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The Lethal Yellowing (16SrIV) Group of Phytoplasmas

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ABSTRACT

Lethal yellowing (LY) is the single most important threat to world coconut palm production. Molecular based tools, and sensitive detection procedures developed over the past decade have permitted great advances in the detection and characterization of the LY phytoplasma. The practice of RFLP analyses of PCR-amplified 16S rRNA sequences with selected restriction endonucleases has resulted in the accurate identification of different strains of the LY group of phytoplasmas associated with plants and insects. The LY phytoplasmas are currently classified as members of the 16SrIV group of phytoplasmas. This progress has facilitated studies on the epidemiology of LY phytoplasma-associated diseases and has made it possible to study LY phytoplasma ecology in greater detail. Here we present an overview of the current status of the LY disease, with emphasis on its geographical distribution, socio-economic impact, vector status, methods of control as well as current advances that have been made in the past decade in the characterization of this group.

Keywords: *Acholeplasma*, *Candidatus*, *Cedusa* sp., *cocos nucifera* L., *Mollicutes*, *Myndus crudus*, 16SrRNA
Abbreviations: Ca., *Candidatus*; **CLDO**, coconut decline; **CLY**, coconut lethal yellowing; **CPD**, coyol palm decline; **CPY**, *Carludovica palmata* leaf yellowing; **EF**, elongation factor; **LDT**, lethal decline Tanzania; **LDN**, lethal decline Nigeria; **LDY**, Yucatán coconut decline; **LY**, lethal yellowing; **PCR**, polymerase chain reaction; **RFLP**, restriction fragment length polymorphism; **rp**, ribosomal protein; **TPD**, Texas Phoenix decline;

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INTRODUCTION

Lethal yellowing (LY) is a fatal disease of coconut palm (*Cocos nucifera* L.) and at least 35 other palm species in the Americas (Harrison *et al*. 1999), and has killed millions of palms in the Caribbean over the past forty years. Lethal yellowing was first observed in the Cayman Islands as early as 1834. Lethal yellowing disease is currently destroying palms in the southern United States, Central America and the Caribbean as well as west and east Africa. In west Africa the diseases caused by the LY group of phytoplasmas have been known by other names such as Cape St. Paul Wilt in Ghana (Dabek *et al*. 1976; Johnson and Harries 1976), Kribi disease in Cameroon (Dollet *et al*. 1977) Kaincopé disease in Togo (Steiner 1976) and Akwa disease in Nigeria (Ekpo and Ojomo 1990). In the east African countries of Tanzania, Kenya and Mozambique, it is known as lethal decline (LD) (Schuiling and Mpunami 1990; Mpunami *et al*. 1996). For the purpose of this paper the term LY will be used regardless of the species of palms affected or the countries affected by this group of phytoplasma.

Coconut lethal yellowing (CLY) disease was first reported in Jamaica in 1884 (Been 1995) and by 1961 approximately 90% of the susceptible Jamaica Tall palms had been lost to CLY. A phytoplasma is the known etiological agent of LY, which kills palms within a matter of months once symptoms are evident (McCoy *et al*. 1983; Cordova *et al*. 2003). Following a latent period of approximately 112 to 262 days (Dabek 1975), symptoms on coconut palms begin with premature nut fall, followed by discoloration (necrosis) of newly emergent inflorescences, a progressive yellowing of successively younger leaves and death of the apical meristem (McCoy *et al*. 1983; Cordova *et al*. 2003).

It is now clear that LY is the single most important disease presently affecting coconut production worldwide and within the last six years, the disease has destroyed most of the Malayan Dwarf palms in eastern Jamaica. Coconut breeding programs are constantly searching for LY resistant germplasm (Ashburner and Been 1997) but pathogen variation may contribute to differential resistance observed among coconut ecotypes (Harrison and Oropeza 1997). The possibility also exists that different vectors might be involved. In this review we present an overview of the current status of the disease caused by the lethal yellowing group of phytoplasmas.

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Invited Review

HISTORY AND GEOGRAPHICAL DISTRIBUTION

The LY group of phytoplasmas is widely distributed. The first report of lethal yellowing was in the Cayman Islands in 1834 (Howard 1983). In Jamaica LY was first observed in 1884 (Howard 1983; Been 1995), then it was known as 'west end bud rot', subsequently, in the 1950's, it was termed lethal yellowing. It has been theorised that LY (then known as *pudricion del cogollo*) was present in Cuba as early as the 1870's (Howard 1983; Llauger *et al*. 2002). In Haiti, the LY disease has been dated as far back as the 1920's and 1930's (Howard 1983) and in the Dominican Republic LY was first reported by Carter in 1962 (Carter and Suah 1964). Leach, in 1946 observed what appeared to be LY on the New Providence Island and theorised that it could have been there for at least 20 years (Leach 1946). A limited inspection of coconuts in 1974 confirmed the presence of 11 LY infected coconuts on the New Providence Island of the Bahamas (Howard 1983). Lethal yellowing was first observed at Key West Florida in the late 1930's and was first found on the Florida mainland in Miami in 1971 (Martinez and Roberts 1967; Seymour *et al*. 1972; McCoy 1976). In 1980, there was the first report of LY infecting date palms in Texas (McCoy 1980). Lethal yellowing was theorised to have reached Mexico in 1977 (Romney and Harries 1978).

Lethal yellowing-type diseases have been known in Ghana, Togo and Cameroon since 1937 (Howard 1983; Dollet *et al*. 1977), in Nigeria a similar disease was seen in the vicinity of Akwa in 1917 and 1955 (Bull 1955). In Tanzania LY was reported as early as 1905, then it was affecting coconuts in Bagamoyo area (Mpunami *et al*. 1999). Lethal yellowing has also been reported in Kenya and Mozambique (Mpunami *et al*. 1999). In 2005, LY was discovered for the first time in Nevis (Myrie *et al*. 2006). The distribution of LY also extends to Trinidad and Tobago, Honduras, Guatemala, Belize, as well as the Turks and Caicos islands (Been, pers. comm.).

