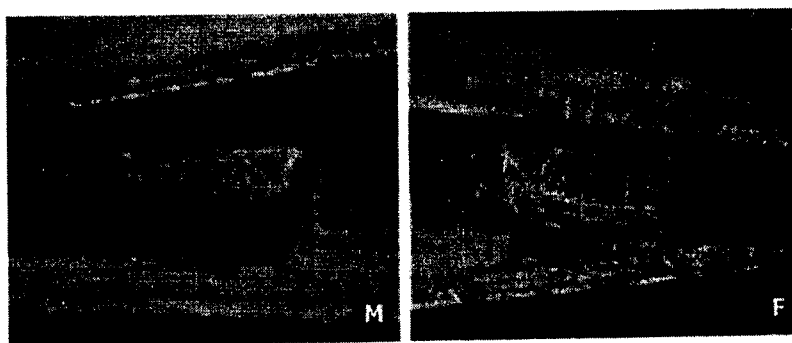

Fig 4. Male (M) and female (F) *Myndus crudus*, a planthopper vector of palm lethal yellowing disease in Florida.

The possibility of an indirect transmission path via infected embryos in mature palm seed has been considered after LY phytoplasma DNA was detected by molecular techniques in embryos of some fruits from diseased Atlantic Tall coconut palms (Cordova *et al.*, 1994; Harrison *et al.*, 1995). In sectioned tissues from positively testing embryos, distribution of phytoplasma DNA was shown by *in situ* PCR to be localized to areas corresponding to the plumule and cells ensheathing it (Cordova *et al.*, 2003). Presence of phytoplasma DNA in embryos of fruits at different stages of development from LY-diseased coconut palms has also been determined by PCR assay (Chumba, 2003). In lieu of these findings, the viability of infected embryos will need to be determined unequivocally before seed transmission can be attributed a role in spread of LY.

5. DIAGNOSTIC TECHNIQUES

In palms, phytoplasma cells are most reliably found in immature leaf bases rich in functional phloem, and, to a lesser extent, in inflorescences and roots. In contrast, they are rarely observed in most mature tissues (Thomas and Norris, 1980). When viewed by EM, cells appear ovoid to filamentous in form enclosed by a trilaminar unit membrane and contain cytoplasm with DNA fibrils and ribosomes (Thomas, 1979). In coconut, nonfilamentous forms average 295 nm in dia. while filamentous forms average 142 nm in dia. and at least 16 mm in length (Waters and Hunt, 1980). Palm tissues preferred for phytoplasma detection by EM techniques also yield DNA that is ideal for analysis by molecular assays. Prior to DNA extraction, large quantities (50-200g) of immature stem apical tissues may be extracted and processed using a modification of a phytoplasma enrichment method described in detail by Kirkpatrick *et al.*, (1995). The procedure utilizes differential centrifugation to produce a phytoplasma-enriched pellet after first homogenizing fresh tissues in an osmotically-augmented grinding buffer using a food blender.

Phytoplasma grinding buffer: Formulation for 1 liter[*]:

$K_2HPO_4.3H_2O$	21.80 g
KH_2PO_4	4.20 g
Sucrose	100.00 g
Fructose	8.00 g
Bovine serum albumin (fraction V)	1.50 g
Polyvinylpyrrolidone (PVP-40)	20.00 g
Ascorbic acid	3.36 g
Disodium EDTA	5.20 g
Adjust to pH 7.3 by addition of NaOH pellets	
Sterile distilled water	to 1 liter

[*]The grinding buffer can be stored as convenient size aliquots at -20°C for several months prior to use.

Immature palm tissues are homogenized in ice-cold grinding buffer at a ratio of 1:4 (g/v). After coarsely filtering the resulting slurry through two layers of cheesecloth, extracts are then clarified by centrifugation at 3,000 g for 10 min at 4°C. Preferably, the clarified supernatent should be filtered through Miracloth (Calbiochem) and then centrifuged at 20,000 g for 30 min to sediment phytoplasma-enriched pellets. After resuspension of each pellet in 2-4 ml of 2% CTAB buffer (Doyle and Doyle, 1990), DNA is extracted according to the protocol described by Kirkparick *et al.*, (1995).