LY SYMPTOMOLOGY IN COCONUTS AND OTHER PALMS

The symptoms of LY disease in coconut palms consist of essentially four stages. The first stage which is sometimes called shelling, involves premature nutfall with most of the nuts having a black or brown water-soaked area under the calyx. The second stage involves the necrosis of the inflorescence. The third stage involves the yellowing of the fronds of the coconut palm (**Fig 1A**). This usually begins with the oldest fronds eventually advancing to the crown of the plant. Fronds that exhibit these symptoms will eventually die and can be easily removed. Death of the bud occurs about halfway through the yellowing sequence. The newly emerged spear leaf will collapse and maybe seen hanging down within the crown. The fourth stage involves complete defoliation, the top of the tree falls away leaving what is known as the telephone pole effect (**Fig 1B**). Affected palms die within 4-6 months of the onset of symptoms, and the disease affects palms at all ages, including transplants as young as 18 months (McCoy *et al*. 1983; Been 1995; Donselman 1999). Similar symptoms have been reported for other yellowing diseases of coconut associated with phytoplasmas.

In palms other than coconuts, the recognition of symptoms of LY disease is more difficult. The first two symptom stages are the same as for the coconut palm (premature nut fall and necrosis of the new inflorescence). The third symptom is the discoloration of the fronds. This stage of the infection varies according to the species of the palm involved and can be divided into two types, those in which the fronds turn golden-yellow before dying, and those in which the fronds turn greyish-brown. Palms in the former symptom category include Talipot (*Corypha umbraculifera*) palms, two species of Pritchardia (*Pritchardia thurstonii*, *Pritchardia pacifica*), Arkury (*Syagrus schizophylla*), Windmill

Fig. 1 Coconut palms showing symptoms of lethal yellowing. (**A**) Stage three, yellowing of the fronds of the coconut. (**B**) Stage 4, complete defoliation-telephone effect. (Pictures courtesy of M Wallace)

(*Trachycarpus fortunei*), Princess (*Dictyosperma album*) and Spindle (*Hyophorbe verschaffeltii*) palms. In these palms, yellowing of the lower fronds is typical, but often one frond will turn yellow before any of the others, giving the appearance of a flag. The fronds remain yellow for various lengths of time before turning brown, breaking at the leaf base junction and dying. In some cases the fronds remain attached to the tree instead of falling to the ground. The yellowing advances from the older to the younger fronds with the spear leaf being the last to turn yellow. Once the spear leaf shows symptoms, the crown dies followed by defoliation leaving a topless trunk (Howard and Harrison 1999).

The second symptom category is best exemplified by the Christmas palm, or Adonidia (*Veitchia merrilli*). The first symptom stages in these palms are also similar to those in LY-infected coconut palms, however, frond discoloration is not as dramatic or easily detected. The first evidence of infection is a brownish 'water mark' along the margin of the pinnae or leaflets, which gradually extends to the entire frond giving it a dried out appearance. Older leaves tend to break easily at the junction of the leaf-base and midrib and younger fronds tend to break within the lower region of the pinnea. Unopened inflorescence may have a distorted or twisted appearance. Death of the bud follows and the entire top falls away leaving a bare trunk (Donselman 1999). A list of palm species affected by the LY group of phytoplasmas can be seen in **Table 1**.

Table 1 List of some palms affected with the LY group of phytoplasmas.

Common names	Botanical names
Manila palm	*Adonidia merrillii*
Ruffle palm	*Aiphanes lindeniana*
Seashore palm	*Allagoptera arenaria*
Dwarf sugar palm	*Arenga engleri*
Palmyra palm	*Borassus flabellifer*
Cluster fishtail plam	*Caryota mitis* Loureiro
Giant fishtail palm	*Caryota rumphiana*
	Chelyocarpus chuco
Buri palm, Gebang palm	*Corypha alata*
Rootspine palm	*Crysophila warsecewiczii*
	Cyphophoenix nucele
Palma de lluvia	*Gaussia attenuata*
Spindle palm	*Hyophorbe verschaffeltii*
Kentia palm	*Howea forsteriana*
Belmore sentry palm	*Howea belmoreana*
Latan palm	*Latania* spp
Coconut palm	*Cocos nucifera*
Chinese fan palm	*Livistona chinensis*
Footstool palm	*Livistona rotundifolia*
Princess palm	*Dictyosperma album*
Cabada palm	*Dypsis cabadae*
Triangle palm	*Neodypsis decaryi*
Mazari palm	*Nannorrhops ritchiana*
Canary Island date palm	*Phoenix canariensis*
Date palm	*Phoenix dactylifera*
Senegal date palm	*Phoenix reclinata*
Cliff date palm	*Phoenix rupicola*
Wild date palm	*Phoenix sylvestris*
Loulu palm	*Pritchardia affinis*
Fiji fan palm, tonga fan palm	*Pritchardia pacifica*
Thurston palm	*Pritchardia thurstonii*
Remota loulu palm	*Pritchardia remota*
Hildebrandt's palm	*Ravenea hildebrantii*
Arikury palm	*Syagrus schizophylla*
Windmill palm	*Trachycarpus fortunei*
Manila palm	*Veitchia merrilli*
	Veitchia arecina
Sunshine palm	*Veitchia mcdanielsi*
	Veitchia montgomeryana

Table adapted from Howard and Harrison 1999.