2% CTAB DNA extraction buffer

2% cetyltrimethylammonium bromide	2 g
1.4 M sodium chloride	8.2 g
20 mM disodium EDTA, pH 8	3 ml of a 0.5 M stock
100 mM Tris-HCl, pH 8	10 ml of a 1 M stock
1% polyvinylpyrrolidone (PVP-40)	1 g
1% mercaptoethanol	1 ml
Sterile water to	100 ml

Such large scale sampling of immature palm tissues is impractical except for experimental purposes. Removal of small samples (1-3 g) of immature, phloem-rich tissues (i.e. unemerged leaf bases, young inflorescence spikelets or leaflet laminae from the newly emerging spear) offers a more practical means for confirming phytoplasma infection in situations where multiple symptomatic palms are involved at locations where they can be felled in order to access and remove these tissues. Typically, samples collected in this manner are either stored at -70°C for later analysis or processed freshly for DNA. Fresh tissues may be processed either by small scale phytoplasma enrichment prior to DNA extraction or alternatively, tissues may be diced and pulverized in liquid nitrogen using a mortar and pestle and an abrasive such as sea washed sand to facilitate the grinding process. For nucleic acid extraction, the resulting powdered tissue preparation should be mixed with 10-15 ml of hot (65°C) 2% CTAB DNA extraction buffer (Doyle and Doyle, 1990), lysed by incubation at 65°C for 30 min, cooled to room temperature and then emulsified with an equal volume of chloroform:isoamyl alcohol (24:1 v/v). After centrifugation at 12,000 g for 15 min, the upper aqueous phase is retained and extraction with chloroform:isoamyl alcohol (24:1 v/v) repeated. Total nucleic acids are recovered from the aqueous phase by precipitation by addition of a 0.8 volume of isopropanol and then sedimented by centrifugation at 12,000 g for 15 min. Upon removal of the isopropanol, nucleic acid pellets are air dried briefly followed by resupension in a small volume (100-250 ml) of TE (10 mM Tris, 1 mM EDTA, pH8) buffer containing RNAse and incubation

for 1 hr at 37°C. Final DNA samples should be quantified by comparison with known amounts of genomic DNA (e.g. uncut l-phage DNA) using standard agarose gel electrophoresis, spectrophotometry (A_{260}) or fluorometry.

5.1 Phytoplasma detection and identification by DNA probe hybridization

Molecular detection of LY phytoplasma was first achieved by DNA hybridization using random cloned *EcoRI* or *Hind*III fragments of phytoplasma genomic DNA isolated from Christmas palm (*Adonidia merrilli*) (Harrison *et al.*, 1992) or windmill palm (*Trachycarpus fortunei*) (Harrison *et al.*, 1994) as probes (Fig. 5). For cloning purposes, phytoplasma DNA was obtained by fractionation from mixtures with host palm DNA using cesium chloride-bisbenzimide density gradient centrifugation as described by Kirkpatrick *et al.* (1995). When used to assess phytoplasma DNA concentrations in palms by dot hybridization analysis, probes were found to vary in both detection sensitivity and specificity (Harrison *et al.*, 1992). Pronounced differences in phytoplasma titers among palm species were evident based on hybridization signals. For example, a comparison of stem apical tissues revealed that these tissues supported 10-12-fold higher phytoplasma concentrations in species such as spindle (*Hyophorbe verschafeltii*), footstool (*Livistona rotundifolia*) and cliff date (*Phoenix rupicola*) palm than in such species as solitary fishtail (*Caryota rumphiana*), roundleaf (*Chelyocarpus chuco*) and lifou (*Cyphophoenix nucele*) palm. The latter concentrations approximated those routinely encountered in coconut palm.

Southern hybridization of LY phytoplasma DNA restriction profiles with cloned probes revealed signals indicative of either single or multiple fragment hybridizations. These observations explained prior variations in detection sensitivity among probes revealed by dot blot analysis of DNAs. Recent studies suggest that multiple fragment hybridizations are indicative of numerous insertion sequence-like elements or transposases (Tpases) (Lee *et al.*, 2005), or multiple copies of gene clusters (Oshima *et al.*, 2003), within phytoplasma genomes. Among LY-affected palm species evaluated in southern Florida, hybridization patterns were identical regardless of the particular probe used (Harrison *et al.*, 1999). This observation supports the concept that LY phytoplasma exists as a group of closely related, or genetically identical, strains within this geographic location.