THE LY PATHOGEN

Phytoplasmas were discovered in 1967 by Japanese scientists studying plants with yellows disease (Doi *et al*. 1967). When the LY disease was first named lethal yellowing, it was thought to be a virus (Nutman and Roberts 1955). In 1972 transmission electron microscopy studies conducted on samples obtained from Jamaica, identified the pathogen to be a mycoplasma (now known as phytoplasma) (Beakbane *et al*. 1972; Heinze *et al*. 1972; Plavsic-Blanjac *et al*. 1972). Phytoplasmas are gram-positive bacteria that form a large monophyletic group within the class *Mollicutes* that are most closely related to the genus *Acholeplasma* (Lim and Sears 1989; Kuske and Kirkpatrick 1992; Sears and Kirkpatrick 1994). They are characterized by the lack of a cell wall, a small genome size (530-1350 kbp) and a low guanine-plus-cytosine (G+C) content (Lee *et al*. 2000). Phytoplasmas have been referred to as being rounded pleiomorphic bodies with an average diameter ranging from 200-800 µm (Lee *at al*. 2000). However other studies have revealed a filamentous morphology as was seen with sections of sieve elements obtained from LY infected tissues in coconut (Waters and Hunt 1980). A concomitant of the lack of cell wall is that mycoplasmas and by extension phytoplasmas have the ability to change forms (Bredt *et al*. 1973). It has been noted that filamentous bodies tend to be predominant in infected plant tissues during the early stages of infection (Lee *at al*. 2000). Phytoplasmas lack several genes that autonomous bacteria such as *E. coli* have for metabolism, an example of which is the lack of genes for the *de novo* synthesis of amino acids, fatty acids or nucleotides (Christensen *et al*. 2005), as a result they cannot be cultured *in vitro*.

PCR-based assays developed in the late 1980's and early 1990's have served to further advance the diagnostics of phytoplasma diseases. These assays provide a much more sensitive means than serological tests or DNA-DNA hybriddization for the detection of phytoplasmas. Over the last 15 years phytoplasma universal as well as group specific primers have been developed based on the highly conserved 16S rRNA gene sequences, 16S-23S intergenic spacer region gene sequences, conserved ribosomal protein (rp) gene and elongation factor EF-Tu (tuf) gene sequences (Lim and Sears 1991; Davis and Lee 1993; Firrao *et al*. 1993; Lee *et*

Table 2 List of phytoplasmas in the genus '*Candidatus* Phytoplasma'.

***Candidatus* Phytoplasma species**	**Phylogenetic group***	**Associated disease**
'*Ca* Phytoplasma asteris'	Aster yellows group (16SrI)	Aster yellows
'*Ca* Phytoplasma japonicum'	Aster yellows group (16SrI)	Japanese Hydrangea phyllody
'*Ca* Phytoplasma aurantifolia'	Peanut witches broom group (16SrII)	Lime witches' broom
'*Ca* Phytoplasma pruni'†	X-disease group (16SrIII)	X-disease
'*Ca* Phytoplasma palmae'†	Coconut lethal yellowing group (16SrIV)	Coconut lethal yellowing (Mexico)
'*Ca* Phytoplasma cocostanzaniae'†	Coconut lethal yellowing group (16SrIV)	Coconut lethal yellowing (Tanzania)
'*Ca* Phytoplasma cocosnigeriae'†	Coconut lethal yellowing group (16SrIV)	Coconut lethal yellowing (Nigeria)
'*Ca* Phytoplasma castaneae'	Coconut lethal yellowing group (16SrIV)	Korea chestnut witches' broom
'*Ca* Phytoplasma ziziphi'	Elm yellows group (16SrV)	Ziziphus jujube witches' broom
'*Ca* Phytoplasma vitis'	Elm yellows group (16SrV)	Flavescence dorée
'*Ca* Phytoplasma ulmi'	Elm yellows group (16SrV)	Elm yellows
'*Ca* Phytoplasma trifolii'	Clover proliferation group (16SrVI)	Clover proliferation
'*Ca* Phytoplasma fraxini'	Ash yellows group (16SrVII)	Ash yellows
'*Ca* Phytoplasma luffae†'	Loofah witches' broom group (16srVIII)	Loofah witches' broom
'*Ca* Phytoplasma phoenicium'	Pigeon pea witches' broom group (16SrIX)	Almond lethal yellows
'*Ca* Phytoplasma mali'	Apple proliferation group (16SrX)	Apple proliferation
'*Ca* Phytoplasma pyri'	Apple proliferation group (16SrX)	Pear decline
'*Ca* Phytoplasma prunorum'	Apple proliferation group (16SrX)	European stone fruit yellows
'*Ca* Phytoplasma spartii'	Apple proliferation group (16SrX)	Spartium witches' broom
'*Ca* Phytoplasma rhamni'	Apple proliferation group (16SrX)	Buckthorn witches' broom
'*Ca* Phytoplasma allocasuarinae'	Apple proliferation group (16SrX)	Allocasuarina yellows
'*Ca* Phytoplasma oryzae'	Rice yellow dwarf group (16SrXI)	Rice yellow dwarf
'*Ca* Phytoplasma australiense'	Stolbour group (16SrXII)	Australian grapevine yellows
'*Ca* Phytoplasma solani'†	Stolbour group (16SrXII)	Stolbur and Bios Noir
'*Ca* Phytoplasma cynodontis'	Bermuda grass white leaf group (16SrXIV)	Bermuda grass white leaf
'*Ca* Phytoplasma brasiliense'	'*Ca*. Phytoplama brasiliense' group (16SrXV)	Brazilian hibiscus witches' broom

* Phylogenetic group according to Lee *et al*. (1998a) *International Journal of Systematic Bacteriology* **48**, 1153-1169.
Table adapted from the IRPCM Phytoplasma/spiroplasma Working Team-Phytoplasma Taxonomy Group (2004)
†Phytoplasma has not yet been fully characterized

al. 1993, 1995; Lorenz *et al*. 1995; Smart *et al*.1996; Guo *et al*. 1998; Lee *et al*. 1998a). A comprehensive classification scheme was developed for phytoplasmas by Lee *et al*. (1998a) which consisted of 14 groups and 38 subgroups. This involved the RFLP analyses on PCR-amplified 16S rDNA sequences with a number of restriction enzymes.

Phylogenetic investigations of phytoplasmas have led to the proposal that the phytoplasma clade should be distinguished at the taxonomic level of a genus, and each subclade (or corresponding 16Sr RNA group) should represent at least a distinct species (Gundersen *et al*. 1994). The International Committee on Systematic Bacteriology implemented a scheme for assigning incompletely described prokaryotes such as phytoplasmas to the provisional status *Candidatus*, this genus now comprises 26 species (**Table 2**) (IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma Taxonomy Group 2004).

The coconut lethal yellowing group of phytoplasmas have been classified as being members of group 16SrIV according to the classification of Lee *et al*. (1998a) and has been divided into four subclades (16SrIV-A, 16SrIV-B, 16SrIV-C and 16SrIV-D). Members of group 16SrIV-A include the palm lethal yellowing phytoplasma, this pytoplasma was obtained from phytoplasma that was infecting coconuts (Atlantic tall and Malayan Dwarf) as well as the Manila palm in Florida (Tymon *et al*. 1998), it is also the type species for the subgroup '*Candidatus* Phytoplasma palmae' (IRPCM). Other phytoplasmas in this subgroup include phytoplasmas detected in infected coconut palms from Honduras, Jamaica, 16 other palm species found in Florida (Harrison *et al*. 2002a), as well as phytoplasmas found in coconut palms in Nevis (Myrie *et al*. 2006).

Phytoplasma isolated from Mexican Yucatán Atlantic Tall coconut palm was found to be distinct from the above mentioned phytoplasmas. It was described as Yucatán coconut lethal decline (LDY) phytoplasma, and was placed in a distinct subgroup, subgroup 16SrIV-B (Tymon *et al*. 1998). Similarly LY phytoplasma isolated from coconuts in Cuba, clustered with the Mexican LY isolate from the Yucatán region and was also placed in subgroup 16SrIV-B (Llauger *et al*. 2002). Analysis of phytoplasma isolated from cayol and coconut palms exhibiting decline symptoms in Honduras revealed a new strain of phytoplasma that was 99.87% similar to the Yucatán coconut lethal decline phytoplasma. This particular strain of phytoplasma (like LDY) could not be ampliflied using the 16SrIV-A group specific (LYF1/ LYR1) primers. On the basis of the above findings, coyol palm decline (CPD) and coconut lethal decline (CLDO) have now been added to the 16SrIV-B clade (Roca *et al*. 2006).

PCR assay was used to detect phytoplasma in Canary Island date palms (*Phoenix canariensis*) displaying symptoms of LY disease in Corpus Christi, Texas. RFLP analysis of the nested PCR products (P1/P7 and 16SF/16SR) revealed that the palm infecting phytoplasmas were uniform and most similar to strains composing the coconut LY group (16SrIV). Sequence analysis of 16S rDNA revealed that the Texas Phoenix palm decline (TPD) phytoplasma was phylogenetically closest to the *Carludovica palmata* leaf yellowing (CPY) phytoplasma. Symptomatic Palma Jipi (*Carludovica plamata*, Cyclanthaceae) plants in Mexico were tested positive using the universal rRNA primers (P1/P7), and negative using LY specific primers (LYF1/ LYR1). RFLP analysis differentiated the *C. palmata* yellows (CPY) phytoplasma from LY and other known phytoplasmas previously characterized by this means. A sequence homology of 99% was obtained between the 16S rDNA of CPY and LY, indicating that the strains are very similar and fall under the same clade (Cordova *et al*. 2000). The rDNA profiles of strains TPD and CPY obtained with *Alu*I were co-identical and distinct from other known 16SrIV group phytoplasmas. On this basis, both strains were classified as members of a new subgroup, 16SrIV-D (Harrison *et al*. 2002b).

Phytoplasmas associated with lethal decline diseases of coconut palm (*C. nucifera*), in Florida, Yucatan region in Mexico and from east and west Africa, were compared using the 16S rRNA genes and the 16S-23S spacer region. Phylogentic analysis confirmed that these coconut phytoplasmas formed a separate cluster within the phytoplasma clade and that the pathogen causing diseases in west Africa formed a new sub-clade within this cluster. Primers developed from the intergenic region for the detection of phytoplasma from east and west Africa did not produce any amplification for LY infected coconuts from the Caribbean, confirming the fact that the phytoplasma from the Caribbean and the Americas are different from those in Africa (Tymon *et al*. 1998). Further work conducted by Mpunami *et al*. (1999) using primer pairs Ghana 813F/AKSR and Rhode forward and reverse primers was able to detect variation in the phytoplasmas obtained from coconuts in east Africa. It was noted that Rhode primers were able to detect phytoplasma in the Nigerian and Tanzanian samples but not in the samples from Mozambique, these were amplified using primers that were specifically designed for phytoplasma in the west. Digestion with *Rsa*I showed that the phytoplasma found in Mozambique varied from those in Nigeria and Tanzania. These phytoplasmas were also placed in the 16SrIV group of phytoplasmas (Mpunami *et al*. 1999), subsequently, phytoplasma strain LDT (lethal decline Tanzania) has been described as species strain '*Candidatus* phytoplasma cocostanzaniae', and phytoplasma strain LDN (lethal decline Nigeria) has now been referred to as '*Candidatus* Phytoplasma cocosnigeriae' (IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma Taxonomy Group 2004) (**Table 2**).