5.2 Phytoplasma detection and identification by PCR

For phytoplasma detection by PCR, universal primer pairs based on conserved regions of the ribosomal RNA gene operon have been developed (Ahrens and Seemüller, 1992; Deng and Hiruki, 1991; Gundersen and Lee; 1993, Smart *et al.*, 1996) which readily amplify rDNA of most, or all,
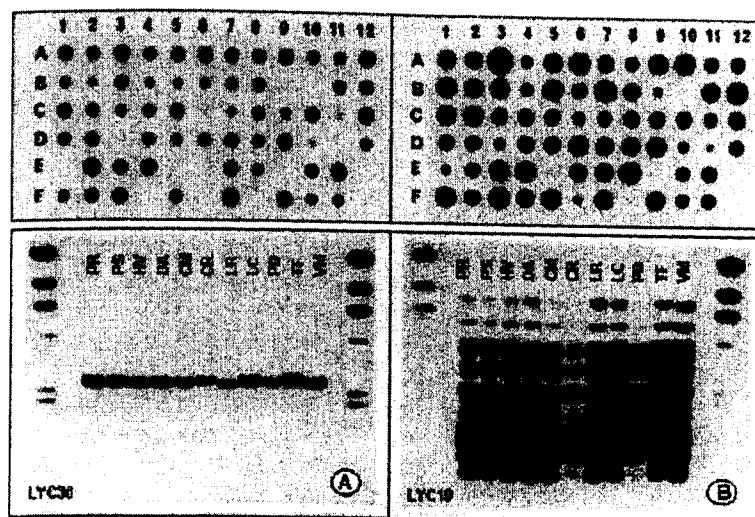
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Fig. 5. Evaluation of replicated dot and Southern bot analysis of DNA samples from various tissues of different palm species with lethal yellowing disease by hybridization with LY phytoplasma DNA probes.

Southern blot DNA samples (~2 mg) were derived from the stem apex of LY-affected palms PR (*Phoenix rupicola*); PS (*P. sylvestris*); HV (*Hyophorbe verschafeltii*); DA (*Dictyosperma album*), CM (*Caryota mitis*); CR (*C. rumphiana*); LR (*Livistona rotundifolia*); LC (*L. chinensis*); PD (*P. dactylifera*), TF (*Trachycarpus fortunei*); VM (*Adonidia merrillii*), digested with *Hind*III endonuclease, and hybridized with probe LYC36 (3.3 kb) or LYC19 (1.2 kb). Each probe represents a *Hind*III fragment of LY phytoplasma cloned from *T. fortunei* and labeled with [^{32}P]dATP by random priming.

phytoplasmas. Occasionally, universal primer pairs have been found to amplify similar rDNA sequences from nontarget gram-positive bacteria (Harrison *et al.*, 2002c; Lee *et al.*, 2002). By comparison, primer pairs 503F (5'-CAG CAG CCG CGG TAA TAC ATA-3') and LY16SR (5'-GCT TAC GCA GTT AGG CTG TC-3') (Harrison *et al.*, 2002b) or LY16Sf (5'-CAT GCA AGT CGA ACG GAA ATC-3') and LY16Sr selectively amplify 16S rRNA gene sequences the LY agent from mixtures with host palm DNA (Harrison *et al.*, 2002a). When used to reamplify products obtained by PCR employing universal rRNA primer pair P1 (5'-AAG AGT TTG ATC CTG GCT CAG GAT T-3') (Deng and Hiruki, 1991) and P7 (5'-CGT CCT TCA TCG GCT CTT-3') (Smart *et al.*, 1996), LY16Sf/LY16Sr prime amplification of rDNA from LY phytoplasma and related strains only in a group (16SrIV)-specific manner. Exclusive detection of 16SrIV-A subgroup strains is made possible by a PCR assay employing nonribosomal primer pair LYF1 (5'-CAT ATT TTA TTT CCT TTG CAA TCT G -3') and LYR1 (5'-

TCG TTT TGA TAA TCT TTC ATT TGA C-3') (Harrison *et al.*, 1994b) and as such provides a precise means of differentiating LY phytoplasma from other 16SrIV subgroup strains.

For detection of LY phytoplasma, PCR amplifications are typically performed for 30 or 35 cycles in 50 ml reaction volumes each containing 50 ng of sample DNA template, 50 ng of each primer, 125 mM of each dNTP, 1U of Taq DNA polymerase and standard PCR buffer containing 1.5 mM $MgCl_2$ (Innis and Gelfand, 1990). Detection sensitivity can be enhanced substantially by performing reamplification (nested) PCR. For example, ultrasensitive detection of the LY agent by nested PCR employing phytoplasma universal rRNA primer pair P1/P7 and LY group (16SrIV)-specific rRNA primer pair LY16Sf/LY16Sr. The former pair prime amplification of a ~1.8 kb rDNA product consisting of almost the entire 16S rRNA gene, the 16-23S intergenic spacer and the 5'-end of the 23S rRNA gene (Schneider *et al.*, 1996) whereas the latter pair prime amplification of a 1,396 bp sequence nested within the 16S rRNA gene of LY phytoplasma (Harrison *et al.*, 2002a). The increased sensitivity attained by nested PCR has enabled consistent detection of the LY agent in small samples of trunk phloem from palms at the onset of foliar symptoms. This capability has facilitated development of a simple and efficient protocol for nondestructively sampling palms (Fig. 6) in order to confirm preliminary diagnoses based on symptomatology (Cordova, 2000; Harrison *et al.*, 1999; 2002a). The vasculature of arborescent monocots such as palms facilitates trunk sampling by the protocol outlined above. Because trunk samples are gathered in the form of shavings, further preparatory grinding of these tissues at the outset of DNA extraction is unnecessary. Instead extraction begins with lysis of sample tissues in 2% CTAB DNA extraction buffer at 65°C. Recovery of total nucleic acids from lysed samples is performed as detailed previously. To ensure that potential inhibtors are removed, further purification of the final DNA sample is a prudent step prior to quantification and use of the sample as template in PCR. Purification is most easily accomplished by using commercially available kits such as Promega's Wizard®DNA Clean-Up System (Cat. #A7280) designed for this purpose.

RFLP analysis of PCR-amplified 16S rDNA provided the first phylogenetically based classification of the phytoplasmas (Ahrens and Seemüller, 1992; Gundersen *et al.*, 1994; Namba *et al.*, 1993) and has since been widely adopted as a method to accurately identify, characterize and determine relationships among these phytopathogenic mollicutes (Lee *et al.*, 1993; 1998). Ideally, for this type of analysis, 50-100 ng (2-4 ml) of products from each final reaction mix are digested separately with 10 or more endonuclease enzymes and, following extended incubation (at least 16 hr), resulting digests are resolved by electrophoresis through 5% or 8% nondenaturing polyacrylamide gels. Alternatively, digests may be separated
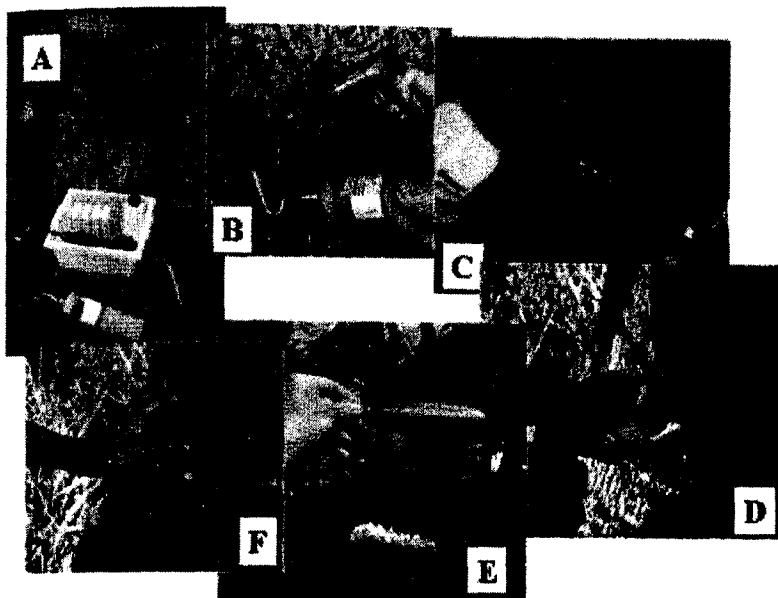
Fig. 6. Trunk sampling protocol for diagnosis of lethal yellowing by PCR
A. Typical equipment used for removing samples of interor tissues from palms include a portable electric drill fitted with a 6.5 inch long (5/16ᵗʰ dia.) bit; propane torch; wash bottle containing water; hammer, clean, sealable plastic bags or screw cap tubes (50 ml) containing DNA extraction buffer; marker pen and golf tees; **B.** Flame sterilization of drill bit using the propane torch; **C.** Cooling of flame sterilized bit with a stream of water prior to sampling; **D.** Removal and collection of interior tissues (2-4 g of shavings) from the palm trunk. Samples are collected in an appropriate clean container such as disposable plastic weigh dishes; **E.** Sample shavings are transferred either into a sterile centrifuge tube containing 15 ml of DNA extraction buffer or, alternatively, into a clean, sealable plastic bag. Bags with samples should be kept on ice to reduce tissue discoloration during transport to the laboratory and then washed from each bag with 15 ml of DNA extraction buffer in preparation for subsequent processing. Alternatively, samples may be stored frozen at -70°C until used. **F.** The hole drilled into the trunk is filled with a colored golf tee to reduce stem bleeding while providing an discernible indicator that a sample has been removed from the palm; **G.** To prevent cross-contamination of samples, the drill bit is washed with water and then flame sterilized prior to sampling the next palm.