Variation in the LY group of phytoplasmas has also been reported in insects. RFLP analysis of nested PCR products (P1/P7 and 16SF/16SR) revealed variation in 6 of the 43 *Cedusa* species of Derbids tested in Jamaica. The others produced bands that were similar to that of CLY in Jamaica and Florida. Variations could be seen using the enzymes *Msp*I, *Taq*I, *Rsa*I, *Hinf*I and *Alu*I. Phylogenetic analyses showed these pytoplasmas forming a new sub-clade within the LY group suggesting that this may be a different LY phytoplasma strain from those mentioned above (Brown *et al*. 2006).

Based on the above results it can be seen that the lethal yellowing 16SrIV group consists of a species complex with variations seen not just in the east and western hemisphere, but also within each region. The LY strain found in Honduras, Jamaica and Florida all showed variations from each other and even in Jamaica, which is a relatively small island variation could been seen in the phytoplasmas found in insect which was different from those seen in coconuts.

The LY group of phytoplasmas have been found in plants other than palms. In Jamaica, the lethal yellowing group of phytoplasmas was found in the weeds *Macroptilium lathyroides* and *Stachytarpheta jamaicensis* (Brown *et al*. 2005). These plants could be possible hosts of the LY group of phytoplasmas in Jamaica. By extention it should be mentioned that phytoplasma belonging to the coconut lethal yellowing group was found in Japanese Chestnut trees (*Castanea crenata* Sieb. and Zucc) in Korea (Jung *et al*. 2002). These trees showed symptoms indicative of witches' broom disease, including abnormally small leaves as well as yellowing of the young leaves. Phylogenetic analysis revealed that they were most closely related to the lethal yellowing group of phytoplasmas, suggesting that they may have a common ancestor. This phytoplasma has been referred to as phytoplasma species '*Candidatus* Phytoplasma castaneae' (Jung *et al*. 2002). This phytoplasma has not been reported in palms.

MOLECULAR BIOLOGY TECHNIQUES IN PHYTOPLASMA DETECTION

Phytoplasmas have resisted all attempts to be cultivated *in vitro* in cell-free media. Development of molecular based tools has not only greatly increased the sensitivity of detection in the host tissue but has also allowed for the

Table 3 Primers used for PCR amplification and sequencing of 16SrRNA genes of phytoplasmas infecting coconuts and other palm species.

Primers	Sequence (5′-3′)	Phytoplasma Group	Reference
P1	AAGAGTTTGATCCTGGCTCAGGATT	Universal	Deng and Hiruki 1991
P7	CGTCCTTCATCGGCTCTT	Universal	Smart *et al*. 1996
P6	CGGTAGGGATACCTTGTTACGACTTA	Universal	Deng and Hiruki 1991
P4	GAAGTCTGCAACTCGACTTC	Universal	Kirkpatrick *et al*. 1994
LYF1	CATATTTTATTTCCTTTGCAATCTG	16SrIV-A only	Harrison *et al*. 1994
LYR1	TCGTTTTGATAATCTTTCATTTGAC	16SrIV-A only	Harrison *et al*. 1994
Rhode F	GAGTACTAAGTGTCGGGGCAA	LD	Rhode *et al*. 1993
Rhode R	AAAAACTCGCGTTTCAGCTAC	LD	Rhode *et al*. 1993
LYDSR	GGTGCCATATATATTAGATTG	Tanzania	Tymon *et al*. 1998
G813F	CTAAGTGTCGGGGGTTTCC	LDG	Tymon *et al*. 1998
AKSR	TTGAATAAGAGGAATGTGG	LDN	Tymon *et al*. 1998
LY16SF	CATGCAAGTCGAACGGAAATC	16SrIV A-D	Harrison *et al*. 2002a
LY16SR	GCTTACGCAGTTAGGCTGTC	16SrIV A-D	Harrison *et al*. 2002a
LY16S-23R	TTGAGAATTTACGTTGTTTATCTAC	16SrIV A-D	Harrison *et al*. 2002c
SN910601	GTTTGATCCTGGCTCAGGATT	Universal	Namba *et al*. 1993
SN910502	AACCCCGAGAACGTATTCACC	Universal	Namba *et al*. 1993
CnWBF1*	CTAGTTTAAAAACAATGCTC	*'Ca* Phytoplasma castaneae'	Jung *et al*. 2002
CnWBR1*	CTCATCTTCCTCCAATTC	*'Ca* Phytoplasma castaneae'	Jung *et al*. 2002

LD = Lethal decline Tanzania; LDN = Lethal decline Nigeria; LDG = Lethal decline Ghana
* = These primers were used to detect non-palm (chestnut) infecting phytoplasma belonging to 16SrIV group of phytoplasmas

Table 4 Showing PCR conditions used for selected phytoplasma universal and LY specific primers.

Primers	PCR programme	Group
P1/P7	Initial 2 min at 95°C followed by 30 cycles each consisting of 30 s at 94°C, 50 s at 54°C,	Universal
P4/P7	90 s at 72°C. Last cycle extension of 10 min at 72°C	
P1/P6	Initial 2 min at 95°C followed by 30 cycles each consisting of 30 s at 94°C, 50 s at 62°C,	Universal
	90 s at 72°C. Last cycle extension of 10 min at 72°C	
SN910601/SN910502	Initial 2 min 94°C followed by 40 cycles each consisting of	Universal
	2 min at 94°C, 2 min at 60°C and 3 min at 72°C. Last cycle extension 72°C for 7 min	
LYF1/LYR1	Initial 2 min 30 s at 94°C followed by 40 cycles each consisting of 30 s 94°C, 50 s at	16SrIV-A only
	60°C, 80 s at 72°C. Last cycle extension of 10 min at 72°C.	
LY16SF/LY16SR†	Initial 2 min 30 s at 94°C followed by 35 cycles each consisting of 30 s 94°C, 50 s at	16SrIV A-D
LY16SF/LY16S-23SR†	60°C, 80 s at 72°C. Last cycle extension of 10 min at 72°C.	
Rhode F/Rhode R	Initial 2 min at 94°C followed by 36 cycles each consisting of 60 s at 94°C, 80 s at 57°C,	LD
	2 min 10 s at 72°C. Last cycle extension at 72°C for 5 min	
G813F/AKSR	Initial 2 min at 95°C followed by 35 cycles each consisting of 30 s at 94°C, 60 s at 53°C,	LDG and LDN
	90s at 72°C. Last cycle extension of 10 min at 72°C	
P1/LDSR	Initial 2 min at 95°C followed by 35 cycles each consisting of 30 s at 94°C, 60 s at 53°C,	LD
	90 s at 72°C. Last cycle extension of 10 min at 72°C	
P1/AKSR	Initial 2 min at 95°C followed by 35 cycles each consisting of 30 s at 94°C, 60 s at 53°C,	LDG and LDN
	90 s at 72°C. Last cycle extension of 10 min at 72°C	
CnWBF1*/ CnWBR1*	Initial 2 min at 94°C followed by 30 cycles each consisting of 2 min at 94°C, 2 min at	*'Ca* Phytoplasma castaneae'
	54°C and 3 min at 72°C. Last cycle extension 72°C for 7 min	