by electrophoresis through size-selection gels using FMC Metaphor or NuSieve agarose. For phytoplasma identification, RFLP analysis of larger PCR products (e.g. P1/P7-primed rDNA) is more desirable as they contain more restriction sites. Identification of unknown phytoplasmas also requires inclusion of comparable products amplified likewise from previously well

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characterized strains. Collectively, RFLP patterns generated by frequently cutting enzymes such as *AluI*, *DdeI*, *HinfI*, *RsaI*, and *Tru9I* (=*MseI*) readily distinguish LY phytoplasma from other phylogentically distinct groups of phytoplasmas. In particular, *AluI* is the single most informative enzyme for differentiating LY phytoplasma from other strains composing group 16SrIV while revealing sequence heterogeneity between 16S rRNA gene copies in member strains Oax1, Gue3 and CPY (Fig. 7). Similarly, RFLP patterns generated by *HinfI* digests of rDNA detect heterogeneous 16S rRNA gene copies in LY phytoplasma genomes (Fig. 8). Resulting five fragment restriction profiles characteristic of subgroup 16SrIV-A strains from Florida, Honduras and Mexico reveals these strains possess two heterogenous 16S rRNA gene copies and differ from three fragment profiles indicative of the strain from Jamaica which possesses either either one or two identical gene copies (Harrison *et al.*, 2002a).

6. PHYLOGENETIC ANALYSIS

Sequence analysis of the 16S rRNA gene (~1,540 bp in length) provides the most informative picture of phylogenetic interrelationships among phytoplasmas. Based on current taxonomic guidelines established by the IRPCM Phytoplasma/Spiroplasma Working Team (2004), sequences >1,200 bp in length is now recommended for this type of analysis. For most 16SrIV group phytoplasmas 16S rRNA gene sequences have been acquired by sequencing entire P1/P7-primed rDNA products which are obtained after 30 or 35 cycles of amplification using the following thermal cycling conditions: denaturation for 1 min at 94°C (2 min, first cycle), annealing for 50 s at 55°C and extension for 2 min at 72°C (10 min, final cycle). Reactions are terminated by cooling to 4°C. In preparation for sequencing, the quantity and quality of amplification products are assessed by electrophoresis through a horizontal 1% agarose gel using TAE (40 mM Tris-acetate, 1 mM EDTA) as running buffer. Typically, a single prominent DNA band corresponding to the phytoplasma rDNA product is evident upon UV transillumination of the gel after ethidium bromide staining. Unused primers and buffer are removed from residual amplification products by passage through spin columns (e.g. QIAquick®, PCR Purification Kit, Qiagen, Valencia, CA) and products then eluted from columns in 30-50 ml of sterile ultrapure water. In cases where multiple bands are observed on gels, components of residual reaction mixtures will need to be electrophoresed through an agarose gel and the phytoplasma rDNA band excised from the gel. DNA is reovered from gel slices by standard procedures using one of several commercially available kits (e.g. Wizard® DNA Clean-Up System , Promega).

Once purified, PCR products may be sequenced directly. Today, this is

Fig. 7. RFLP analysis of rDNA amplified from 16SrIV group phytoplasma strains by PCR employing primers 503F and LY16Sr.
Footnote to Figure 7.
*Alu*I endonuclease patterns of phytoplasmas from coconut with typical LY symptoms in: Florida (Fla-LY), Belize (Bel-LY), Honduras (Hon-LY), Mexico (Mex-LY), Jamaica (Jam-LY); other 16SrIV group strains from coconut palms in Dominican Republic (DR1-5) and southern Mexico (Oax1; Gue1-2; CLD); and the arborescent monocot *C. palmata* (CPY) in southern Mexico.
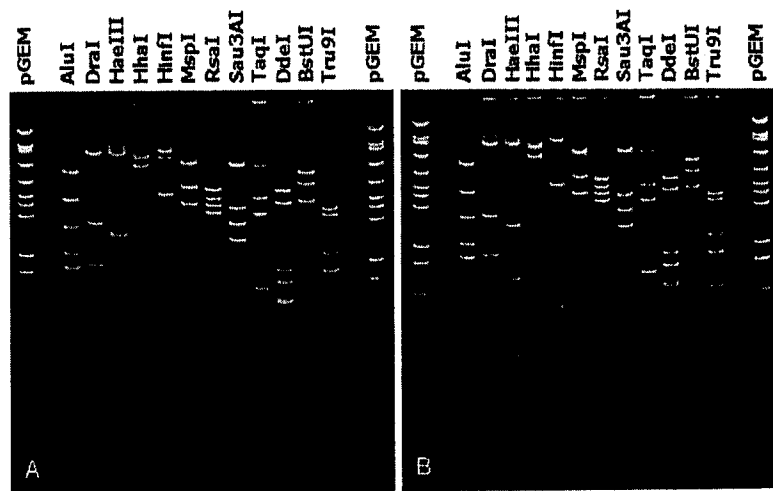

Fig. 8. RFLP analysis of rDNA amplified by P1/P7-primed PCR from Florida (A) and Jamaican (B) strains of LY phytoplasma.

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typically accomplished with BigDye® Terminator cycle sequencing chemistry on automated equipment at sequencing service laboratories. Full-length sequencing on both strands of rDNA products amplified from LY phytoplasma and other 16SrIV subgroup members by P1/P7-primed PCR are listed in Table 2. Alternatively, after purification, phytoplasma rDNA products are archived by cloning into a plasmid vector (e.g. TOPO TA Cloning® kit, Invitrogen, Carlsbad, CA) and then sequenced.

Table 2. Oligonucleotide primers for direct sequencing of rDNA products (~1/8 kb) amplified from group 16SrIV phytoplasmas by PCR incorporating primer pair P1/P7

Primer name	Sequence
P1	5′-AAGAGTTTGATCCTGGCTCAGGATT-3′
503F	5′-CAGCAGCCGCGGTAATACATA-3′
1033F	5′-TTGTCGTCAGCTCGTGTC-3′
503R	5′-TATGTATTACCGCGGCTGCTG-3′
1033R	5′-GACACGAGCTGACGACAA-3′
P7	5′-CGTCCTTCATCGGCTCTT-3′

For identification purposes, phytoplasma 16S rRNA gene sequences generated by these methods may be compared directly with >200 published phytoplasma 16S rRNA sequences publicly available in GenBank and other nucleotide databases using a compilation programs such as Pileup (Genetics Computer Group, Madison, WI) or Clustal (Higgins and Sharp, 1988). Pairwise similarities (% homology) between known and unknown phytoplasma sequences is computed by Gap analysis (GCG). Sequences should be analyzed further using phylogenetic software such as Phylogenetic Analysis Using Parsimony (PAUP) (Swofford, 1993) or MEGA3 (Kumar *et al.*, 2004) from which more inclusive information concerning interrelationships among phytoplasmas, such as group 16SrIV strains, may be displayed in the form of a dendrogram or phylogenetic tree (Fig. 3).

7. MANAGEMENT APPROACHES

A rehabilitation program in Jamaica based on replacement of the local highly susceptible Jamaica tall ecotype lost to LY with resistant Malayan dwarf and hybrid MayPan (Malayan dwarf x Panama tall) resulted in an island-wide recovery of the coconut industry by the early 1980s (Been, 1995). Both have also been widely used in replanting efforts in Florida and Mexico. Unusually high losses of Malayan dwarfs to LY at certain localized

sites in Jamaica and Florida were subsequently recognized during the mid-1980s (Howard *et al.*, 1987). A reevaluation of resistance data compiled for Malayan dwarfs and MayPans in field trials conducted in Jamaica identified significant environmental and genotype x environmental effects that influence the performance of both cultivars to LY although factors responsible for these effects were not determined (Ashburner and Been, 1997). Given our present understanding of the disease, use of resistant ecotypes and hybrids is the only practical long-term solution to LY (Harries, 2001). Promising levels of resistance have also been identified in other coconut ecotypes that include 'Chowghat Green Dwarf', 'Fiji Dwarf', 'Red Spicata Dwarf', 'Sri Lanka Yellow Dwarf', and 'King' (Harries, 1995), but, in contrast to Malayan dwarf and hybrid MayPan, these ecotypes have not been commercially exploited.