† = Primers used in nested PCR analysis
* = These primers were used to detect non-palm (chestnut) infecting phytoplasma belonging to 16SrIV group of phytoplasmas

identification and classification of phytoplasmas (Lim and Sears 1989; Lee *et al*. 1998a; Seemüller *et al*. 1998; Lee *et al*. 2000). Over the past 15 years numerous molecular techniques have been developed and applied to phytoplasma research. One of the most important techniques that have been applied to date is polymerase chain reaction (PCR). The conservative nature of ribosomal DNA across all prokaryotic organisms makes it possible for total DNA from plants and insects to be used as a template for short synthetic primers. Universal primers have been designed that amplify sequences common to all phytoplasma and has been used in the determination of the presence of phytoplasmal DNA (Deng and Hiruki 1991; Namba *et al*. 1993; Kirkpatrick *et al*. 1994; Smart *et al*. 1996). Some of these primers can be seen in **Table 3**. Within the LY group of phytoplasmas there are primers that are specific for only group 16SrIV-A phytoplasmas (primer pair LYF1/LYR1) as well as those that amplify DNA from the Caribbean, Mexico, Florida and Central America (group 16SrIV A-D) (**Tables 3, 4**). There are also primers that are designed specifically for the LY group of phytoplasmas in the African continent. These include the Rhode primers (Rhode *et al*. 1993) and primer LYDSR (**Table 4**) which amplifies a member of the LY group of phytoplasmas (LD) in Tanzania. The primers G813F and AKSR were designed to detect members of the LY group of phytoplasmas (LDG and LDN) found in Ghana and Nigeria (**Table 4**).

Nested PCR was developed to increase the specificity of the PCR detection process, it involves the amplification by one primer pair, followed by amplification with a second more specific primer pair or one that amplifies a smaller sequence. This has been utilized extensively in the study of the LY group of phytoplasmas and has facilitated its detection in plants and insects that have a very low titre of the pathogen (Brown *et al*. 2005, 2006) (**Table 4**). Multiplex nested PCR (Daire *et al*. 1997) has also been used in the detection and differentiation of different phytoplasma groups. The intensive use of restriction fragment polymorphism (RFLP) analyses of polymerase chain reaction (PCR)-amplified conserved genes, have contributed to the establishment of the phylogeny and taxonomy of phytoplasmas (Deng and Hiruki 1990; Lee *et al*. 1993; Schneider *et al*. 1993). It has been particular useful in the differentiation of the different strains of the LY group of phytoplasmas regionally as well as locally (Tymon *et al*. 1998; Mpunami *et al*. 1999, 2000; Cordova *et al*. 2000; Harrison *et al*. 2002a, 2002b, 2002c; Brown *et al*. 2006). Recently the technique of real-time PCR has been utilised in quantifying the movement and multiplication of phytoplasma in plants (Christensen *et al*. 2004, Baric and Dalla-Via 2004; Wei *et al*. 2004; Crosslin *et al*. 2006; Saracco *et al*. 2006)

Heteroduplex mobility assay (HMA) which involves the hybridization of amplicons from study samples to amplicons from reference strains and its resolution by electrophoresis,

has been used in the differentiation of closely related strains of phytoplasmas in plants as well as insects (Palmano and Firrao 2000; Wang and Hiruki 2000). *In situ* PCR (on fixed and sectioned plants and on whole insects) was developed by Webb *et al*. (1999), using 20- to 24-mer oligonucleotide primers. Their findings were in strong agreement with previous immunochemical and electron microscopic studies. *In situ* PCR allows for a more efficient and effective study of the biology and epidemiology of multiple infections in a single host and of the events leading to transovarial transmission (Hanboonsong *et al*. 2002). *In situ* PCR was also used in the detection of phytoplasma DNA in the embryos from coconut palms with lethal yellowing disease (Cardova *et al*. 2003). Denaturing gradient electrophoresis (DGGE) has been used to estimate the levels of sequence diversity in some phytoplasma isolates (Ceranic-Zagorac and Hiruki 1996). In DGGE individual bands of amplified PCR products are separated on the basis of their decreasing electrophoretic mobility in a linear urea/formamide gel gradient or by a linear temperature gradient. This method, however is not used extensively in phytoplasma analysis. A serological technique has been developed that involves putting a phytoplasmal gene fragment into a bacteria vector (*Escherichia coli*) that subsequently expresses the genes that produce an abundance of pure proteins from which antibodies are made. Wei *et al*. 2004, developed antibodies to the SecA membrane protein, which is unique to bacteria and central to the process of protein secretion from cell membranes. The SecA antibodies react to the phytoplasma allowing the detection and monitoring of the progress of a phytoplasma infection in plants.