To discourage the inadvertent spread of LY, commercial movement of living palms and palm seeds from LY-affected to disease-free areas is generally not permitted. However, quarantine requirements vary according to the localities that are involved. Technical guidelines for the safe movement of coconut germplasm from LY-affected areas for research but not for commercial purposes have been developed (Frison *et al.*, 1993) under the auspices of the International Plant Genetic Resources Institute (http://www.ipgri.cgiar.org). Other measures, such as the eradication of affected palms, have not reduced the spread of LY but slight reductions were achieved by insecticide suppression of vector populations in experimental areas in Florida (Howard and McCoy, 1980). However, insecticidal control on a broad scale would be too costly, environmentally deleterious and likely result in resistant vectors. The nymphal stages of *M. crudus* develop on grasses and the adults feed on palms. Thus, ground cover management has been investigated as a method of reducing populations of this insect. All turfgrasses popular in Florida, including St. Augustine, Bahia, Bermuda and Zoyzia grasses proved to be favorable development hosts (Howard, 1989; 1990). Several grass selections tested are poor development hosts under experimental conditions but do not have desirable turf grass qualities. Dicotyledonous ground covers do not support development of *M. crudus* (Howard, 1999), and can be used in coconut plantations in the tropics, but are unlikely to become popular as understory plantings in residential or urban landscape settings.

Preventative or therapeutic antibiotic treatments using oxytetracycline-HCL (OTC) administered by trunk injection to palms (Hunt *et al.*, 1974; McCoy, 1982) is an effective method for control of LY in coconut and other susceptible palm species. Used proactively in mandatory inoculation zones as part of an integrated management program in Collier County, southwestern Florida, OTC treatments have been largely successful in suppressing local spread of LY thereby protecting most of the county's local

population of about 80,000 vulnerable Atlantic tall coconuts for the last 30 years (Fedelem, 2000). However, due to cost considerations, OTC treatments are not a viable means of disease control in commercial coconut production.

No losses due to LY has ever been confirmed in species of palms native to Florida, Cuba, Jamaica, Hispaniola, or Yucatan, Mexico, areas where LY is now endemic. Native palms are therefore good choices for landscaping in Florida and elsewhere where LY is known to occur. Native palms frequently planted as ornamentals in ladnscape and amenity plantings include cabbage palmetto (*Sabal palmetto*), royal palm (*Roystonea regia*), Paurotis palm (*Acoelorraphe wrightii*), Florida thatch palm (*Thrinax radiata*), Key thatch palm (*T. morrisii*). However, it was recently confirmed by PCR assay that *T. radiata* and Mexican silver palm (*Coccothrinax redii*), two native species commonly found throughout the Yucatan peninsula, both harbor LY phytoplasma while remaining symptomless (Narvaez *et al.*, 2005). That populations of these two native species provide a persistent source of the pathogen suggests they could play an important role in the continued dispersion of the disease in LY-endemic Yucatan.

Severe outbreaks of a disease confirmed as LY have occurred around Montego Bay, Ocho Rios and Buff Bay where up to two-thirds of Malayan dwarfs and hybrid MayPans at these localities have been killed within the last six years (Harrison *et al.*, 2002a). This newest development has prompted considerable speculation about the reasons for such unexpected losses at these sites. Reminiscent in intensity to the original epiphytotic of the 1970s, there is great concern that recurrent losses reflect an erosion of LY disease resistance in coconut due to the emergence of a new pathotype of the LY agent.

Recent technological innovations have made genome sequencing efforts possible at ever more reasonable cost. A nucleotide sequence survey of the LY phytoplasma genome is in progress to identify genes and gene sequence tags characteristic of phenotypic variability. This effort represents the first in-depth comprehensive molecular study with the intent of understanding the genetic basis for parasitism and pathogenicity in this organism. Knowledge of these traits will provide significant benefits for those concerned with coconut improvement and development of integrated disease management practices.

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