TRANSMISSION

The insect order Hemiptera is the single most successful order of phytoplasma vectors. This group of insects possesses at least four characteristics that make its members efficient vectors of phytoplasma. The first feature is their hemimetabolous nature. Both adults and nymphs can be found in the same location and are known to feed in a similar manner, as a result both can transmit the phytoplasma (Weintraub and Beanland 2006). The second feature is the fact that they feed on certain specific plant tissues which makes them efficient vectors of pathogens residing in those tissues. Their feeding is nondestructive which in turn promotes the successful inoculation of the plant vascular tissue without damaging the conductive tissues which would elicit certain defensive responses (Weintraub and Beanland 2006). The third feature is the propagative and persistent relationship that exists between these insects and the phytoplasma. Lastly, these insects tend to have obligate symbiotic prokaryotes that are passed to the offspring by transovarial transmission of phytoplasmas (Weintraub and Beanland 2006). Examples of transovarial transmission of phytoplasmas could be seen in the transmission of mulberry dwarf phytoplasma (Kawakita *et al*. 2000), the sugarcane whiteleaf phytoplasma (Hanboonsong *et al*. 2002) and the transmission of '*Candidatus* Phytoplasma prunorum' (Tedeschi *et al*. 2006).

Phytoplasmas are phloem limited, as a result only phloem-feeding insects can potentially acquire and transmit the pathogen. Phytoplasmas are transmitted in a persistent manner. Within the groups of phloem-feeding insects only a small number have been confirmed as vectors of phytoplasmas. They fall primarily into three taxonomic groups, Membracoidea, Fulgoromorpha and Sternorrhyncha. Within the superfamily Membracoidea, all known vectors to date have been confined to Cicadellidae. In the group Fulgoromorpha, four families of vector species have been found. These include Cixiidae, Delphacidae, Derbidae, and one species in the Flatidae. All the potential vectors of LY to date have been confined to these families. In the group Sternorrhyncha only two genera in Psyllidae have been confirmed as vectors (Weintraub and Beanland 2006).

The relationship between phytoplasmas and their vectors is not fully understood. Following acquisition there is a latency period, during which the phytoplasma replicates in the body of the vector (Purcell 1982; Kirkpatrick 1992). The phytoplasma is then thought to enter the epithelial cells of the midgut and replicate within a vesicle or pass between two midgut cells and through the basement membrane to enter the haemocoel (Purcell 1982; Kirkpatrick 1992). The phytoplasmas circulate in the haemolymph where they replicate. In order for the phytoplasma to be transmitted it must enter specific cells of the salivary glands and the levels in the posterior acinar cells must accumulate to a high level before it can be transmitted. An insect is classified as a 'dead-end' host if the phytoplasma is not able to pass into the haemolymph. Both insects and plants are natural hosts of phytoplasmas (Lee *et al*. 1998b). The host range in insect vectors varies with phytoplasma strains. Some phytoplasmas have low insect vector specificity wheras others have high insect vector specificity (Lee *et al*. 1998b). The LY group of phytoplasmas can be classified as having a very high insect vector specificity as the only vector that has been identified to date is *M. crudus*.

The cixiid *M. crudus* was shown in transmission trials to be a vector of LY in Florida (Howard *et al*. 1983). In Jamaica, during a search for vectors of LY, leafhoppers were found to predominate in the undergrowth, while planthoppers (Fulgoridae) were the most prevalent group on palms (Dabek 1981). Then, the cixiid, *Myndus crudus*, was the most abundant planthopper, and was the prime suspect of LY in Jamaica, although extensive transmission trails failed to confirm this possibility (Schuiling *et al*. 1976; Eden-Green 1978; Eden-Green and Schuiling 1978; Dabek 1981). In a recent study conducted by Brown *et al*. (2006), the LY group of phytoplasmas was detected in 30% (13/43) of the *Cedusa* species of Derbids analysed. It was also noted that variation in the phytoplasma could be seen in 6 of the insects that were tested positive for LY. These findings have serious implications for LY epidemiology. The fact that a new member of the *Cedusa* species has been discovered coupled with the variation in the RFLP banding pattern of the DNA, raises the possibility that the *Cedusa* species of Derbids could be a vector of phytoplasmas associated with CLY disease. It may also explain the recent spread of LY in Jamaica. Prior to the onset of the epidemic there was no mention of this particular species of Derbids, however in recent times the Derbids are now found in greater abundance on coconuts than *M. crudus* (Brown *et al*. 2006).

Mpunami *et al*. (2000) in looking at potential vectors of LD in Tanzania was able to use PCR to identify two species of insects infected with the phytoplasma, they are *Diastrombus mkurangai* and the *Meenoplus* sp. At total of 8 *D. mkurangai* and 4 *Meenoplus* spp. out of a total of 5000 insects were tested positive. This is equivalent to 0.16%. Based on these results, it was assumed by the investigators that LD phytoplasma is not readily acquired by putative vectors. With the exception of the work done by Howard *et al*. (1983) no vector has been found for LY outside of Florida. However, the search continues.

Plant to plant transmission of phytoplasmas can occur in three ways. These include inoculation by vector insects during feeding, vegetative propagation of infected plant material or by grafting (Kirkpatrick 1992). Although infestation of floral tissues by these obligate, pleomorphic mollicutes has been demonstrated (Clark *et al*. 1986), it has been generally accepted that seed transmission is unlikely because phloem sieve elements of plants in which phytoplasmas reside lack any direct connection to seeds. Also, yellows disease symptoms often include floral or seed abnormalities (premature nut fall in the case of coconut) thereby preventing production of viable seed once infection has occurred (McCoy *et al*. 1989). However, a report on detection of phytoplasmas in both seed and seedling progeny of alfalfa plants affected by alfalfa witches,-broom disease indicated that seed transmission in certain plant host-phytoplasma pathosystems is indeed possible (Khan *et al*. 2002). Cordova *et al*. (2003) conducted *in situ* PCR on 72 embryos, 13 of

which were found to have the LY phytoplasma. The presence of phytoplasma DNA in embryo tissues is suggestive of the potential for seed transmission which remains to be definitively demonstrated.

ECONOMIC IMPACT

The economic impact of the disease caused by the LY group of phytoplasmas has been tremendous. Coconut production in Belize is not supported by a strong institutional structure as can be seen by the fact that coconuts are grown mainly in sporadic stands rather than intensive plantations. Losses in coconut production to LY were estimated at 25% in the cays and 75% on the mainland (Quiroz 2002). The coconut industry in the Dominican Republic produces approximately 250 million coconuts. In 2002, though LY was present in the Dominican Republic, the coconut growing areas were relatively free of the disease (Vargas 2002). In Haiti the economic impact of LY has been great. In 1979, there were approximately 1.5 million coconut trees producing in the region of 60 million fruits a year, in 1994 this figure dropped to 500,000 trees yielding approximately 30 million fruits a year. Lethal yellowing is responsible for the death of 80% of the coconut trees of the western region of Haiti, 75% of those in the northwest region and is one of the main causes of death of coconuts in the northern region (Donis 2002).

Lethal yellowing reached Honduras in 1995 and to date over 90% of the coconut palm population of the Honduran Atlantic coast which consists mainly of the Atlantic Tall variety has been destroyed (Doyle 2001). Coconut is a major source of income for farmers in Ghana who have been severely affected by the LY disease. Since 1964, LY has being devastating western and central Ghana and in twenty years more than 6,500 hectares have been devastated. To date only the Sri Lanka Green Dwarf seems to be resistant to the disease, however, trials have shown that it does not adapt well to growing conditions in Ghana (Mariau *et al*. 1996).

The coconut industry plays an important role in Jamaica's agricultural sector and is directly supported by 9500 farmers with five to six million trees (Been 1995). Of the estimated six million coconut palms that were growing in Jamaica in 1961, 90% were lost to lethal yellowing (Been 2002). In 1988 there was a resurgence of the disease, and now LY is the single most important disease affecting coconuts in Jamaica. The disease is lethal and fast spreading. It has been noted that the LY of the 1970's affected mainly bearing trees, however the LY of the 1990's and 2000's affects both bearing and non bearing trees (Myrie 2002) which makes it particularly devastating. Mexico is ranked as the sixth largest producer of coconuts in the world. Coconut production is important from an economic and social perspective with some 70,000 families relying on its cultivation. Since the detection of LY in the Yucatán Peninsula in 1977, it has contributed to the destruction of 650,000 palms (Mora-Aguilera 2002). In Tanzania the coconut palm is an important perennial oil crop to farmers on the coastal belt. LD has killed 8 million palms or 38% of the total palm population over a period of 30 years (Schuiling *et al*. 1992; Mugini 2002). In the Key West area of Florida approximately 75% of the coconut palms were destroyed between 1950 and 1965. By 1983, the epidemic had destroyed an estimated 100,000 coconut palms and thousands of palms of other species in mainland Florida (Howard and Harrison 1999).

CONTROL

One of the main hindrances in the control of LY is the lack of understanding of the pathogen itself. Due to the nature of the plant involved, not much work has been able to be conducted. Hence very little information is available on the plant-pathogen interaction. With the exception of *M. crudus* in Florida, the vector of the LY group of phytoplasmas in Africa and elsewhere is still not known. This poses a great problem as one is not sure what one is fighting against. The best course of action is the development of tolerant or resistant varieties. This was demonstrated in Jamaica in 1974 when a cross between the Malayan Dwarf and the Panama Tall produced the MayPan hybrid that was shown to be resistant to LY for 15 years. This resistance has now been broken and no cultivar currently being cultivated in the northeast where the disease is active has shown any sign of possessing a high or any level of resistance (Wallace 2002).

Habitat management can remove the incidence of pests; this involves the practice of systematic rouging of weeds and grasses. Certain grasses have been shown to be hosts of *M. crudus*, the elimination of these weeds and grasses would remove any potential breeding condition or alternate hosts of the phytoplasma. The type of mulching materials used around coconut trees influences the abundance of *M. crudus*. Fewer nymphs were found around trees mulched with coarse materials such as pine bark nuggets (Howard and Oropeza 1998). The prompt elimination and burning of LY infected trees on coconut farms is critical as this will serve to remove the inoculum. Good farm practices such as the proper fertilizing and care of the coconuts will result in healthy plants that will be better able to withstand the disease than an unhealthy plant. Antibiotic application of oxytetracycline-HCL could be used to suppress LY symptoms (McCoy *et al*. 1976), but single palm application is very expensive, hence it is not feasible at the moment. Turpenes also have been used in the control of LY, however it is still in the experimental phase (Been pers. comm.). In the medium to long term, an active replanting program-med should be implemented (as is done in Jamaica), where tolerant coconut varieties are imported from countries that are not affected by LY and placed in nurseries after which they are put out in the field. No single method will control the disease but a combination of the methods mentioned above will make it more manageable.

CONCLUDING REMARKS

Molecular biology tools have assisted greatly in the identification and classification of the lethal yellowing group of phytoplasmas. The variability of the LY phytoplasmas found in the *Cedusa* sp. of Derbids coupled with the fact that the LY group of phytoplasmas were found in weeds in coconut growing areas opens the door to the possibility of a vector other than *M. crudus* being involved in the spread of LY (at least in Jamaica). The variability of the LY group of phytoplasmas in the different regions affected by the disease points to the fact that different strains of the disease exist. There is no single solution for the pandemic of LY, however what we can do is to approach the problem with an open mind, tackling it from all angles and letting the molecular tools work for us. The search for vectors is critical, as well as information on the plant-pathogen and pathogen-vector interactions, then and only then will we begin to understand this disease and by extension its control.

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